Journal of Medicinal Chemistry

Article

pubs.acs.org/jmc

The Bivalent Ligand Approach Leads to Highly Potent and Selective Acylguanidine-Type Histamine H_2 Receptor Agonists†

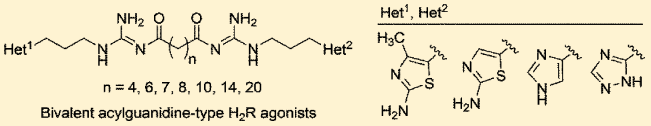
Tobias Birnkammer,‡ Anja Spickenreither,‡ Irena Brunskole,‡ Miroslaw Lopuch,‡ Nicole Kagermeier,‡ Günther Bernhardt,‡ Stefan Dove,‡ Roland Seifert,§ Sigurd Elz,‡ and Armin Buschauer*,‡

‡Department of Pharmaceutical/Medicinal Chemistry, Faculty of Chemistry and Pharmacy, University of Regensburg, Universitätsstrasse 31, D-93053 Regensburg, Germany

§Institute of Pharmacology, Medical School of Hannover, D-30625 Hannover, Germany

Supporting Information

ABSTRACT: Bivalent histamine H_2 receptor (H_2R) agonists were synthesized by connecting pharmacophoric 3-(2-amino-4-methylthiazol-5-yl)-, 3-(2-aminothiazol-5-yl)-, 3-(imidazol-4-yl)-, or 3-(1,2,4-triazol-5-yl)propylguanidine moieties by N^G-acylation with alkanedioic acids of various chain lengths. The compounds were investigated for H_2R agonism in GTPase and [^{35}S]GTPγS binding assays at guinea pig (gp) and human (h) H_2R-Gsα_S fusion proteins including various H_2R mutants, at the isolated gp right atrium, and in GTPase assays for activity on recombinant H_1, H_3, and H_4 receptors. The bivalent ligands are H_2R partial or full agonists, up to 2 orders of magnitude more potent than monovalent acylguanidines and, with octanedioyl or decanedioyl spacers, up to 4000 times more potent than histamine at the gpH_2R. In contrast to their imidazole analogues, the aminothiazoles are highly selective for H_2R vs other HR subtypes. Compounds with (theoretically) sufficient spacer length (20 CH_2 groups) to simultaneously occupy two orthosteric binding sites in H_2R dimers are nearly inactive, whereas the highest potency resides in compounds with considerably shorter spacers. Thus, there is no evidence for interaction with H_2R dimers. The high agonistic potency may result from interaction with an accessory binding site at the same receptor protomer.



INTRODUCTION

The histamine H_2 receptor (H_2R), a member of class A G-protein-coupled receptors (GPCRs), is a well-established target for the treatment of gastric and duodenal ulcers using antagonists such as cimetidine or famotidine.[1,2] H_2R agonists are important pharmacological tools to study the physiological and pathophysiological role of this histamine receptor. Although numerous compounds were described as H_2R agonists decades ago, after discovery of the histamine H_3 (H_3R) and H_4 receptor (H_4R), the H_2R selectivity of compounds such as 5-methylhistamine,[1] dimaprit,[3] impromidine[4] (**3**, Figure 1), or arpromidine[5] and related imidazolylpropylguanidines[6] turned out to be compromised.[7−9] Most strikingly, 5-methylhistamine is nowadays considered as selective for the H_4R.[7] Recently, in search for H_2R agonists derived from guanidine-type compounds such as **3**,[10] N^G-acylated hetarylpropylguanidines (e.g., **4**) were identified in our laboratory as a new class of potent H_2R agonists with considerably reduced basicity.[10,11] These acylguanidines proved to be highly selective for the H_2R, when the imidazole ring was replaced with an amino(methyl)thiazole moiety[10] as in amthamine[12] (**2**). The structure−activity relationships revealed that even space-filling substituents at the guanidine group were well tolerated. This prompted us to explore the applicability of the bivalent ligand approach, based on the working hypothesis that such compounds should possess increased H_2R agonistic potency and could be useful to study H_2R dimers.[13]

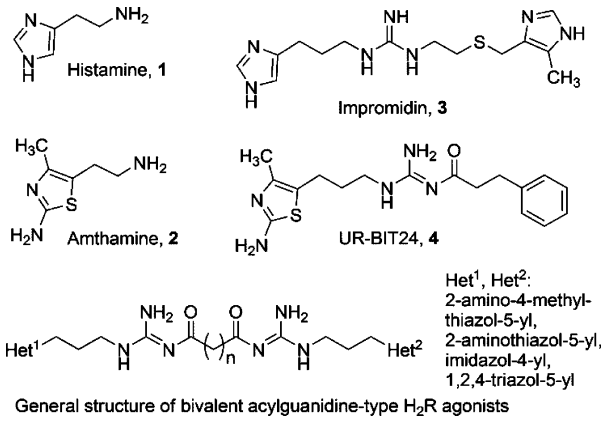


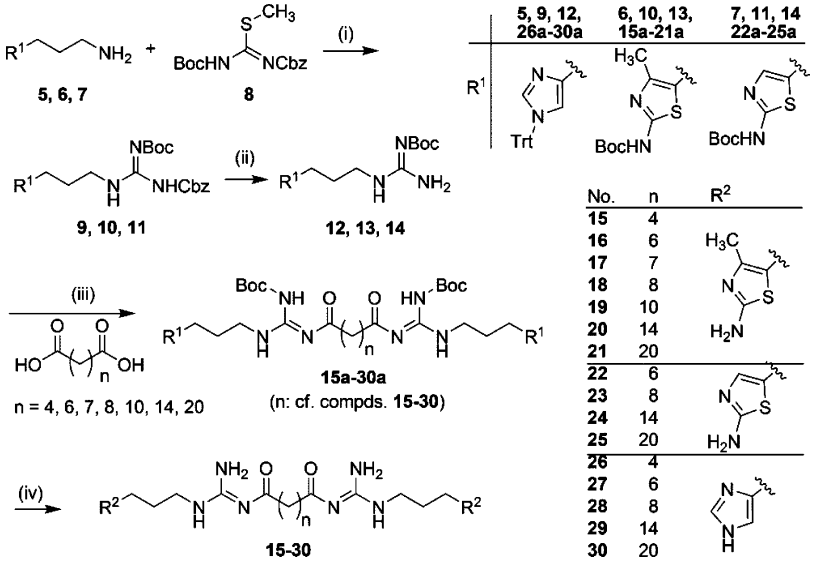
Figure 1. Histamine, selected H_2R agonists, and general structure of bivalent hetarylpropylguanidines.

Over the past few decades the understanding of GPCR structure and function has been challenged by the discovery that GPCRs are able to form homo- and hetero-oligomeric complexes.[14−17] The existence of homo- and heterodimers has been demonstrated for several class A GPCRs including opioid receptors,[18−20] adrenergic receptors,[21] somatostatin receptors,[22,23] dopaminergic receptors,[24−26] muscarinergic recep-

Received: August 23, 2011
Published: January 5, 2012

 © 2012 American Chemical Society

Scheme 1. General Procedure for the Preparation of the Boc-Protected Hetarylpropylguanidine Building Blocks 12−14 and the Synthesis of the Symmetrical Bivalent Acylguanidines 15−30[a]



[a]Reagents and conditions: (i) $HgCl_2$ (2 equiv), NEt_3 (3 equiv), DCM/abs, 48 h, rt; (ii) H_2, Pd/C (10%), MeOH/THF (1:1), 8 bar, 8−9 days (**12**, **13**) or 3−4 days (**14**), rt; (iii) EDAC (1 equiv), HOBt (1 equiv), DIEA (1 equiv), DCM/abs, 16 h, rt; (iv) 20% TFA, DCM/abs, 3−5 h, rt.

tors,[27,28] and the histamine receptor subtypes.[13,29−34] The term bivalent ligand is widely used and refers to molecules containing two sets of pharmacophoric entities linked through a spacer.[35−37] It is assumed that duplication of the pharmacophoric groups according to the bivalent ligand approach leads to a supra-additive increase in potency compared to the corresponding monovalent ligand..[35,36] This concept has been studied for various GPCRs,[37] for instance, for opioid receptors[38] or aminergic GPCRs such as serotonin or dopamine receptor subtypes,[39−42] in more detail. The bivalent ligand approach in the design of ligands targeting GPCRs has proven to be promising to improve not only potency and selectivity but also the pharmacokinetic profile of compounds.[43]

For opioid receptors, the distance between two recognition sites of a contact dimer with a TM5/TM6 interface is about 22−27 Å, as suggested from molecular modeling.[35] In an approach to explore the structural requirements of putative bivalent H_2R agonists we synthesized and pharmacologically investigated compounds with two pharmacophoric hetarylpropylguanidine entities linked at the N^G-nitrogen atoms with dicarboxylic acids as spacers with lengths between 6 and 27 Å (Figure 1).

CHEMISTRY

The bivalent acylguanidine-type compounds were preferentially synthesized by analogy with the procedures developed for the N^G-acylation of monovalent imidazolyl- and aminothiazolylpropylguanidines.[10,11] The primary amines **5**−**7** were treated with the orthogonally protected isothiourea **8**, yielding the guanidines **9**−**11**, which were subjected to hydrogenolytic cleavage of the benzyloxycarbonyl (Cbz) group to give the *tert*-butoxycarbonyl (Boc) protected 2-aminothiazolylpropylguanidines **12**[10] and **13**[10] as well as the N^G-Boc-,N^{Im}-Trt-protected imidazolylpropylguanidine **14**[44] in good yields (Scheme 1). To obtain the designated symmetrical bivalent ligands **15**−**30**, the mono Boc-protected hetarylpropylguanidines **12**−**14** were coupled to alkanedioic acids of various length using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC), hydroxybenzotriazole (HOBt), and *N*,*N*-diisopropylethylamine (DIEA) as standard coupling reagents to yield the protected compounds **15a**−**30a**. Thereby, the Boc-protected guanidine (**12**−**14**), at its terminal position (N^G), reacts similarly to a primary amine but at lower reaction rate. Finally, removal of the protecting groups under acidic conditions gave the symmetrical acylguanidines **15**−**30** (Scheme 1), which were purified by preparative RP-HPLC.

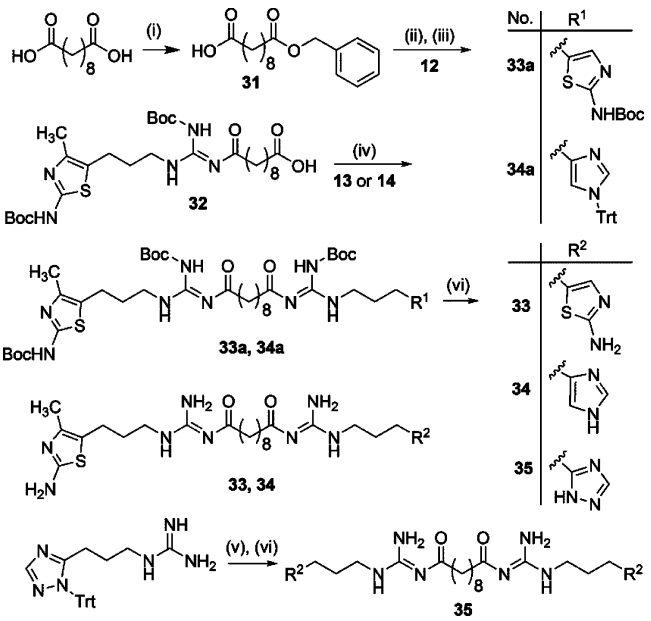
The unsymmetrical ligands **33** and **34** were synthesized as depicted in Scheme 2, using the decanedioyl spacer. To reduce the formation of byproduct, one carboxylic function of the dicarboxylic acid was capped with a benzyl group, and the resulting 10-benzyloxy-10-oxodecanoic acid (**31**) was coupled to the 2-amino-4-methylthiazol-5-ylpropylguanidine building block **12**. After hydrogenolysis of the benzyl ester group, the free carboxylic group of **32** was coupled to the Boc-protected guanidine building blocks **13** and **14**, respectively. In addition, the bivalent N^G-acylated 1,2,4-triazol-5-ylpropylguanidine **35** was synthesized starting from the corresponding Trt-protected triazolylpropylguanidine building block (cf. Scheme 2).[45] In contrast to the aforementioned acylation steps, this guanidine building block was deprotonated with NaH and coupled to decanedioic acid, which was activated by CDI, to yield the Trt-protected precursor **35a**. Final deprotection with trifluoroacetic acid (TFA) and purification by preparative RP-HPLC yielded the acylguanidines **33**−**35**.

RESULTS AND DISCUSSION

The synthesized compounds were examined for histamine H_2R agonism on human (h) and guinea pig (gp) H_2 receptors in steady-state GTPase assays using membranes of Sf9 insect cells expressing hH_2R-G$s\alpha_S$ and gpH_2R-G$s\alpha_S$ fusion proteins, respectively (Table 1).[46] By use of this approach, agonists of Gs-coupled receptors can be studied directly at the level of receptor/G-protein coupling with high sensitivity.[47,48] In addition, selected compounds were investigated on the isolated spontaneously beating guinea pig right atrium[1] as a pharmacological standard model for the functional character-

dx.doi.org/10.1021/jm201128q | J. Med. Chem. 2012, 55, 1147−1160

Scheme 2. Synthesis of Building Block 32 and Bivalent Acylguanidines 33−35[a]



[a]Reagents and conditions: (i) BnOH (1 equiv), DCC (1.2 equiv), DMAP (cat.), THF/abs, 48 h, rt; (ii) EDAC (1 equiv), HOBt (1 equiv), DIEA (1 equiv), DCM/abs, 16 h, rt; (iii) H_2, Pd/C (10%), MeOH, 1 h, rt; (iv) EDAC (1 equiv), HOBt (1 equiv), DIEA (1 equiv), DCM/abs, 16 h, rt; (v) CDI (1.2 equiv), NaH (60% dispersion in mineral oil) (2 equiv), THF/abs, 3 h, rt; (vi) 20% TFA, DCM/abs, 3 h, rt.

ization of H_2R ligands (positive chronotropic response) (Table 2) and in the GTPγS binding assay on gpH_2R-Gsα_S fusion proteins. The GTPγS binding assay is a valid alternative to the GTPase assay and yields similar results.[49] In addition to occupation of the orthosteric binding site, interactions of the bivalent ligands with individual amino acids or sequences in other regions of the same receptor protomer, especially N-terminus and extracellular loops, are conceivable. Different potencies at human and guinea pig H_2R orthologues might reflect such interactions. Therefore, selected bivalent ligands were examined on H_2R mutants/chimera, in which Cys-17 and Ala-271 in the hH_2R were replaced by Tyr-17 and Asp-271 as in the gpH_2R (hH_2R-C17Y-A271D-Gsα_S, hH_2R-C17Y-Gsα_S),[50,51] the four different amino acids in the e2 loop were reciprocally mutated (hH_2R-gpE2-Gsα_S, gpH_2R-hE2-Gsα_S),[52] and the N-terminus of the hH_2R was replaced by the N-terminus of the gpH_2R (hH_2R-gpNT-Gsα_S) (Figure 8, data in Supporting Information). Moreover, the histamine receptor subtype selectivities of representative compounds were explored in GTPase assays using recombinant human histamine H_1, H_3, and H_4 receptors (Table 3). In the following, on the basis of the data from functional assays, the terms potency (expressed as pEC_{50} of an agonist) and efficacy (intrinsic activity of an agonist, expressed as E_{max} relative the maximal response induced by histamine) and antagonistic activity (expressed as pK_B) characterize agonists and antagonists, respectively.[53]

Agonism at Human and Guinea Pig H_2R-Gsα_S Fusion Proteins. Pharmacophore duplication led to potent partial to full agonists in the GTPase assay at hH_2R-Gsα_S and gpH_2R-Gsα_S fusion proteins (Table 1). Investigations of three different series of symmetrical compounds containing two (2-amino-4-methylthiazolyl)propylguanidine entities (**15**−**21**), two (2-aminothiazolyl)propylguanidine entities (**22**−**25**), or two imidazolylpropylguanidine entities (**26**−**30**) revealed the following results: When increasing the spacer length from 4 to 20 C-atoms, covering a distance of ∼6 to ∼27 Å between the carbonyl groups, the highest potencies were obtained with octanedioyl to decanedioyl spacers at both the hH_2R-Gsα_S ($pEC_{50} \leq 8.2$) and the gpH_2R-Gsα_S fusion proteins ($pEC_{50} \leq 9.4$). Further extension of the spacer length resulted in a significant drop in potency or in a complete loss of agonistic activity at hH_2R-Gsα_S and switch to H_2R antagonism (pK_B values: **21**, 6.1; **25**, 5.8; **30**, 6.6).

Homobivalent 2-aminothiazoles lacking the 4-methyl substituent showed slightly decreased potencies but increased efficacies compared to their methylated analogues (**22** vs **16**, **23** vs **18**, and **24** vs **20**) at both hH_2R-Gsα_S and gpH_2R-Gsα_S. Compounds **22** and **23** were full agonists at gpH_2R-Gsα_S. Compared to the corresponding 2-amino-4-methylthiazoles, most imidazoles (**27**−**30**) were nearly equipotent at hH_2R-Gsα_S and slightly less potent at gpH_2R-Gsα_S. Compound **35** bearing two 1,2,4-triazole rings showed up to 2 orders of magnitude lower potencies compared to the corresponding aminothiazoles **18**, **23** and the imidazole **28** at hH_2R-Gsα_S and gpH_2R-Gsα_S, respectively. Furthermore, the imidazoles revealed the highest efficacies among the four structural classes, resulting in full agonists at gpH_2R-Gsα_S.

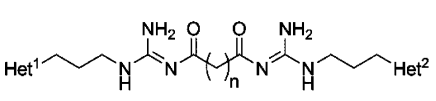
To further elaborate the contribution of the different pharmacophores with respect to potency and efficacy, two unsymmetrical compounds with one 2-amino-4-methylthiazole moiety (**33** and **34**) were investigated (Figure 2). Interestingly, the potencies at both receptors were always between the potencies of the symmetrical analogues (compare **33** with **18** and **23**, **34** with **18** and **28**). In contrast, the efficacies of **33** and **34** were close to the high efficacies of the corresponding "bis-imidazole" **28** and "bis-aminothiazole" **23**, respectively. In conclusion, both heterocycles of the unsymmetrical compounds nearly additively contribute to potency, whereas efficacy seems to be determined by the "more efficacious moiety".

In agreement with previous studies on monovalent acylguanidine-type H_2R agonists, all bivalent compounds exhibit higher potencies and efficacies at gpH_2R-Gsα_S relative to hH_2R-Gsα_S (see Figure 3).[46,55] Interestingly, compounds **15**, **16**, **18**, and **22** are 20−76 times more potent at the gpH_2R-Gsα_S compared to hH_2R-Gsα_S and exhibit the highest selectivity toward gpH_2R-Gsα_S among all the acylguanidines from our laboratory. Compounds **16**, **18**, and **34** (EC_{50} values at gpH_2R-Gsα_S: 0.63, 0.39, and 0.51 nM, respectively) are the most potent acylguanidine-type H_2R agonists identified in the GTPase assay.

When membrane preparations are used, G-proteins are directly accessible to the investigated compounds; i.e., the possibility of H_2R-receptor-independent G-protein activation[56−58] has to be taken into account. Therefore, selected compounds were investigated in the presence of the H_2R antagonist famotidine in GTPase assays as shown for **33** in Figure 4. At both, hH_2R-Gsα_S and gpH_2R-Gsα_S, **33**-stimulated GTP hydrolysis was inhibited in a concentration-dependent manner by famotidine, confirming the measured GTPase activity to be stimulated via the H_2R. The calculated K_B values of famotidine (52 ± 22 and 65 ± 32 nM, Figure 4) determined against **33** at hH_2R-Gsα_S and gpH_2R-Gsα_S, respectively, are comparable to data obtained from GTPase assays using

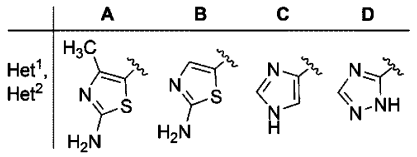
dx.doi.org/10.1021/jm201128q | J. Med. Chem. 2012, 55, 1147−1160

Table 1. Agonist Efficacies and Potencies of Bivalent Acylguanidines and Reference Compounds at hH_2R-Gsα_S and gpH_2R-Gsα_S Fusion Proteins Expressed in Sf9 Cell Membranes[a]



15-30, 33-35

	Het^1	Het^2
15-21	A	A
22-25	B	B
26-30	C	C
33	A	B
34	A	C
35	D	D



		hH_2R-Gsα_S			gpH_2R-Gsα_S			
compd	n	E_{max} ± SEM	$pEC_{50}/(pK_B)$ ± SEM	Pot_{rel}	E_{max} ± SEM	pEC_{50} ± SEM	Pot_{rel}	$EC_{50}(hH_2R\text{-}Gs\alpha_S)/EC_{50}(gpH_2R\text{-}Gs\alpha_S)$
1[51]		1.00	5.90 ± 0.09	1.0	1.00	5.92 ± 0.09	1.0	1.16
2[51]		0.91 ± 0.02	6.72 ± 0.10	6.6	1.04 ± 0.01	6.72 ± 0.09	6.3	1.00
4[10]		0.79 ± 0.02	7.69 ± 0.13	61.7	0.76 ± 0.02	8.13 ± 0.05	162.2	2.75
15	4	0.68 ± 0.03	7.24 ± 0.22	21.9	0.90 ± 0.05	8.59 ± 0.30	467.7	22.39
16	6	0.62 ± 0.03	7.32 ± 0.23	26.3	0.81 ± 0.03	9.20 ± 0.16	1905	75.97
17	7	0.48 ± 0.04	7.35 ± 0.13	28.2	0.90 ± 0.06	8.56 ± 0.16	436.5	16.24
18	8	0.53 ± 0.04	8.11 ± 0.25	162.2	0.79 ± 0.07	9.41 ± 0.15	3090	19.90
19	10	0.46 ± 0.04	7.78 ± 0.17	75.9	0.66 ± 0.05	8.57 ± 0.32	446.7	6.17
20	14	0.12 ± 0.02	7.59 ± 0.22	49.0	0.51 ± 0.02	7.46 ± 0.01	34.7	0.74
21	20		6.11 ± 0.15[b]		0.58 ± 0.02	6.48 ± 0.37	3.6	
22	6	0.79 ± 0.03	7.51 ± 0.02	40.7	1.00 ± 0.03	8.87 ± 0.28	891.3	22.89
23	8	0.75 ± 0.03	7.67 ± 0.07	58.9	0.94 ± 0.01	8.30 ± 0.22	239.9	4.27
24	14	0.14 ± 0.01	7.03 ± 0.13	13.5	0.59 ± 0.01	7.23 ± 0.19	20.4	1.58
25	20		5.77[b]		0.36 ± 0.01	6.69 ± 0.01	5.9	
26	4	0.68 ± 0.04	6.67 ± 0.34	5.9	1.00 ± 0.02	7.96 ± 0.07	109.7	19.51
27	6	0.77 ± 0.12	7.25 ± 0.16	22.4	1.18 ± 0.01	8.49 ± 0.33	371.5	17.35
28	8	0.81 ± 0.02	8.21 ± 0.07	204.2	0.98 ± 0.05	8.94 ± 0.16	1047	5.36
29	14	0.29 ± 0.08	7.61 ± 0.18	51.3	0.85 ± 0.10	7.70 ± 0.26	60.4	1.23
30	20		6.57 ± 0.07[b]		0.19 ± 0.03	7.46 ± 0.12	34.7	
33	8	0.75 ± 0.04	7.86 ± 0.11	91.2	0.89 ± 0.04	8.46 ± 0.30	346.7	3.98
34	8	0.76 ± 0.05	8.12 ± 0.04	166.0	1.01 ± 0.03	9.29 ± 0.10	2344	14.88
35	8	0.49 ± 0.03	6.82 ± 0.05	10.5	0.95 ± 0.04	7.99 ± 0.02	117.5	14.79

[a]Steady state GTPase activity in Sf9 membranes expressing hH_2R-Gsα_S and gpH_2R-Gsα_S was determined as described.[46] Reaction mixtures contained ligands at concentrations from 0.1 nM to 10 μM as appropriate to generate saturated concentration–response curves. Data were analyzed by nonlinear regression and were best fit to sigmoidal concentration–response curves. Typical basal GTPase activities ranged between ~0.5 and 2.5 pmol·mg^{-1}·min^{-1}, and activities stimulated by histamine (**1**) at a concentration of 100 μM ranged between ~2 and 13 pmol·mg^{-1}·min^{-1}. The intrinsic activity (E_{max}) of **1** was determined by nonlinear regression and was set to 1.0. The E_{max} values of other agonists were referenced to this value. For determination of antagonism, reaction mixtures contained histamine (**1**) (100 nM) and ligands were at concentrations from 10 nM to 1 mM. Data were analyzed by nonlinear regression and were best fitted to sigmoidal concentration–response curves. Typical basal GTPase activities ranged between ~1.5 and 2.5 pmol·mg^{-1}·min^{-1}, and activities stimulated by **1** (10 μM) ranged between ~3.5 and 4.5 pmol·mg^{-1}·min^{-1}. Data shown are the mean ± SEM of two to six independent experiments performed in duplicate. The relative potency of **1** was set to 1.0, and the potencies of other agonists were referenced to this value. [b]No agonistic activity. IC_{50} values were converted to pK_B values using the Cheng–Prusoff equation.[54]

histamine as the H_2R agonist (reported K_B values: hH_2R-Gsα_S, 48 ± 10 nM; gpH_2R-Gsα_S, 38 ± 3 nM).[59]

For comparison, examples of acylguanidines were additionally investigated in GTPγS binding assays using membrane preparations of Sf9 cells expressing the gpH_2R-Gsα_S fusion protein (cf. Figure 5). The determined pEC_{50} values and the intrinsic activities are in good agreement with the data from the GTPase assay.

Histamine H_2R Agonism on the Guinea Pig Right Atrium. In addition to the studies on membrane preparations, representative bivalent H_2R agonists were investigated on the isolated spontaneously beating guinea pig right atrium as a more complex, well established standard model for the characterization of H_2R ligands. The obtained data (Table 2) are largely comparable with the results from the GTPase assays on the gpH_2R-Gsα_S fusion protein in terms of both potencies and intrinsic activities. The structure–activity relationships are similar to those derived from the GTPase assay. However, the agonist potency of the long chain members of the series (viz. **19**, **20**, and **21**) decreases substantially in the organ assay compared with the GTPase assay. The combination of two hetarylpropylguanidine moieties with octanedioyl, nonanedioyl, or decanedioyl spacer (**16**, **17**, **18**, and **28**) leads to the most potent agonists at the guinea pig right atrium known so far, surpassing up to 4000 times the potency of histamine in increasing heart rate. In contrast to the GTPase assay, the equilibration on the guinea pig atrium was extremely slow. For the generation of cumulative concentration–response curves, the incubation periods after addition of the H_2R agonists at concentrations below 10 nM had to be extended to 120–180 min. The positive chronotropic response was mediated by the H_2R, since it could be blocked by the H_2R antagonist cimetidine (10–300 μM, data not shown). Characteristic concentration–response curves are shown in Figure 6 for histamine, compound **15** alone, and **15** in the presence of cimetidine.

Are the Binding Sites Located at Two Protomers of an H_2R Dimer or at a Single H_2R Protomer? The structure–

dx.doi.org/10.1021/jm201128q | J. Med. Chem. 2012, 55, 1147–1160

Table 2. Histamine H_2 Receptor Agonism at the Spontaneously Beating Guinea Pig Right Atrium

compd	pEC_{50} ± SEM[a]	Pot_{rel}[b]	E_{max} ± SEM[c]
1	6.00 ± 0.02	1.0	1.0
2[60]	6.21 ± 0.09	1.6	0.95 ± 0.02
15	8.59 ± 0.07	389	0.88 ± 0.03
16	9.61 ± 0.03	4070	0.64 ± 0.03
17	9.08 ± 0.05	1210	0.71 ± 0.05
18	8.93 ± 0.12	847	0.62 ± 0.03
19	6.56 ± 0.05	3.66	0.46 ± 0.04
20	6.26 ± 0.14	1.82	0.53 ± 0.11
21	5.10 ± 0.13	0.13	0.62 ± 0.07
28	9.22 ± 0.06	1640	0.91 ± 0.04

[a] pEC_{50} was calculated from the mean corrected shift ΔpEC_{50} of the agonist curve relative to the histamine reference curve by equation $pEC_{50} = 6.00 + \Delta pEC_{50}$; data shown are the mean ± SEM of three to five experiments. [b] Potency relative to histamine (**1**) (=1). [c] Intrinsic activity: maximal response relative to the maximal increase in heart rate induced by the reference compound histamine (=1.0).

activity relationships of bivalent H_2R agonistic acylguanidines, resulting from GTPase, GTPγS binding, and guinea pig right atrium assays, are not compatible with the possible role of such ligands as compounds "bridging" the recognition (orthosteric) sites of receptor dimers (cf. Figure 7). The spacers of the highly potent agonists **16**–**19**, **22**, **23**, **27**, and **28** are too short to simultaneously occupy two H_2R protomers. The presumed optimal spacer length of ∼22–27 Å may be attained only by compounds **21**, **25**, and **30** (n = 20, carbonyl–carbonyl distance of 26.4 Å with fully extended chain). However, spacers with 14 and 20 carbon atoms result in weak agonism (gpH_2R-Gsα_S) or loss of agonistic activity and conversion to antagonism (hH_2R-Gsα_S). Thus, the remarkable increase in potency compared to monovalent H_2R agonists is presumably due to interaction with an accessory (allosteric?) binding site at the same receptor molecule rather than to occupation of two protomers of a receptor dimer (cf. Figure 7). In fact, many bivalent GPCR ligands with drastically increased activities relative to the

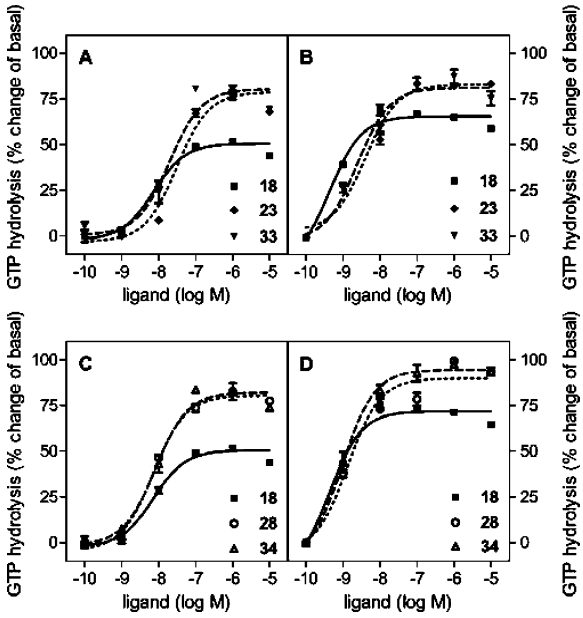


Figure 2. Histamine H_2 receptor agonism of the symmetrical bivalent ligands **18** and **23** compared to the unsymmetrical bivalent ligand **33** in membranes expressing hH_2R-Gsα_S (A) and gpH_2R-Gsα_S (B) and H_2R agonism of the symmetrical bivalent ligands **18** and **28** compared to the unsymmetrical bivalent ligand **34** at hH_2R-Gsα_S (C) and gpH_2R-Gsα_S (D). Data are from two to six representative experiments performed in duplicate, expressed as percentage change in GTPase activity relative to the maximum effect induced by histamine (100 μM).

Table 3. Histamine Receptor Subtype Selectivity of Selected Bivalent Ligands: Agonistic, Antagonistic or Inverse Agonistic Effects at hH_1R + RGS4, hH_2R-Gsα_S, hH_3R + Gα_{i2} + G$\beta_1\gamma_2$ + RGS4. and hH_4R-RGS19 + Gα_{i2} + G$\beta_1\gamma_2$ Expressed in Sf9 Cell Membranes[a]

		hH_2R		hH_3R		hH_4R	
compd	hH_1R (pK_B)	pEC_{50} (pK_B)	E_{max}	pEC_{50} (pK_B)	E_{max}	pEC_{50} (pK_B)	E_{max}
15	(<6.00)	7.24 ± 0.22	0.68 ± 0.03	(<5.00)		(<6.00)	
16	(<6.00)	7.32 ± 0.23	0.62 ± 0.03	(<5.00)		(<6.00)	
18	(6.01 ± 0.07)	8.11 ± 0.25	0.53 ± 0.04	(<5.00)		(<6.00)	
20	(<6.00)	7.59 ± 0.22	0.12 ± 0.02	(<6.00)		(<6.00)	
22	(<6.00)	7.51 ± 0.02	0.79 ± 0.03	(6.36 ± 0.11)		(<6.00)	
23	(<6.00)	7.67 ± 0.07	0.75 ± 0.03	(<5.00)		(<6.00)	
26	(6.13 ± 0.22)	6.67 ± 0.34	0.68 ± 0.04	<5.00	−0.22 ± 0.03	7.10 ± 0.12	0.42 ± 0.01
27	(6.70 ± 0.07)	7.25 ± 0.16	0.77 ± 0.12	8.38 ± 0.11	0.37 ± 0.08	7.38 ± 0.02	0.51 ± 0.04
28	(6.32 ± 0.16)	8.21 ± 0.07	0.81 ± 0.02	8.75 ± 0.06	0.63 ± 0.08	8.07 ± 0.19	0.44 ± 0.05
29	(<6.00)	7.61 ± 0.18	0.29 ± 0.08	<6.00	−1.02 ± 0.02	6.47 ± 0.04	−0.29 ± 0.09
30	(<6.00)	(6.57 ± 0.07)		6.35 ± 0.03	−0.77 ± 0.02	<6.00	−0.86 ± 0.02
33	(<6.00)	7.86 ± 0.11	0.75 ± 0.04	(<5.00)		(<5.00)	
34	(6.27 ± 0.19)	8.12 ± 0.04	0.76 ± 0.05	8.54 ± 0.02	0.68 ± 0.06	8.07 ± 0.09	0.52 ± 0.03

[a] Antagonism (pK_B), agonism, and inverse agonism (pEC_{50}, in parentheses), determined in steady state GTPase activity assays using Sf9 membranes expressing hH_1R + RGS4, hH_2R-Gsα_S, hH_3R + Gα_{i2} + G$\beta_1\gamma_2$ + RGS4, or hH_4R-RGS19 + Gα_{i2} + G$\beta_1\gamma_2$ as described.[46] Reaction mixtures contained ligands at concentrations from 0.1 nM to 1 mM as appropriate to generate saturated concentration–response curves. For antagonism, reaction mixtures contained histamine (**1**) (100 nM) and ligands were at concentrations from 10 nM to 1 mM. Data were analyzed by nonlinear regression and were best fitted to sigmoidal concentration–response curves. Typical basal GTPase activities ranged between ∼1.5 and 2.5 pmol·mg^{-1}·min^{-1}, and activities stimulated by **1** (10 μM) ranged between ∼3.5 and 4.5 pmol·mg^{-1}·min^{-1}. Data shown are mean values of one to six experiments performed in duplicate. Efficacy (E_{max}) relative to the maximal response of **1** was set to 1.00. Negative E_{max} values refer to inverse agonistic effects.

dx.doi.org/10.1021/jm201128q | J. Med. Chem. 2012, 55, 1147–1160

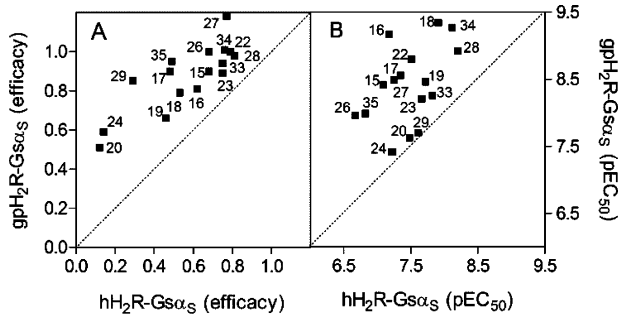
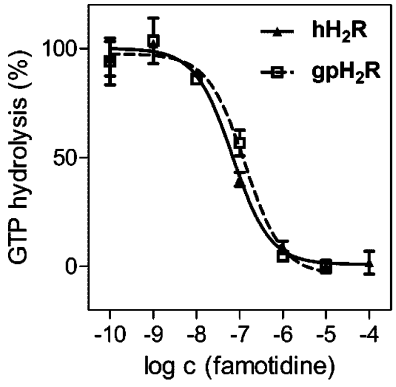


Figure 3. Efficacies (E_{max}) and agonistic potencies of compounds **15**–**20**, **22**–**24**, **26**–**28**, and **33**–**35** at hH_2R-$Gs\alpha_S$ in comparison with gpH_2R-$Gs\alpha_S$ as determined in the steady-state GTPase assay. The dotted lines represent the line of identity. (A) Plot of efficacies at gpH_2R-$Gs\alpha_S$ vs hH_2R-$Gs\alpha_S$. (B) Plot of pEC_{50} at gpH_2R-$Gs\alpha_S$ vs hH_2R-$Gs\alpha_S$.

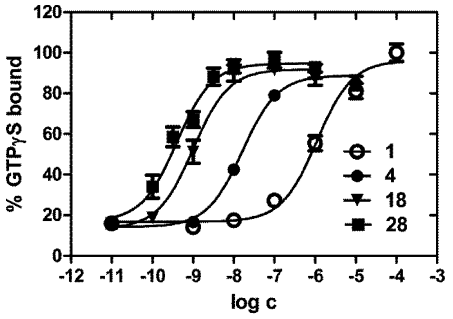


	K_B [nM]
hH_2R-$Gs\alpha_S$	52 ± 22
gpH_2R-$Gs\alpha_S$	65 ± 32

Figure 4. Concentration-dependent inhibition of GTP hydrolysis by famotidine using **33** as the H_2R agonist at 10 and 1 nM at the hH_2R-$Gs\alpha_S$ (solid line) and the gpH_2R-$Gs\alpha_S$ fusion proteins (dashed line), respectively. Data points are the mean of a representative experiment performed in duplicate.

monovalent parent compounds in spite of insufficient linker lengths for bridging of receptor protomers have been reported.[35,43,61,62] In the unsymmetrical derivatives **33** and **34** with potencies between those of their symmetrical analogues, each of the two different pharmacophoric entities may interact with the orthosteric and the accessory sites, respectively, suggesting two binding modes depending on the recognition pathway.

Agonistic Activities on Histamine H_2R Mutants/Chimera. Unlike small H_2R agonists such as histamine (**1**) and amthamine (**2**) (Figure 1), which are full agonists at human and guinea pig H_2 receptors, all investigated bivalent ligands were significantly more potent and efficacious at the gpH_2R relative to the hH_2R in the GTPase assay (cf. Table 1). These differences may result from species-dependent interactions with both the orthosteric and the putative accessory binding site. The latter probably resides in the extracellular domain, and amino acids in the e2 loop are possible candidates for interacting with bivalent ligands. On the basis of the crystal structure of rhodopsin,[21] the participation of various residues in the e2 loop to ligand binding was proposed[63] and already



	pEC_{50}	E_{max}
1	5.96 ± 0.08	1.00
4	7.80 ± 0.07	0.92 ± 0.03
18	9.00 ± 0.09	0.99 ± 0.04
28	9.40 ± 0.10	0.98 ± 0.06

Figure 5. Histamine H_2 receptor agonism of representative bivalent ligands **18** and **28** compared to the monovalent ligand **4** and histamine (**1**) in the GTPγS binding assay using membranes expressing gpH_2R-$Gs\alpha_S$ fusion proteins. Data points are the mean of two (**4**, **18**) or three (**1**, **28**) independent experiments performed in duplicate or triplicate, analyzed by nonlinear regression for best fit to sigmoidal concentration–response curves.

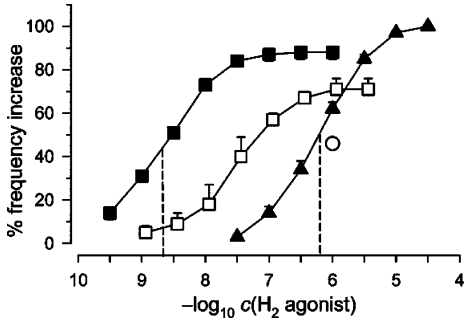


Figure 6. Concentration–response curves on the isolated guinea pig right atrium. Histamine (▲, N = 4), **15** alone (■, corrected mean leftward shift ΔpEC_{50} = 2.59 ± 0.07, relative potency of 389, E_{max} = 0.88 ± 0.03, N = 4), and **15** in the presence of cimetidine (□, 10 μM, pA_2 = 6.09 ± 0.08, N = 2). As expected, cimetidine (100 μM, 60 min incubation time) also led to a fading of the maximum response induced by **15** (■, 1 μM) to 46 ± 2% (○).

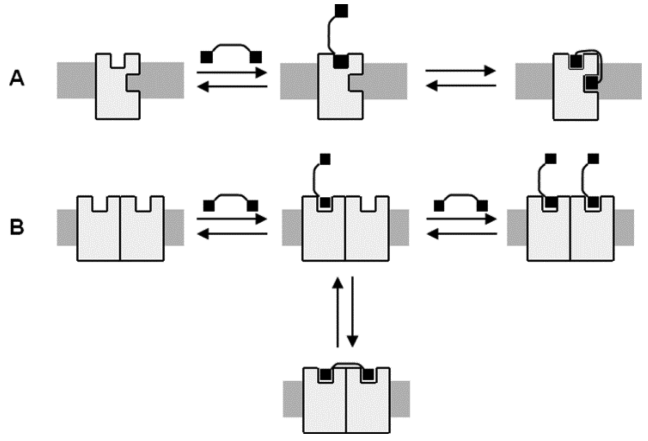


Figure 7. Bivalent ligand binding to (A) a GPCR with an accessory binding site or to (B) a GPCR dimer (according to Portoghese et al.[35]).

dx.doi.org/10.1021/jm201128q | J. Med. Chem. 2012, 55, 1147–1160

experimentally demonstrated for some members of class A GPCRs.[64,65] The recently resolved crystal structures of the turkey β_1- and the human β_2-adrenergic receptor indicate a certain contribution of a phenylalanine in the e2 loop to agonist and antagonist binding,[66−68] but this residue belongs to the orthosteric site. Since the e2 loops of the hH_2R and the gpH_2R differ by only four amino acids outside the orthosteric pocket (hH_2R; G167, H169, T171, S172 vs gpH_2R; D167, D169, I171, V172), reciprocal mutation (hH_2R-gpE2-Gsα_S, gpH_2R-hE2-Gsα_S) is an approach to probe whether species selectivity of bivalent ligands depends on an accessory function of e2. Application of this approach to *N*-[3-(1*H*-imidazol-4-yl)-propyl]guanidines and N^G-acylated analogues indicated that the e2 loop does not contribute to species selectivity of monovalent H_2R agonists.[52] Investigations of selected bivalent acylguanidines on the reciprocal mutants led to rather ambivalent results. All investigated compounds exhibited similar potencies and efficacies at mutant hH_2R-gpE2-Gsα_S and wild-type hH_2R-Gsα_S (for a summary of the data, see Supporting Information). At mutant gpH_2R-hE2-Gsα_S the compounds are equally efficacious compared to the wild-type gpH_2R-Gsα_S. However, the pEC_{50} values are significantly reduced by 0.5−0.9 in the case of all 2-amino-4-methylthiazolyl compounds (**16**, **18**, **34**) except **20** and **33**, whereas the potencies of imidazolyl and 2-aminothiazolyl derivatives remain nearly unchanged (Figure 8A). Hence, these results do not indicate direct interactions of the mutated residues with the bivalent ligands. However, the integrity of the e2 loop seems to be necessary for high-affinity gpH_2R binding of bivalent 2-amino-4-methylthiazoles. It is not obvious whether the detrimental effect of the mutations is directly based on the modification of an accessory site in the extracellular region or indirectly because of conformational changes of the orthosteric site.

Furthermore, as predicted by H_2R models and verified by site-directed mutagenesis studies, the preference of the guanidine-type agonists for the gpH_2R is strongly dependent on two amino acids, Tyr-17 and Asp-271 in TM 1 and TM 7, respectively, which are thought to stabilize an active receptor conformation via direct or through-water interactions.[50,51] Cys-17 and Ala-271 in the hH_2R cannot fulfill this function. Investigations of selected bivalent acylguanidines on H_2R mutants (Figure 8B), in which Cys-17 and Ala-271 of the hH_2R were replaced by the corresponding amino acids Tyr-17 and Asp-271 of the gpH_2R (hH_2R-C17Y-A271D-Gsα_S, hH_2R-C17Y-Gsα_S), confirmed that both Tyr-17 in TM1 and Asp-271 in TM7 or at least Asp-271 are key residues for highly potent and efficacious H_2R activation. The single Cys-17-Tyr mutation has only slight (**1**, **2**, **16**, **22**, **23**, **28**, **33**, **34**) or in some cases even detrimental effects (**18**, **20**) on hH_2R potency and efficacy.

Because of the low homology in the N-terminus of human and guinea pig H_2Rs, the potential role of this extracellular region with respect to the species-specific pharmacological profile of bivalent acylguanidines was also studied. Investigation at a chimeric hH_2R possessing the N-terminus of the gpH_2R (hH_2R-gpNT-Gsα_S) revealed that the N-terminus does not contribute to the species-selective effects (Figure 8C and Supporting Information).

Histamine Receptor Subtype Selectivity. To determine the histamine receptor subtype selectivity profile (human H_2R vs H_1R, H_3R, H_4R), representative compounds were investigated in GTPase assays on recombinant human H_1, H_3, and H_4 receptors for agonism and antagonism (Table 3). The imidazolylpropylguanidine moiety proved to be a privileged structure in terms of interactions with histamine receptors. Such substances, initially designed as H_2R agonists,[11,69] were previously identified as model compounds for the development of H_3R and H_4R ligands.[8,70] By contrast, recently reported monovalent N^G-acylated aminothiazolylpropylguanidine-type H_2R agonists proved to be devoid of agonistic and antagonistic activities or to have only negligible effects on histamine receptors other than the H_2R.[10] This also holds for bivalent ligands: the investigated compounds containing two 2-aminothiazole moieties (**15**, **16**, **18**, **20**, **22**, **23**, and **33**) showed only very weak antagonistic effects on H_1, H_3, and H_4 histamine receptors. By contrast, compounds containing at least one imidazole ring (**26**−**30** and **34**) showed, in addition to H_2R agonism, significant agonistic, antagonistic, or inverse agonistic activities at the other histamine receptor subtypes, depending on the spacer length. While imidazolylpropylguanidines with octane (**27**) and decanedioyl (**28**, **34**) spacers turned out to be highly potent hH_3R and hH_4R partial agonists in the low

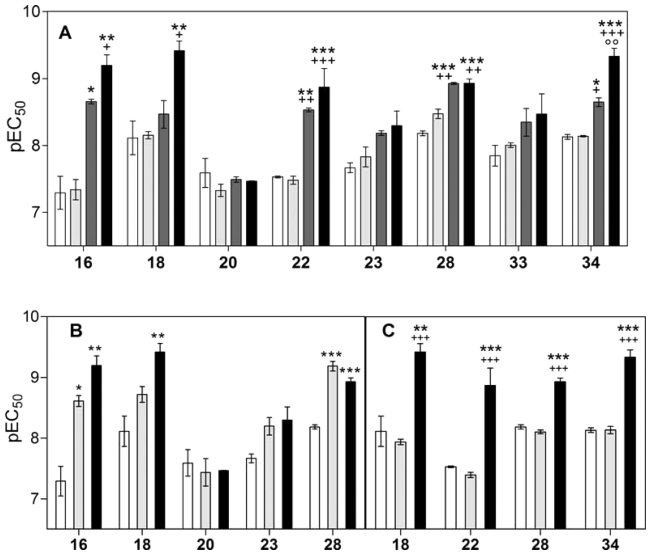


Figure 8. Comparison of the agonistic potencies of selected bivalent ligands at wild-type and mutant human and guinea pig H_2 receptors as determined in GTPase assays. Data shown are the mean ± SEM of two to five independent experiments performed in duplicate. pEC_{50} values were compared with each other using one-way ANOVA, followed by Bonferroni's multiple comparison test. (A) pEC_{50} values of **16**, **18**, **20**, **22**, **23**, **28**, **33**, and **34** at hH_2R-Gsα_S (□) vs hH_2R-gpE2-Gsα_S (light-gray column) vs gpH_2R-hE2-Gsα_S (medium-gray column) vs gpH_2R-Gsα_S (■) fusion proteins. pEC_{50} values are significantly different for (∗) hH_2R-Gsα_S, (+) hH_2R-gpE2-Gsα_S, and (○) gpH_2R-hE2-Gsα_S: one symbol, $p < 0.05$; two symbols, $p < 0.01$; three symbols, $p < 0.001$; 95% confidence interval. (B) pEC_{50} values of **16**, **18**, **20**, **23**, and **28** at hH_2R-Gsα_S (□) vs hH_2R-C17Y-A271D-Gsα_S double mutant (light-gray column) vs gpH_2R-Gsα_S (■). The sensitivity of the hH_2R-C17Y-A271D-Gsα_S double mutant against agonist stimulation is shifted from that of the gpH_2R isoform. The asterisk (∗) indicates that pEC_{50} is significantly different for hH_2R-Gsα_S: one symbol, $p < 0.05$; two symbols, $p < 0.01$; three symbols, $p < 0.001$; 95% confidence interval. (C) pEC_{50} values of **18**, **22**, **28**, and **34** at hH_2R-Gsα_S (□) vs hH_2R-gpNT-Gsα_S (light-gray column) vs gpH_2R-Gsα_S (■) fusion proteins. pEC_{50} values are significantly different for (∗) hH_2R-Gsα_S, (+) hH_2R-gpNT-Gsα_S: one symbol, $p < 0.05$; two symbols, $p < 0.01$; three symbols, $p < 0.001$; 95% confidence interval.

dx.doi.org/10.1021/jm201128q | *J. Med. Chem.* 2012, 55, 1147−1160

nanomolar range, elongation of the spacer (**29**, n = 14; **30**, n = 20) resulted in a switch to inverse agonism. Hence, by analogy with amthamine[12] as an H_2R selective analogue of histamine, the replacement of the imidazolyl with an aminothiazolyl moiety strongly favors the selectivity for the H_2R in the case of both monovalent[10] and bivalent N^G-acylated hetarylpropylguanidines.

CONCLUSION

The application of the bivalent ligand approach to acylguanidines resulted in novel hH_2R and gpH_2R agonists that may serve as pharmacological tools for more detailed investigations of the H_2R. The combination of two hetarylpropylguanidine moieties with octanedioyl or decanedioyl spacers led to the most potent agonists at the guinea pig right atrium known so far, surpassing up to 4000 times the potency of histamine in increasing heart rate. However, the results of this study, in particular the structure–activity relationships with respect to the spacer length, do not support the hypothesis of simultaneous occupation of the orthosteric recognition sites of neighboring protomers. The spacer optimum suggests that the remarkable increase in potency compared to monovalent H_2R agonists is due to interaction with an accessory binding site at the same receptor molecule. To explore the topology of this putative site, further investigations with H_2R mutants and novel unsymmetrical bivalent ligands are necessary. In addition, the preparation of a structurally related bivalent radioligand could be helpful to determine the ligand–receptor stoichiometry.

EXPERIMENTAL SECTION

Chemistry. General Conditions. Commercially available reagents were purchased from Acros Organics (Geel, Belgium), Lancaster Synthesis GmbH (Frankfurt, Germany), Sigma-Aldrich Chemie GmbH (München, Germany), Alfa Aesar GmbH & Co. KG (Karlsruhe, Germany), Iris Biotech GmbH (Marktredwitz, Germany), or Merck (Darmstadt, Germany) and used as received. Where indicated, reactions were carried out under a dry, oxygen-free argon atmosphere. All solvents used were of analytical grade or distilled before use. THF and Et_2O were distilled over Na. CH_2Cl_2 was predried over $CaCl_2$ or distilled from P_2O_5 and stored under argon atmosphere over 3 Å molecular sieves. Column chromatography was carried out using Merck silica gel Geduran 60 (0.063–0.200) and Merck silica gel 60 (0.040–0.063) for flash column chromatography. Reactions were monitored by thin layer chromatography (TLC) on Merck silica gel 60 F_{254} aluminum sheets and spots were visualized with UV light at 254 nm.

Nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR) spectra were recorded on a Bruker Avance 300 spectrometer with perdeuterated solvents. The chemical shift δ is given in parts per million (ppm) with reference to the chemical shift of the residual protic solvent compared to tetramethylsilane (δ = 0 ppm). Multiplicities were specified with the following abbreviations: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and br (broad signal) as well as combinations thereof. The multiplicity of carbon atoms (^{13}C NMR) was determined by DEPT 135 and DEPT 90 (distortionless enhancement by polarization transfer): "+" primary and tertiary carbon atom (positive DEPT 135 signal), "–" secondary carbon atom (negative DEPT 135 signal), "quat" quaternary carbon atom. Mass spectrometry analysis (MS) was performed on a Finnigan MAT 95, a Finnigan SSQ 710A, and a Finnigan ThermoQuest TSQ 7000 spectrometer. Melting points (mp) were measured on a Büchi 530 electrically heated copper block apparatus using an open capillary and are uncorrected.

Preparative HPLC was performed with a pump model K-1800 (Knauer, Berlin, Germany). The column was either a Eurosphere-100 (250 mm × 32 mm) (Knauer) or a Nucleodur-100 C_{18}ec (250 mm × 21 mm) (Macherey & Nagel, Düren, Germany), which were attached to the UV detector model K-2000 (Knauer). UV detection was done at 254 and 210 or 220 nm. The temperature was 25 °C and the flow rate 37 mL/min (Eurosphere-100) or 20 mL/min (Nucleodur-100 C_{18}ec). The mobile phase was 0.1% TFA in Millipore water and MeCN. Analytical HPLC was performed on a system from Thermo Separation Products equipped with a SN400 controller, P4000 pump, an AS3000 autosampler, and a Spectra Focus UV/vis detector. Stationary phase was either a Eurosphere-100 C_{18} (250 mm × 4.0 mm, 5 μm) column (Knauer) or a Nucleodur-C_{18}HTec (250 mm × 4.0 mm, 5 μm) column (Macherey-Nagel) thermostated at 30 °C. For the mobile phase, gradients of MeCN/TFA (0.05% aq) were used (flow rate of 0.75 mL min^{-1}). The gradient mode was as follows: 0 min, MeCN/TFA (0.05% aq) 10:90; 20 min, 60:40; 20–23 min, 95:5; 33 min, 95:5. Absorbance was detected at 210 nm. t_0(Eurosphere-100 C_{18}) = 3.318 min; t_0(Nucleodur-C_{18}HTec) = 2.675 min; $k' = (t_R - t_0)/t_0$. Compound purities were calculated as the percentage peak area of the analyzed compound by UV detection at 210 nm. The purities (see Supporting Information) of the bivalent H_2R agonists used for pharmacological investigation were ≥95% except for compounds **15** (93%), **17** (92%), and **33** (94%).

Preparation of the Guanidine Building Blocks 12, 13, and 14. General Procedure for the Guanidinylation Reaction. Compounds **5**, **6**, **7**, and **8** were prepared according to the literature.[10,11] To a suspension of **5**, **6**, or **7** (1 equiv), **8** (1 equiv), and $HgCl_2$ (2 equiv) in DCM/abs was added NEt_3 (3 equiv), and the mixture was stirred at ambient temperature for 48 h. Subsequently, EtOAc was added and the precipitate filtered over Celite. The crude product was purified by flash chromatography (PE/EtOAc, 80/20 v/v) to give the Boc- and Cbz-protected guanidines **9** and **10** and the Boc-, Cbz-, and Trt-protected guanidine **11**.

General Procedure for the Hydrogenolytic Cleavage of Cbz Groups. To a solution of **9**, **10**, or **11** in a mixture of THF/MeOH (1:1) was added Pd/C (10%), and the mixture was hydrogenated at 8 bar for 3–4 days. The catalyst was removed by filtration over Celite and washed with MeOH. The solvent was removed in vacuo.

***tert*-Butyl 5-[3-(2-*tert*-Butoxycarbonylguanidino)propyl]-4-methylthiazol-2-ylcarbamate (12).[10]** **12** was prepared from **9** (5.8 g, 10.6 mmol) and 6 g of Pd/C (10%) in a mixture of 160 mL of THF/MeOH (1:1) according to the general procedure, yielding **12** as a colorless foamlike solid (4.38 g, 100%), mp =113 °C. ^{1}H NMR (CD_3OD) δ (ppm): 3.23 (t, ^3J = 6.9 Hz, 2H, CH_2-NH), 2.75 (t, ^3J = 7.5 Hz, 2H, Thiaz-5-CH_2), 2.17 (s, 3H, Thiaz-4-CH_3), 1.86 (m, 2H, Thiaz-5-CH$_2$CH_2), 1.52 (s, 9H, C(CH_3)$_3$), 1.47 (s, 9H, C(CH_3)$_3$). ES-MS (DCM/MeOH + NH_4OAc) m/z (%): 414 (MH^+, 100); $C_{18}H_{31}N_5O_4S$ (413.53).

***tert*-Butyl 5-[3-(2-*tert*-Butoxycarbonylguanidino)propyl]-thiazol-2-ylcarbamate (13).[10]** **13** was prepared from **10** (5.8 g, 10.6 mmol) and 6 g of Pd/C (10%) in a mixture of 160 mL of THF/MeOH (1:1) according to the general procedure, yielding **13** as a colorless foamlike solid (3.39 g, 75%). ^{1}H NMR ($CDCl_3$) δ (ppm): 7.03 (s, 1H, Thiaz-4-*H*), 3.26 (t, ^3J = 6.9 Hz, 2H, CH_2-NH), 2.84 (t, ^3J = 7.2 Hz, 2H, Thiaz-5-CH_2), 1.95 (m, 2H, Thiaz-5-CH$_2$CH_2), 1.55 (s, 9H, C(CH_3)$_3$), 1.47 (s, 9H, C(CH_3)$_3$). ES-MS (DCM/MeOH + NH_4OAc) m/z (%): 400 (MH^+, 100); $C_{17}H_{29}N_5O_4S$ (399.50).

***2*-(*tert*-Butoxycarbonyl)-1-[3-(1-trityl-1*H*-imidazol-4-yl)-propyl]guanidine (14).[44]** The title compound was prepared from **11** (1.5 g, 2.33 mmol) and 1 g of Pd/C (10%) in a mixture of 60 mL of THF/MeOH (1:1) according to the general procedure, yielding **14** as a colorless foamlike solid (1.05 g, 88%). ^{1}H NMR ($CDCl_3$) δ (ppm): 7.34–7.10 (m, 16H, Im-2-*H*, CPh$_3$), 6.57 (s, 1H, Im-5-*H*), 3.41 (m, 2H, CH_2NH$_2$), 2.56 (m, 2H, Im-4-CH_2), 1.86 (m, 2H, Im-4-CH$_2$CH_2), 1.46 (s, 9H, C(CH_3)$_3$). ES-MS (DCM/MeOH + NH_4OAc) m/z (%): 510 (MH^+, 100); $C_{31}H_{35}N_5O_2$ (509.64).

Preparation of the Boc-Protected Bivalent Acylguanidines 15a–30a. General Procedure. DIEA (1 equiv) was added to a solution of carboxylic acid (0.5 equiv), EDAC (1 equiv), and HOBt monohydrate (1 equiv) in DCM/abs under argon and stirred for 15 min. A solution of **12**, **13**, or **14** (1 equiv) in DCM/abs was added, and the mixture was stirred overnight at room temperature. The solvent

was removed under reduced pressure. EtOAc and water were added to the residue. The organic phase was separated and the aqueous layer extracted two times with EtOAc. After drying over $MgSO_4$, the organic solvent was removed in vacuo. The crude product was purified by flash chromatography (PE/EtOAc, 70/30 to 50/50 v/v) unless otherwise indicated.

N^1,N^{10}-Bis((*tert*-butoxycarbonylamino)[3-[2-(*tert*-butoxycarbonyl)amino-4-methylthiazol-5-yl]propylamino]-methylene)decanediamide (18a). The title compound was prepared from decanedioic acid (100 mg, 0.5 mmol), EDAC (190 mg, 1 mmol), HOBt monohydrate (150 mg, 1 mmol), DIEA (0.17 mL, 1 mmol) in 5 mL of DCM/abs and **12** (410 mg, 1 mmol) in 5 mL of DCM/abs according to the general procedure, yielding **18a** (0.23 g, 46%) as a colorless foamlike solid. ^{1}H NMR ($CDCl_3$) δ (ppm): 12.40 (s, 2H, N*H*), 9.02 (t, ^3J = 5.2 Hz, 2H, CH_2NH), 3.45 (m, 4H, CH_2NH), 2.70 (t, ^3J = 7.4 Hz, 4H, Thiaz-5-CH_2), 2.35 (t, ^3J = 7.5 Hz, 4H, $COCH_2$), 2.21 (s, 6H, Thiaz-4-CH_3), 1.87 (m, 4H, Thiaz-5-CH_2CH_2), 1.66 (m, 4H, $COCH_2CH_2$), 1.51 (s, 18H, $C(CH_3)_3$), 1.49 (s, 18H, $C(CH_3)_3$), 1.32 (m, 8H, $(CH_2)_4$). ES-MS (DCM/MeOH + NH_4OAc) *m*/*z* (%): 993 (MH^+, 100); $C_{46}H_{76}N_{10}O_{10}S_2$ (992.5).

N^1,N^{10}-Bis((*tert*-butoxycarbonylamino)[3-[2-(*tert*-butoxycarbonyl)aminothiazol-5-yl]propylamino]methylene)-decanediamide (23a). The title compound was prepared from decanedioic acid (50 mg, 0.25 mmol), EDAC (95 mg, 0.5 mmol), HOBt monohydrate (77 mg, 0.5 mmol), DIEA (0.08 mL, 0.5 mmol) in 5 mL of DCM/abs and **13** (200 mg, 0.5 mmol) in 5 mL of DCM/abs according to the general procedure, yielding **23a** (0.20 g, 54%) as a brown oil. ^{1}H NMR ($CDCl_3$) δ (ppm): 7.04 (s, 2H, Thiaz-4-*H*), 3.48 (m, 4H, CH_2NH), 2.79 (m, 4H, Thiaz-5-CH_2), 2.34 (m, 4H, $COCH_2$), 1.93 (m, 4H, Thiaz-5-CH_2CH_2), 1.65 (m, 4H, $COCH_2CH_2$), 1.56 (s, 18H, $C(CH_3)_3$), 1.50 (s, 18H, $C(CH_3)_3$), 1.32 (m, 8H, $(CH_2)_4$). ES-MS (DCM/MeOH + NH_4OAc) *m*/*z* (%): 965.5 (MH^+, 100); $C_{44}H_{72}N_{10}O_{10}S_2$ (964.5).

N^1,N^{10}-Bis[(*tert*-butoxycarbonylamino)[3-(1-trityl-1*H*-imidazol-4-yl)propylamino]methylene]decanediamide (28a). The title compound was prepared from decanedioic acid (100 mg, 0.5 mmol), EDAC (190 mg, 1 mmol), HOBt monohydrate (150 mg, 1 mmol), DIEA (0.17 mL, 1 mmol) in 5 mL of DCM/abs and **14** (510 mg, 1 mmol) in 5 mL of DCM/abs according to the general procedure (flash chromatography $CHCl_3$/MeOH, 95/5 v/v), yielding **28a** (0.18 g, 30%) as a yellow oil. ^{1}H NMR ($CDCl_3$) δ (ppm): 7.33−7.12 (m, 32H, Im-2-*H*, CPh_3), 6.53 (d, ^4J = 1.0 Hz, 2H, Im-5-*H*), 3.43 (m, 4H, CH_2NH), 2.59 (t, ^3J = 7.6 Hz, 4H, Im-4-CH_2), 2.34 (m, 4H, $COCH_2$), 1.90 (m, 4H, Im-4-CH_2CH_2), 1.65 (m, 4H, $COCH_2CH_2$), 1.49 (s, 18H, $C(CH_3)_3$), 1.27 (m, 8H, $(CH_2)_4$). ES-MS (DCM/MeOH + NH_4OAc) *m*/*z* (%): 1185 (MH^+, 100); $C_{72}H_{84}N_{10}O_6$ (1184.66).

Compounds **15a**−**17a**, **19a**−**22a**, **24a**−**27a**, **29a**, and **30a** were prepared by analogy (see Supporting Information).

Preparation of the Boc-Protected Bivalent Acylguanidines 33a and 34a. 10-Benzyloxy-10-oxodecanoic Acid (31).[71] Phenylmethanol (0.27 g, 0.25 mL, 2.47 mmol) was dropwise added to a cooled suspension of decanedioic acid (0.5 g, 2.47 mmol) and DMAP (cat.) in 3 mL of THF/abs. A solution of DCC (0.61 g, 2.96 mmol) in 3 mL of THF/abs was dropwise added to this mixture and stirred for 72 h at ambient temperature. Subsequently, 1,1-dicyclohexylurea was filtered and the solvent removed under reduced pressure. The crude product was subjected to flash chromatography (PE/EtOAc, 90/10 v/v) to obtain **31** (0.34 g, 47%) as a colorless semisolid. ^{1}H NMR ($CDCl_3$) δ (ppm): 10.88 (s, 1H, COO*H*), 7.34 (m, 5H, Ar-*H*), 5.11 (s, 2H, CH_2-Ar), 2.34 (m, 4H, $COCH_2$), 1.61 (m, 4H, $COCH_2CH_2$), 1.29 (s, 8H, $(CH_2)_4$). ^{13}C NMR ($CDCl_3$) δ ppm: 179.80 (quat *C*OOH), 173.72 (quat *C*=O), 136.12 (quat Ar-*C*), 128.55 (+, Ar-*C*H), 128.18 (+, Ar-*C*H), 66.11 (−, *C*H_2-Ar), 34.30 (−, *C*H_2COOH), 34.04 (−, CO*C*H_2), 29.02 (−, *C*H_2), 28.96 (−, *C*H_2), 24.90 (−, $COCH_2$*C*H_2), 24.64 (−, *C*H_2CH_2COOH). EI-MS (70 eV) *m*/*z* (%): 292 ($M^{+\bullet}$, 30); $C_{17}H_{24}O_4$ (292.37).

10-((*tert*-Butoxycarbonylamino)[3-[2-(*tert*-butoxycarbonylamino)-4-methylthiazol-5-yl]propylamino]methyleneamino)-10-oxodecanoic Acid (32). Compound **32** was prepared from **31** (150 mg, 0.5 mmol), EDAC (95 mg, 0.5 mmol), HOBt monohydrate (80 mg, 0.5 mmol), DIEA (0.09 mL, 0.5 mmol) in 2.5 mL of DCM/abs and **12** (210 mg, 0.5 mmol) in 2.5 mL of DCM/abs according to the general procedure, yielding the Bzl-protected compound as a yellow oil, which was immediately dissolved in 10 mL of MeOH and hydrogenated with Pd/C as catalyst for 1 h at room temperature. After filtration over Celite, the solvent was removed under reduced pressure to obtain **32** (0.21 g, 70%) as a colorless foamlike solid. ^{1}H NMR ($CDCl_3$) δ (ppm): 3.47 (m, 2H, CH_2NH), 2.70 (t, ^3J = 7.1 Hz, 2H, Thiaz-5-CH_2), 2.33 (m, 4H, CH_2COOH, $COCH_2$), 2.16 (s, 3H, Thiaz-4-CH_3), 1.88 (m, 2H, Thiaz-5-CH_2CH_2), 1.64 (m, 4H, $COCH_2CH_2$, CH_2CH_2COOH), 1.53 (s, 9H, $C(CH_3)_3$), 1.49 (s, 9H, $C(CH_3)_3$), 1.33 (s, 8H, $(CH_2)_4$). ES-MS (DCM/MeOH + NH_4OAc) *m*/*z* (%): 598 (MH^+, 100); $C_{28}H_{47}N_5O_7S$ (597.77).

N^1-((*tert*-Butoxycarbonylamino){3-[2-(*tert*-butoxycarbonylamino)-4-methylthiazol-5-yl]propylamino]methylene)-N^{10}-((*tert*-butoxycarbonylamino)[3-[2-(*tert*-butoxycarbonylamino)-thiazol-5-yl]propylamino]methylene)decanediamide (33a). The title compound was prepared from **32** (135 mg, 0.23 mmol), EDAC (44 mg, 0.23 mmol), HOBt monohydrate (35 mg, 0.23 mmol), DIEA (0.04 mL, 0.23 mmol) in 3 mL of DCM/abs and **13** (92 mg, 0.23 mmol) in 2 mL of DCM/abs according to the general procedure, yielding **33a** (0.12 g, 57%) as a brown oil. ^{1}H NMR ($CDCl_3$) δ (ppm): 7.05 (s, 1H, Thiaz-4-*H*), 3.47 (m, 4H, CH_2NH), 2.75 (m, 4H, Thiaz-5-CH_2), 2.34 (m, 4H, $COCH_2$), 2.21 (s, 3H, Thiaz-4-CH_3), 1.91 (m, 4H, Thiaz-5-CH_2CH_2), 1.65 (m, 4H, $COCH_2CH_2$), 1.54 (s, 18H, $C(CH_3)_3$), 1.50 (s, 18H, $C(CH_3)_3$), 1.32 (m, 8H, $(CH_2)_4$). ESI-MS: *m*/*z* (rel intens, %): 979.6 (MH^+, 100); $C_{45}H_{74}N_{10}O_{10}S_2$ (978.50).

N^1-{(tert-Butoxycarbonylamino)[3-(1-trityl-1*H*-imidazol-4-yl)propylamino]methylene]-N^{10}-((*tert*-butoxycarbonylamino)-[3-[2-(*tert*-butoxycarbonylamino)-4-methylthiazol-5-yl]-propylamino]methylene)decanediamide (34a). The title compound was prepared from **32** (179 mg, 0.3 mmol), EDAC (57 mg, 0.3 mmol), HOBt monohydrate (46 mg, 0.3 mmol), DIEA (0.05 mL, 0.3 mmol) in 3 mL of DCM/abs and **14** (120 mg, 0.3 mmol) in 2 mL of DCM/abs according to the general procedure, yielding **34a** (0.07 g, 24%) as a brown oil. ^{1}H NMR ($CDCl_3$) δ (ppm): 8.82 (s, 1H, Im-2-*H*), 7.37−7.22 (m, 16H, Im-5-*H*, CPh_3), 3.38 (m, 4H, CH_2NH), 2.84 (t, ^3J = 7.7 Hz, 2H, Im-4-CH_2), 2.71 (t, ^3J = 7.4 Hz, 2H, Thiaz-5-CH_2), 2.47 (m, 4H, $COCH_2$), 2.18 (s, 3H, Thiaz-4-CH_3), 2.03 (m, 2H, Im-4-CH_2CH_2), 1.90 (m, 2H, Thiaz-5-CH_2CH_2), 1.66 (m, 4H, $COCH_2CH_2$), 1.52 (s, 18H, $C(CH_3)_3$), 1.35 (m, 8H, $(CH_2)_4$). ESI-MS: *m*/*z* (rel intens, %): 989.7 (MH^+, 100); $C_{54}H_{72}N_{10}O_6S$ (988.54).

Preparation of the Trt-Protected Bivalent Acylguanidine 35a. N^1,N^{10}-Bis{amino[3-(1-trityl-1*H*-1,2,4-triazol-5-yl)-propylamino]methylene}decanediamide (35a). To a solution of CDI (195 mg, 1.2 mmol) in DMF (7 mL), decanedioic acid (100 mg, 0.5 mmol) was added. The mixture was stirred under argon for 1 h. In a second flask, 3-(1-trityl-1*H*-1,2,4-triazol-5-yl)propylguanidine[45] (410 mg, 1 mmol) and NaH (60% dispersion in oil) (80 mg, 2 mmol) in DMF (7 mL) under argon were heated to 30−35 °C for 45 min, and the mixture was then allowed to cool to room temperature. The two mixtures were combined and stirred for 4 h at ambient temperature. The solvent was removed in vacuo and the crude product was purified by flash chromatography ($CHCl_3$/MeOH/NH_3, 95/3/2 v/v/v) to obtain **35a** (300 mg, 60%) as pale white foamlike solid. ^{1}H NMR (CD_3OD) δ (ppm): 8.01 (s, 2H, Triaz-3-*H*), 7.37−7.05 (m, 30H, CPh_3), 3.14 (t, ^3J = 7.6 Hz, 4H, CH_2NH), 2.88 (m, 4H, Triaz-5-CH_2), 2.41 (t, ^3J = 7.5 Hz, 4H, $COCH_2$), 1.96 (m, 4H, Triaz-5-CH_2CH_2), 1.63 (m, 4H, $COCH_2CH_2$), 1.29 (m, 8H, $(CH_2)_4$). ES-MS (DCM/MeOH + NH_4OAc) *m*/*z* (%): 987.7 (MH^+, 10), 494.4 ($(M+2H)^{2+}$, 100); $C_{60}H_{66}N_{12}O_2$ (987.25).

Preparation of Deprotected Bivalent Acylguanidines 15−30 and 33−35. General Procedure. TFA (20%) was added to a solution of the protected acylguanidines **15a**−**30a** and **33a**−**35a** in DCM/abs, and the mixture was stirred at ambient temperature until the protecting groups (Boc, Trt) were removed (3−5 h). Subsequently, the solvent was evaporated in vacuo and the residue was purified by preparative RP-HPLC and lyophilized. All compounds were obtained as trifluoroacetic acid salts.

N^1,N^{10}-Bis[[3-(2-amino-4-methylthiazol-5-yl)propylamino]-(amino)methylene]decanediamide Tetratrifluoroacetate (18). The title compound was prepared from **18a** (200 mg, 0.19 mmol) in 5

dx.doi.org/10.1021/jm201128q | *J. Med. Chem.* 2012, 55, 1147−1160

mL of DCM/abs and 1 mL of TFA according to the general procedure, yielding **18** as a colorless foamlike solid (120 mg, 57%). ^{1}H NMR (CD_3OD) δ (ppm): 3.35 (t, ^3J = 6.8 Hz, 4H, CH_2NH), 2.70 (t, ^3J = 7.5 Hz, 4H, Thiaz-5-CH_2), 2.45 (t, ^3J = 7.4 Hz, 4H, $COCH_2$), 2.16 (s, 6H, Thiaz-4-CH_3), 1.90 (m, 4H, Thiaz-5-CH_2CH_2), 1.63 (m, 4H, $COCH_2CH_2$), 1.33 (m, 8H, $(CH_2)_4$). ^{13}C (CD_3OD) δ (ppm): 177.57 (quat **C**=O), 170.43 (quat Thiaz-2-**C**), 155.41 (quat **C**=NH), 132.59 (quat Thiaz-4-**C**), 118.33 (quat Thiaz-5-**C**), 41.56 (−, CH_2NH), 37.75 (−, $COCH_2$), 30.13 (−, Thiaz-5-CH_2CH_2), 29.90 (−, CH_2), 29.68 (−, CH_2), 25.46 (−, $COCH_2CH_2$), 23.64 (−, Thiaz-5-CH_2), 11.46 (+, Thiaz-5-CH_3). HREIMS: m/z for ($[C_{26}H_{44}N_{10}O_2S_2 + H]^+$) calcd 593.3163, found 593.3161. Prep HPLC: MeCN/0.1% TFA/aq (25/75). HPLC: k' = 2.28 (t_R = 10.90 min), purity = 100%; $C_{26}H_{44}N_{10}O_2S_2$·4TFA (1048.78).

***N*^{1},*N*^{10}-Bis[[3-(2-aminothiazol-5-yl)propylamino](amino)-methylene]decanediamide Tetratrifluoroacetate (23).** The title compound was prepared from **23a** (200 mg, 0.2 mmol) in 5 mL of DCM/abs and 1 mL of TFA according to the general procedure, yielding **23** as a yellow-brown oil (100 mg, 49%). ^{1}H NMR (CD_3OD) δ (ppm): 7.01 (s, 2H, Thiaz-4-*H*), 3.37 (t, ^3J = 7.1 Hz, 4H, CH_2NH), 2.77 (t, ^3J = 7.1 Hz, 4H, Thiaz-5-CH_2), 2.47 (t, ^3J = 7.4 Hz, 4H, $COCH_2$), 1.95 (m, 4H, Thiaz-5-CH_2CH_2), 1.65 (m, 4H, $COCH_2CH_2$), 1.35 (m, 8H, $(CH_2)_4$). ^{13}C (CD_3OD) δ (ppm): 176.47 (quat **C**=O), 172.43 (quat Thiaz-2-**C**), 155.92 (quat **C**=NH), 125.55 (quat Thiaz-5-**C**), 123.27 (+, Thiaz-4-**C**), 40.66 (−, CH_2NH), 36.83 (−, $COCH_2$), 30.43 (−, CH_2), 28.80 (−, CH_2), 28.74 (−, Thiaz-5-CH_2-CH_2), 24.41 (−, Thiaz-5-CH_2), 24.08 (−, $COCH_2CH_2$). HRLSIMS: m/z for ($[C_{24}H_{40}N_{10}O_2S_2 + H]^+$) calcd 565.2855, found 565.2855. Prep HPLC: MeCN/0.1% TFA/aq (10/90−50/50). HPLC: k' = 2.13 (t_R = 10.37 min), purity = 100%; $C_{24}H_{40}N_{10}O_2S_2$·4TFA (1020.36).

***N*^{1},*N*^{10}-Bis[[3-(1*H*-imidazol-4-yl)propylamino](amino)-methylene]decanediamide Tetratrifluoroacetate (28).** The title compound was prepared from **28a** (160 mg, 0.13 mmol) in 10 mL of DCM/abs and 2 mL of TFA according to the general procedure, yielding **28** as a pale yellow oil (30 mg, 23%). ^{1}H NMR (CD_3OD) δ ppm: 8.81 (d, ^4J = 1.3 Hz, 2H, Im-2-*H*), 7.37 (d, ^4J = 0.9 Hz, 2H, Im-5-*H*), 3.38 (t, ^3J = 6.9 Hz, 4H, CH_2NH), 2.84 (t, ^3J = 7.6 Hz, 4H, Im-4-CH_2), 2.47 (t, ^3J = 7.4 Hz, 4H, $COCH_2$), 2.03 (m, 4H, Im-4-CH_2CH_2), 1.65 (m, 4H, $COCH_2CH_2$), 1.35 (m, 8H, $(CH_2)_4$). ^{13}C (CD_3OD) δ (ppm): 177.45 (quat **C**=O), 155.42 (quat **C**=NH), 134.97 (+, Im-2-**C**), 134.33 (quat Im-4-**C**), 117.14 (+, Im-5-**C**), 41.54 (−, CH_2NH), 37.75 (−, $COCH_2$), 30.17 (−, Im-4-CH_2), 29.95 (−, Im-4-CH_2CH_2), 27.98 (−, $COCH_2CH_2CH_2CH_2$), 25.45 (−, $COCH_2CH_2CH_2$), 22.56 (−, $COCH_2CH_2$). HREIMS: m/z for ($[C_{24}H_{40}N_{10}O_2 + H]^+$) calcd 501.3408, found 501.34199. Prep HPLC: MeCN/0.1% TFA/aq (15/85−30/70). HPLC: k' = 1.91 (t_R = 9.66 min), purity = 96%; $C_{24}H_{40}N_{10}O_2$·4TFA (956.60).

***N*^{1}-[[3-(2-Amino-4-methylthiazol-5-yl)propylamino](amino)-methylene]-*N*^{10}-[[3-(2-aminothiazol-5-yl)propylamino]-(amino)methylene]decanediamide Tetratrifluoroacetate (33).** The title compound was prepared from **33a** (110 mg, 0.11 mmol) in 10 mL of DCM/abs and 2 mL TFA according to the general procedure, yielding **33** as a colorless foamlike solid (30 mg, 26%). ^{1}H NMR (CD_3OD) δ (ppm): 7.01 (s, 1H, Thiaz-4-*H*), 3.37 (m, 4H, CH_2NH), 2.74 (m, 4H, Thiaz-5-CH_2), 2.46 (t, ^3J = 7.41 Hz, 4H, $COCH_2$), 2.18 (s, 3H, Thiaz-4-CH_3), 1.93 (m, 4H, Thiaz-5-CH_2CH_2), 1.65 (m, 4H, $COCH_2CH_2$), 1.35 (m, 8H, $(CH_2)_4$). ^{13}C NMR (CD_3OD) δ (ppm): 177.41 (quat **C**=O), 171.82 (quat Thiaz-2-**C**), 155.29 (quat **C**=NH), 126.36 (quat Thiaz-5-**C**), 123.37 (+, Thiaz-4-**C**), 118.44 (quat Thiaz-5-**C**), 41.47 (−, CH_2NH), 37.76 (−, $COCH_2$), 30.20 (−, CH_2), 29.97 (−, Thiaz-5-CH_2CH_2), 25.45 (−, $COCH_2CH_2$), 24.89 (−, Thiaz-5-CH_2), 11.45 (+, Thiaz-4-CH_3). HRLSIMS: m/z for ($[C_{25}H_{42}N_{10}O_2S_2 + H]^+$) calcd 579.3012, found 579.3006. Prep HPLC: MeCN/0.1% TFA/aq (10/90−35/65); $C_{25}H_{42}N_{10}O_2S_2$·4TFA (1034.88).

***N*^{1}-[[3-(Imidazol-4-yl)propylamino](amino)methylene]-*N*^{10}-[[3-(2-amino-4-methylthiazol-5-yl)propylamino](amino)-methylene]decanediamide Tetratrifluoroacetate (34).** The title compound was prepared from **34a** in 5 mL of DCM/abs and 1 mL of TFA according to the general procedure, yielding **34** as a colorless oil (70 mg, 24%). ^{1}H NMR (CD_3OD) δ (ppm): 8.82 (s, 1H, Im-2-*H*), 7.37 (s, 1H, Im-5-*H*), 3.38 (m, 4H, CH_2NH), 2.84 (t, ^3J = 7.7 Hz, 2H, Im-4-CH_2), 2.71 (t, ^3J = 7.41 Hz, 2H, Thiaz-5-CH_2), 2.47 (m, 4H, $COCH_2$), 2.18 (s, 3H, Thiaz-4-CH_3), 2.03 (m, 2H, Im-4-CH_2CH_2), 1.90 (m, 2H, Thiaz-5-CH_2CH_2), 1.66 (m, 4H, $COCH_2CH_2$), 1.35 (m, 8H, $(CH_2)_4$). ^{13}C NMR (CD_3OD, 400 MHz, HSQC, HMBC) δ (ppm): 177.37 (quat **C**=O), 155.64 (quat **C**=NH), 134.96 (quat Thiaz-**C**-4), 118.46 (quat Thiaz-**C**-5), 117.09 (+, Im-5-**CH**), 41.56 (−, CH_2NH), 37.76 (−, $COCH_2$), 30.13 (−, CH_2), 29.96 (−, Thiaz-5-CH_2-CH_2), 28.10 (−, Im-4-CH_2CH_2), 25.41 ($COCH_2CH_2$), 23.60 (−, Thiaz-5-CH_2), 22.54 (−, Im-4-CH_2), 11.41 (+, Thiaz-4-CH_3). HRLSIMS: m/z for ($[C_{25}H_{42}N_{10}O_2S + H]^+$) calcd 547.3291, found 547.3299. Prep HPLC: MeCN/0.1% TFA/aq (20/80−50/50). HPLC: k' = 1.87 (t_R = 9.51 min), purity = 94%; $C_{25}H_{42}N_{10}O_2S$·4TFA (1002.32).

***N*^{1},*N*^{10}-Bis{[3-(1*H*-1,2,4-triazol-5-yl)propylamino](amino)-methylene}decanediamide Tetratrifluoroacetate (35).** The title compound was prepared from **35a** (300 mg, 0.31 mmol) in 10 mL of DCM/abs and 2 mL of TFA according to the general procedure, yielding **35** (85 mg, 29%) as pale yellow oil. ^{1}H NMR (CD_3OD) δ (ppm): 8.54 (s, 2H, Triaz-3-*H*), 3.42 (t, ^3J = 7.2 Hz, 4H, CH_2NH), 2.94 (t, ^3J = 7.4 Hz, 4H, Triaz-5-CH_2), 2.46 (t, ^3J = 7.4 Hz, 4H, $COCH_2$), 2.11 (m, 4H, Triaz-5-CH_2CH_2), 1.66 (m, 4H, $COCH_2CH_2$), 1.36 (m, 4H, $(CH_2)_4$). ^{13}C NMR (CD_3OD) δ (ppm): 177.22 (quat **C**=O), 163.55 (quat Triaz-5-**C**), 155.41 (quat **C**=NH), 138.37 (+, Triaz-3-**C**), 41.62 (−, CH_2NH), 37.79 (−, $COCH_2$), 30.19 (−, CH_2), 29.96 (−, CH_2), 26.90 (−, Triaz-5-CH_2CH_2), 25.41 (−, $COCH_2CH_2$), 24.11 (−, Triaz-5-CH_2). HRLSIMS: m/z for ($[C_{22}H_{38}N_{12}O_2 + H]^+$) calcd 503.3319, found 503.3304. Prep HPLC: MeCN/0.1% TFA/aq (20/80−50/50). HPLC: k' = 1.77 (t_R = 7.41 min), purity = 100%; $C_{22}H_{38}N_{12}O_2$·4TFA (958.7).

Compounds **15**−**17**, **19**−**22**, **24**−**27**, **29**, and **30** were prepared by analogy (see Supporting Information).

Pharmacological Methods. Materials. Histamine dihydrochloride was purchased from Alfa Aesar GmbH & Co. KG (Karlsruhe, Germany). Amthamine[12] was from Tocris Bioscience (Avonmouth, Bristol, U.K.). Compound **4**[10] was synthesized in our laboratory, and cimetidine was from Sigma-Aldrich Chemie GmbH (Munich, Germany). [γ-^{32}P]GTP and [γ-^{33}P]GTP were synthesized according to a previously described method.[72] [^{32}P]P_i (8500−9100 Ci/mmol orthophosphoric acid) and [^{33}P]P_i (3000 Ci/mmol orthophosphoric acid) were purchased from Hartmann Analytic (Braunschweig, Germany). All unlabeled nucleotides, glycerol 3-phosphate dehydrogenase, triose phosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, and lactate dehydrogenase were from Roche (Mannheim, Germany). 3-Phosphoglycerate kinase and L-α-glycerol phosphate were from Sigma-Aldrich Chemie GmbH (Munich, Germany). Unlabeled GTPγS was from Roche (Mannheim, Germany), and [^{35}S]GTPγS was from Hartmann Analytics GmbH (Braunschweig, Germany). GF/B filters were from Brandel (Gaithersburg, MD)

Histamine H_2 Receptor Assay on Isolated Guinea Pig Right Atrium (Spontaneously Beating).[1] Guinea pigs of either sex (250−500 g) were sacrificed by a blow on the neck and exsanguinated. The heart was rapidly removed, and the right atrium was quickly dissected and set up isometrically in Krebs−Henseleit's solution under a diastolic resting force of approximately 5 mN in a jacketed 20 mL organ bath of 32.5 °C as previously described. The bath fluid (composition [mM]: NaCl 118.1, KCl 4.7, $CaCl_2$ 1.8, $MgSO_4$ 1.64, KH_2PO_4 1.2, $NaHCO_3$ 25.0, glucose 5.0, sodium pyruvate 2.0) was equilibrated with 95% O_2 and 5% CO_2 and additionally contained (*RS*)-propranolol (0.3 μM) to block β-adrenergic receptors. Stock solutions (10 mM) and all dilutions of bivalent ligands (1, 0.1, and 0.01 mM) were made in freshly prepared bath fluid instead of distilled water in order to prevent absorption at glass surfaces. Experiments were started after 30 min of continuous washing and an additional equilibration period of 15 min. Two successive curves for histamine displayed a significant desensitization of 0.13 ± 0.02 (N = 16 control

organs). This value was used to correct each individual experiment. For agonists, two successive concentration−frequency curves were established: the first for histamine (0.1−30 μM) and the second for the agonist under study in the absence or presence of cimetidine (10 μM, 30 min of incubation time). Furthermore, the sensitivity to 30, 100, or 300 μM cimetidine was routinely checked at the end of each H_2R agonist concentration−effect curve, and a significant reduction of frequency was observed. Relative potency of the agonist under study was calculated from the corrected pEC_{50} difference (ΔpEC_{50}). pEC_{50} values are given relative to the long-term mean value for histamine (pEC_{50} = 6.00) in our laboratory (pEC_{50} = 6.00 + ΔpEC_{50}).

Determination of Histamine Receptor Agonism and Antagonism in GTPase Assays. Generation of Recombinant Baculoviruses, Cell Culture, and Membrane Preparation. Recombinant baculoviruses encoding human H_1R or a fusion protein of the human H_2R with $Gs\alpha_S$ or a fusion protein of the guinea pig H_2R with $Gs\alpha_S$ or the human H_3R or a fusion protein of the human H_4R with RGS19 or fusion proteins of mutant H_2Rs with $Gs\alpha_S$ (hH_2R-C17Y-A271D-$Gs\alpha_S$ or hH_2R-C17Y-$Gs\alpha_S$ or hH_2R-gpE2-$Gs\alpha_S$ or gpH_2R-hE2-$Gs\alpha_S$ or hH_2R-gpNT-$Gs\alpha_S$) were prepared as described,[13,50−52] using the BaculoGOLD transfection kit (BD Pharmingen, San Diego, CA) according to the manufacturer's instructions. For construction of the cDNA for hH_2R-gpNT-$Gs\alpha_S$ see Supporting Information. Sf9 cells were cultured in 250 or 500 mL disposable Erlenmeyer flasks at 28 °C under rotation at 150 rpm in Insect-Xpress medium (Lonza, Velviers, Belgium) supplemented with 5% (v/v) fetal calf serum (Biochrom, Berlin, Germany) and 0.1 mg/mL gentamicin (Lonza, Walkersville, MD). Cells were maintained at a density of (0.5−6.0) × 10^6 cells/mL. After initial transfection, high-titer virus stocks were generated by two sequential virus amplifications. In the first amplification, cells were seeded at 2.0 × 10^6 cells/mL and infected with a 1:100 dilution of the supernatant from the initial transfection. Cells were cultured for 7 days, resulting in the lysis of the entire cell population. The supernatant was harvested and stored under light protection at 4 °C. In a second amplification, cells were seeded at a densitiy of 3.0 × 10^6 cells/mL and infected with a 1:20 dilution of the supernatant from the first amplification. Cells were cultured for 48 h, and the supernatant was harvested. After a 48 h culture period, the majority of cells showed signs of infections (e.g., altered morphology, viral inclusion bodies), whereas most of the cells were still intact. The supernatant from the second amplification was stored under light protection at 4 °C and used as routine virus stock for infections to obtain membrane preparations. For membrane preparation, cells were sedimented by centrifugation (1000 rpm, 5 min, rt) and suspended in fresh medium at a density of 3.0 × 10^6 cells/mL. Cells were infected with 1:100 dilutions of high-titer baculovirus stocks encoding the various histamine receptors, histamine receptor fusion proteins, G-protein subunits, and RGS proteins. Cells were cultured for 48 h before membrane preparation. Sf9 membranes were prepared as described,[73] using 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/mL benzamidine, and 10 μg/mL leupeptin as protease inhibitors. Membranes were suspended in binding buffer (12.5 mM $MgCl_2$, 1 mM EDTA, and 75 mM Tris-HCl, pH 7.4) and stored at −80 °C until use. Protein concentrations were determined using the DC protein assay kit (Bio-Rad, München, Germany).

Steady-State GTPase Activity Assay with Sf9 Insect Cell Membranes Expressing Histamine H_1, H_2, H_3, or H_4 Receptors or H_2R Mutants. Membranes were thawed, sedimented, and resuspended in 10 mM Tris-HCl, pH 7.4. In the cases of the H_1R and H_2R, Sf9 membranes expressing either H_1R isoforms plus RGS4 or H_2R-$Gs\alpha_S$ fusion proteins were used.[50,74] H_3R-stimulated GTP hydrolysis was determined with membranes coexpressing human H_3R, mammalian $G\alpha_{i2}$, $G\beta_1\gamma_2$, and RGS4. Human H_4R activity was measured with membranes coexpressing an H_4R-RGS19 fusion protein with $G\alpha_{i2}$ and $G\beta_1\gamma_2$. Activity on H_2R mutants was measured with hH_2R-C17Y-A271D-$Gs\alpha_S$, hH_2R-C17Y-$Gs\alpha_S$, hH_2R-gpE2-$Gs\alpha_S$, gpH_2R-hE2-$Gs\alpha_S$, and hH_2R-gpNT-$Gs\alpha_S$ fusion proteins.[51,52] Assay tubes contained Sf9 membranes (5−20 μg of protein/tube), $MgCl_2$ (H_1R, H_2R, 1.0 mM; H_3R, H_4R, 5.0 mM), 100 μM EDTA, 100 μM ATP, 100 nM GTP, 100 μM adenylyl imidodiphosphate, 5 mM creatine phosphate, 40 μg of creatine kinase, and 0.2% (w/v) bovine serum albumin in 50 mM Tris-HCl, pH 7.4, as well as ligands at various concentrations. In H_4R assays, NaCl (final concentration of 100 mM) was included. Reaction mixtures (80 μL) were incubated for 2 min at 25 °C before the addition of 20 μL of [γ-^{32}P]GTP (0.1 μCi/tube) or [γ-^{33}P]GTP (0.05 μCi/tube). Reactions were conducted for 20 min at 25 °C and terminated by the addition of 900 μL of slurry consisting of 5% (w/v) activated charcoal suspended in 50 mM NaH_2PO_4, pH 2.0. Charcoal absorbs nucleotides but not P_i. Charcoal-quenched reaction mixtures were centrifuged for 7 min at room temperature at 13000*g*. An amount of 600 μL of the supernatant fluid was removed, and $^{32}P_i$ or $^{33}P_i$ was determined by Cerenkov or liquid scintillation counting, respectively. Enzyme activities were corrected for spontaneous hydrolysis of [γ-^{32}P]GTP or [γ-^{33}P]GTP, determined in tubes containing all components described above, plus a high concentration of unlabeled GTP (1 mM) to prevent enzymatic hydrolysis of the labeled nucleotides in the presence of Sf9 membranes. Spontaneous [γ-^{32}P]GTP or [γ-^{33}P]GTP hydrolysis was <1% of the total amount of the labeled nucleotides. The experimental conditions chosen ensured that not more than 10% of the total amount of added [γ-^{32}P]GTP and [γ-^{33}P]GTP was converted to $^{32}P_i$ and $^{33}P_i$, respectively. All experimental data were analyzed by nonlinear regression with the Prism 5 program (GraphPad Software, San Diego, CA).

[^{35}S]GTPγS Binding Assay. [^{35}S]GTPγS binding assays[75,76] were performed as previously described for the H_2R[13,46] using Sf9 insect cell membranes expressing the gpH_2R-$Gs\alpha_S$ fusion protein. The respective membranes were thawed and sedimented by a 10 min centrifugation at 4 °C and 13000*g*. Membranes were resuspended in binding buffer (12.5 mM $MgCl_2$, 1 mM EDTA, and 75 mM Tris-HCl, pH 7.4). Each assay tube contained Sf9 membranes (15−30 μg of protein/tube), 1 μM GDP, 0.05% (w/v) bovine serum albumin, 0.2 nM [^{35}S]GTPγS, and the investigated ligands at various concentrations in binding buffer (total volume 250 μL). Incubations were conducted for 90 min at 25 °C, and shaking was at 250 rpm. Bound [^{35}S]GTPγS was separated from free [^{35}S]GTPγS by filtration through GF/B filters, followed by three washes with 2 mL of binding buffer (4 °C) using a Brandel harvester. Filter-bound radioactivity was determined after an equilibration phase of at least 12 h by liquid scintillation counting. The experimental conditions chosen ensured that no more than 10% of the total amount of [^{35}S]GTPγS added was bound to filters. Nonspecific binding was determined in the presence of 10 μM unlabeled GTPγS.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthetic procedures and analytical data for compounds **15a**−**17a**, **19a**−**22a**, **24a**−**27a**, **29a**, **30a**, **15**−**17**, **19**−**22**, **24**−**27**, **29**, and **30a**, purity data and retention times (HPLC data) of all target compounds, and construction of the cDNA for hH_2R-gpNT-$Gs\alpha_S$. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +49-941 9434827. Fax: +49-941 9434820. E-mail: armin.buschauer@chemie.uni-regensburg.de.

■ ACKNOWLEDGMENTS

The authors are grateful to Maria Beer-Krön, Christine Braun, and Kerstin Röhrl for expert technical assistance. This work was supported by the Graduate Training Program (Graduiertenkolleg) GRK 760, "Medicinal Chemistry: Molecular Recognition, Ligand−Receptor Interactions", of the Deutsche Forschungsgemeinschaft. The contribution of Tobias Birnkammer to this

project has been awarded by the European Histamine Research Society ("Young Investigators Award" 2011).

DEDICATION

†This paper is dedicated to Prof. Dr. Dr. Dr. h.c. Walter Schunack, in memoriam.

ABBREVIATIONS USED

abs, absolute; aq, aqueous; Boc, *tert*-butoxycarbonyl; Cbz, benzyloxycarbonyl; CDI, 1,1′-carbonyldiimidazole; DCM, dichloromethane; DIEA, *N*,*N*-diisopropylethylamine; DMF, dimethylformamide; DMSO, dimethylsulfoxide; e2, second extracellular; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; EI-MS, electron-impact ionization mass spectrometry; E_{max}, efficacy; ES-MS, electrospray ionization mass spectrometry; GAIP, Gα interacting protein (corresponds to RGS19 protein); GPCR, G-protein-coupled receptor; G$\beta_1\gamma_2$, G protein β_1- and γ_2-subunit; Gα_i, α-subunit of the G_i protein that mediates inhibition of adenylyl cyclase; Gsα_s, α-subunit (short splice variant) of the Gs protein that mediates stimulation of adenylyl cyclase; H_1R, histamine H_1 receptor; H_2R, histamine H_2 receptor; H_3R, histamine H_3 receptor; H_4R, histamine H_4 receptor; hH_1R, human histamine H_1 receptor; hH_2R, human histamine H_2 receptor; H_2R-gpNT, N-terminus of the guinea pig H_2 receptor; GTP, guanosine 5′-triphosphate; hH_2R-Gsα_S, fusion protein of the human histamine H_2 receptor and the short splice variant of Gsα; hH_3R, human histamine H_3 receptor; hH_4R, human histamine H_4 receptor; hH_4R-RGS19, fusion protein of the human histamine H_4 receptor and RGS19; HOBt, hydroxybenzotriazole; HPLC, high performance liquid chromatography; HR-MS, high resolution mass spectrometry; *k*′, capacity factor; LSI-MS, liquid-secondary-ion mass spectrometry; rel pot, potency relative to histamine; RGS, regulator of G protein signaling proteins; RP-HPLC, reverse phase HPLC; rt, room temperature; SEM, standard error of the mean; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TM, transmembrane domain of a GPCR; t_R, retention time; Trt, trityl

REFERENCES

(1) Black, J. W.; Duncan, W. A. M.; Durant, C. J.; Ganellin, C. R.; Parsons, E. M. Definition and antagonism of histamine H_2-receptors. *Nature* **1972**, *236*, 385−390.

(2) Hill, S. J.; Ganellin, C. R.; Timmerman, H.; Schwartz, J. C.; Shankley, N. P.; Young, J. M.; Schunack, W.; Levi, R.; Haas, H. L. International Union of Pharmacology. XIII. Classification of histamine receptors. *Pharmacol. Rev.* **1997**, *49*, 253−278.

(3) Durant, G. J.; Ganellin, C. R.; Parsons, M. E. Dimaprit, [*S*-[3-(*N*,*N*-dimethylamino)propyl]isothiourea]. A highly specific histamine H_2-receptor agonist. Part 2. Structure−activity considerations. *Inflammation Res.* **1977**, *7*, 39−43.

(4) Durant, G. J.; Ganellin, C. R.; Hills, D. W.; Miles, P. D.; Parsons, M. E.; Pepper, E. S.; White, G. R. The histamine H2 receptor agonist impromidine: synthesis and structure−activity considerations. *J. Med. Chem.* **1985**, *28*, 1414−1422.

(5) Buschauer, A. Synthesis and in vitro pharmacology of arpromidine and related phenyl(pyridylalkyl)guanidines, a potential new class of positive inotropic drugs. *J. Med. Chem.* **1989**, *32*, 1963−1970.

(6) Buschauer, A.; Friese-Kimmel, A.; Baumann, G.; Schunack, W. Synthesis and histamine H_2 agonistic activity of arpromidine analogues: replacement of the pheniramine-like moiety by non-heterocyclic groups. *Eur. J. Med. Chem.* **1992**, *27*, 321−330.

(7) Lim, H. D.; van Rijn, R. M.; Ling, P.; Bakker, R. A.; Thurmond, R. L.; Leurs, R. Evaluation of histamine H_1-, H_2-, and H_3-receptor ligands at the human histamine H_4 receptor: identification of 4-methylhistamine as the first potent and selective H_4 receptor agonist. *J. Pharmacol. Exp. Ther.* **2005**, *314*, 1310−1321.

(8) Igel, P.; Schneider, E.; Schnell, D.; Elz, S.; Seifert, R.; Buschauer, A. N(G)-acylated imidazolylpropylguanidines as potent histamine H4 receptor agonists: selectivity by variation of the N(G)-substituent. *J. Med. Chem.* **2009**, *52*, 2623−2627.

(9) Igel, P.; Dove, S.; Buschauer, A. Histamine H4 receptor agonists. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 7191−7199.

(10) Kraus, A.; Ghorai, P.; Birnkammer, T.; Schnell, D.; Elz, S.; Seifert, R.; Dove, S.; Bernhardt, G.; Buschauer, A. N^G-Acylated aminothiazolylpropylguanidines as potent and selective histamine H_2 receptor agonists. *ChemMedChem* **2009**, *4*, 232−240.

(11) Ghorai, P.; Kraus, A.; Keller, M.; Gotte, C.; Igel, P.; Schneider, E.; Schnell, D.; Bernhardt, G.; Dove, S.; Zabel, M.; Elz, S.; Seifert, R.; Buschauer, A. Acylguanidines as bioisosteres of guanidines: NG-acylated imidazolylpropylguanidines, a new class of histamine H2 receptor agonists. *J. Med. Chem.* **2008**, *51*, 7193−7204.

(12) Eriks, J. C.; Van der Goot, H.; Sterk, G. J.; Timmerman, H. Histamine H_2-receptor agonists. Synthesis, in vitro pharmacology, and qualitative structure−activity relationships of substituted 4- and 5-(2-aminoethyl)thiazoles. *J. Med. Chem.* **1992**, *35*, 3239−3246.

(13) Houston, C.; Wenzel-Seifert, K.; Bürckstümmer, T.; Seifert, R. The human histamine H2-receptor couples more efficiently to Sf9 insect cell Gs-proteins than to insect cell Gq-proteins: limitations of Sf9 cells for the analysis of receptor/Gq-protein coupling. *J. Neurochem.* **2002**, *80*, 678−696.

(14) George, S. R.; O'Dowd, B. F.; Lee, S. P. G-Protein-coupled receptor oligomerization and its potential for drug discovery. *Nat. Rev. Drug Discovery* **2002**, *1*, 808−820.

(15) Nikbin, N.; Edwards, C.; Reynolds, C. A. G-Protein coupled receptor dimerization. *Iran. J. Pharmacol. Ther.* **2003**, *2*, 1−11.

(16) Terrillon, S.; Bouvier, M. Roles of G-protein-coupled receptor dimerization. *EMBO Rep.* **2004**, *5*, 30−34.

(17) Smith, N. J.; Milligan, G. Allostery at G protein-coupled receptor homo- and heteromers: uncharted pharmacological landscapes. *Pharmacol. Rev.* **2010**, *62*, 701−725.

(18) McVey, M.; Ramsay, D.; Kellett, E.; Rees, S.; Wilson, S.; Pope, A. J.; Milligan, G. Monitoring receptor oligomerization using time-resolved fluorescence resonance energy transfer and bioluminescence resonance energy transfer. *J. Biol. Chem.* **2001**, *276*, 14092−14099.

(19) Cvejic, S.; Devi, L. A. Dimerization of the delta opioid receptor: implication for a role in receptor internalization. *J. Biol. Chem.* **1997**, *272*, 26959−26964.

(20) Jordan, B. A.; Devi, L. A. G-Protein-coupled receptor heterodimerization modulates receptor function. *Nature* **1999**, *399*, 697−700.

(21) Angers, S.; Salahpour, A.; Joly, E.; Hilairet, S.; Chelsky, D.; Dennis, M.; Bouvier, M. Detection of β_2-adrenergic receptor dimerization in living cells using bioluminescence resonance energy transfer (BRET). *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 3684−3689.

(22) Grant, M.; Kumar, U. The role of G-proteins in the dimerisation of human somatostatin receptor types 2 and 5. *Regul. Pept.* **2010**, *159*, 3−8.

(23) Duran-Prado, M.; Malagon, M. M.; Gracia-Navarro, F.; Castano, J. P. Dimerization of G protein-coupled receptors: new avenues for somatostatin receptor signalling, control and functioning. *Mol. Cell. Endocrinol.* **2008**, *286*, 63−68.

(24) Scarselli, M.; Novi, F.; Schallmach, E.; Lin, R.; Baragli, A.; Colzi, A.; Griffon, N.; Corsini, G. U.; Sokoloff, P.; Levenson, R.; Vogel, Z.; Maggio, R. D2/D3 dopamine receptor heterodimers exhibit unique functional properties. *J. Biol. Chem.* **2001**, *276*, 30308−30314.

(25) Lee, S. P.; So, C. H.; Rashid, A. J.; Varghese, G.; Cheng, R.; Lanca, A. J.; O'Dowd, B. F.; George, S. R. Dopamine D1 and D2 receptor co-activation generates a novel phospholipase C-mediated calcium signal. *J. Biol. Chem.* **2004**, *279*, 35671−35678.

(26) Lukasiewicz, S.; Polit, A.; Kedracka-Krok, S.; Wedzony, K.; Mackowiak, M.; Dziedzicka-Wasylewska, M. Hetero-dimerization of

serotonin 5-HT(2A) and dopamine D(2) receptors. *Biochim. Biophys. Acta* **2010**, *1803*, 1347−1358.
(27) Zeng, F.; Wess, J. Molecular aspects of muscarinic receptor dimerization. *Neuropsychopharmacology* **2000**, *23*, S19−S31.
(28) Hern, J. A.; Baig, A. H.; Mashanov, G. I.; Birdsall, B.; Corrie, J. E.; Lazareno, S.; Molloy, J. E.; Birdsall, N. J. Formation and dissociation of M1 muscarinic receptor dimers seen by total internal reflection fluorescence imaging of single molecules. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 2693−2698.
(29) Bakker, R. A.; Dees, G.; Carrillo, J. J.; Booth, R. G.; Lopez-Gimenez, J. F.; Milligan, G.; Strange, P. G.; Leurs, R. Domain swapping in the human histamine H_1 receptor. *J. Pharmacol. Exp. Ther.* **2004**, *311*, 131−138.
(30) van Rijn, R. M.; Chazot, P. L.; Shenton, F. C.; Sansuk, K.; Bakker, R. A.; Leurs, R. Oligomerization of recombinant and endogenously expressed human histamine H_4 receptors. *Mol. Pharmacol.* **2006**, *70*, 604−615.
(31) Carrillo, J. J.; Pediani, J.; Milligan, G. Dimers of class A G protein-coupled receptors function via agonist-mediated trans-activation of associated G proteins. *J. Biol. Chem.* **2003**, *278*, 42578−42587.
(32) Fukushima, Y.; Asano, T.; Takata, K.; Funaki, M.; Ogihara, T.; Anai, M.; Tsukuda, K.; Saitoh, T.; Katagiri, H.; Aihara, M.; Matsuhashi, N.; Oka, Y.; Yazaki, Y.; Sugano, K. Role of the C terminus in histamine H_2 receptor signaling, desensitization, and agonist-induced internalization. *J. Biol. Chem.* **1997**, *272*, 19464−19470.
(33) Nguyen, T.; Shapiro, D. A.; George, S. R.; Setola, V.; Lee, D. K.; Cheng, R.; Rauser, L.; Lee, S. P.; Lynch, K. R.; Roth, B. L.; O'Dowd, B. F. Discovery of a novel member of the histamine receptor family. *Mol. Pharmacol.* **2001**, *59*, 427−433.
(34) Shenton, F. C.; Hann, V.; Chazot, P. L. Evidence for native and cloned H_3 histamine receptor higher oligomers. *Inflammation Res.* **2005**, *54*, S48−S49.
(35) Portoghese, P. S. From models to molecules: opioid receptor dimers, bivalent ligands, and selective opioid receptor probes. *J. Med. Chem.* **2001**, *44*, 2259−2269.
(36) Portoghese, P. S. Bivalent ligands and the message−address concept in the design of selective opioid receptor antagonists. *Trends Pharmacol. Sci.* **1989**, *10*, 230−235.
(37) Shonberg, J.; Scammells, P. J.; Capuano, B. Design strategies for bivalent ligands targeting GPCRs. *ChemMedChem* **2011**, *6*, 963−974.
(38) Erez, M.; Takemori, A. E.; Portoghese, P. S. Narcotic antagonistic potency of bivalent ligands which contain β-naltrexamine. Evidence for simultaneous occupation of proximal recognition sites. *J. Med. Chem.* **1982**, *25*, 847−849.
(39) Decker, M.; Lehmann, J. Agonistic and antagonistic bivalent ligands for serotonin and dopamine receptors including their transporters. *Curr. Top. Med. Chem.* **2007**, *7*, 347−353.
(40) Halazy, S.; Perez, M.; Fourrier, C.; Pallard, I.; Pauwels, P. J.; Palmier, C.; John, G. W.; Valentin, J. P.; Bonnafous, R.; Martinez, J. Serotonin dimers: application of the bivalent ligand approach to the design of new potent and selective 5-HT(1B/1D) agonists. *J. Med. Chem.* **1996**, *39*, 4920−4927.
(41) Kühhorn, J.; Hübner, H.; Gmeiner, P. Bivalent dopamine D2 receptor ligands: synthesis and binding properties. *J. Med. Chem.* **2011**, *54*, 4896−4903.
(42) Perez, M.; Jorand-Lebrun, C.; Pauwels, P. J.; Pallard, I.; Halazy, S. Dimers of 5HT1 ligands preferentially bind to 5HT1B/1D receptor subtypes. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1407−1412.
(43) Halazy, S. G-Protein coupled receptors bivalent ligands and drug design. *Expert Opin. Ther. Pat.* **1999**, *9*, 431−446.
(44) Kraus, A. Highly Potent, Selective Acylguanidine-Type Histamine H2 Receptor Agonists: Synthesis and Structure−Activity Relationships. Doctoral Thesis. University of Regensburg, Regensburg, Germany, 2007; http://epub.uni-regensburg.de/10699/.
(45) Igel, P. Synthesis and Structure−Activity Relationships of N(G)-Acylated Arylalkylguanidines and Related Compounds as Histamine Receptor Ligands: Searching for Selective H_4R Agonists. Doctoral Thesis, University of Regensburg, Regensburg, Germany, 2008; http://epub.uni-regensburg.de/12093/.
(46) Wenzel-Seifert, K.; Kelley, M. T.; Buschauer, A.; Seifert, R. Similar apparent constitutive activity of human histamine H_2-receptor fused to long and short splice variants of $G_{sα}$. *J. Pharmacol. Exp. Ther.* **2001**, *299*, 1013−1020.
(47) Seifert, R.; Wenzel-Seifert, K.; Kobilka, B. K. GPCR-Galpha fusion proteins: molecular analysis of receptor-G-protein coupling. *Trends Pharmacol. Sci.* **1999**, *20*, 383−389.
(48) Schneider, E. H.; Seifert, R. Sf9 cells: a versatile model system to investigate the pharmacological properties of G protein-coupled receptors. *Pharmacol. Ther.* **2010**, *128*, 387−418.
(49) Wenzel-Seifert, K.; Seifert, R. Molecular analysis of beta(2)-adrenoceptor coupling to G(s)-, G(i)-, and G(q)-proteins. *Mol. Pharmacol.* **2000**, *58*, 954−966.
(50) Kelley, M. T.; Bürckstümmer, T.; Wenzel-Seifert, K.; Dove, S.; Buschauer, A.; Seifert, R. Distinct interaction of human and guinea pig histamine H_2-receptor with guanidine-type agonists. *Mol. Pharmacol.* **2001**, *60*, 1210−1225.
(51) Preuss, H.; Ghorai, P.; Kraus, A.; Dove, S.; Buschauer, A.; Seifert, R. Mutations of Cys-17 and Ala-271 in the human histamine H_2 receptor determine the species selectivity of guanidine-type agonists and increase constitutive activity. *J. Pharmacol. Exp. Ther.* **2007**, *321*, 975−982.
(52) Preuss, H.; Ghorai, P.; Kraus, A.; Dove, S.; Buschauer, A.; Seifert, R. Point mutations in the second extracellular loop of the histamine H_2 receptor do not affect the species-selective activity of guanidine-type agonists. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **2007**, *376*, 253−264.
(53) Neubig, R. R.; Spedding, M.; Kenakin, T.; Christopoulos, A. International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification. XXXVIII. Update on terms and symbols in quantitative pharmacology. *Pharmacol. Rev.* **2003**, *55*, 597−606.
(54) Cheng, Y.; Prusoff, W. H. Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem. Pharmacol.* **1973**, *22*, 3099−3108.
(55) Xie, S.-X.; Kraus, A.; Ghorai, P.; Ye, Q.-Z.; Elz, S.; Buschauer, A.; Seifert, R. N^1-(3-Cyclohexylbutanoyl)-N^2-[3-(1*H*-imidazol-4-yl)-propyl]guanidine (UR-AK57), a potent partial agonist for the human histamine H_1- and H_2-receptors. *J. Pharmacol. Exp. Ther.* **2006**, *317*, 1262−1268.
(56) Klinker, J. F.; Hagelüken, A.; Grünbaum, L.; Heilmann, I.; Nürnberg, B.; Harhammer, R.; Offermanns, S.; Schwaner, I.; Ervens, J.; Wenzel-Seifert, K.; Müller, T.; Seifert, R. Mastoparan may activate GTP hydrolysis by Gi-proteins in HL-60 membranes indirectly through interaction with nucleoside diphosphate kinase. *Biochem. J.* **1994**, *304* (Part2), 377−383.
(57) Hagelüken, A. G., L.; Klinker, J. F.; Nürnberg, B.; Harhammer, R.; Schultz, G.; Leschke, C.; Schunack, W.; Seifert, R. Histamine receptor-dependent and/or -independent activation of guanine nucleotide-binding by histamine and 2-substituted histamine derivatives in human leukemia (HL-60) and human erythroleukemia (HEL) cells. *Biochem. Pharmacol.* **1995**, *49*, 901−914.
(58) Seifert, R.; Hagelüken, A.; Hoer, A.; Hoer, D.; Grünbaum, L.; Offermanns, S.; Schwaner, I.; Zingel, V.; Schunack, W.; Schultz, G. The H1 receptor agonist 2-(3-chlorophenyl)histamine activates Gi proteins in HL-60 cells through a mechanism that is independent of known histamine receptor subtypes. *Mol. Pharmacol.* **1994**, *45*, 578−586.
(59) Preuss, H.; Ghorai, P.; Kraus, A.; Dove, S.; Buschauer, A.; Seifert, R. Constitutive activity and ligand selectivity of human, guinea pig, rat, and canine histamine H_2 receptors. *J. Pharmacol. Exp. Ther.* **2007**, *321*, 983−995.
(60) Nederkoorn, P. H. J.; Lenthe, J. H.; Goot, H.; Donné-Op den Kelder, G. M.; Timmerman, H. The agonistic binding site at the histamine H_2 receptor. I. Theoretical investigations of histamine

binding to an oligopeptide mimicking a part of the fifth transmembrane α-helix. *J. Comput.-Aided Mol. Des.* **1996**, *10*, 461−478.
(61) Messer, W. S. Jr. Bivalent ligands for G protein-coupled receptors. *Curr. Pharm. Des.* **2004**, *10*, 2015−2020.
(62) Perez, M.; Pauwels, P. J.; Fourrier, C.; Chopin, P.; Valentin, J. P.; John, G. W.; Marien, M.; Halazy, S. Dimerization of sumatriptan as an efficient way to design a potent, centrally and orally active 5-HT1B agonist. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 675−680.
(63) Shi, L.; Javitch, J. A. The binding site of aminergic G protein-coupled receptors: the transmembrane segments and second extracellular loop. *Annu. Rev. Pharmacol. Toxicol.* **2002**, *42*, 437−467.
(64) Kim, J.; Jiang, Q.; Glashofer, M.; Yehle, S.; Wess, J.; Jacobson, K. A. Glutamate residues in the second extracellular loop of the human A2a adenosine receptor are required for ligand recognition. *Mol. Pharmacol.* **1996**, *49*, 683−691.
(65) Scarselli, M.; Li, B.; Kim, S.-K.; Wess, J. Multiple residues in the second extracellular loop are critical for m_3 muscarinic acetylcholine receptor activation. *J. Biol. Chem.* **2007**, *282*, 7385−7396.
(66) Rasmussen, S. G. F.; Choi, H. J.; Fung, J. J.; Pardon, E.; Casarosa, P.; Chae, P. S.; DeVree, B. T.; Rosenbaum, D. M.; Thian, F. S.; Kobilka, T. S.; Schnapp, A.; Konetzki, I.; Sunahara, R. K.; Gellman, S. H.; Pautsch, A.; Steyaert, J.; Weis, W. I.; Kobilka, B. K. Structure of a nanobody-stabilized active state of the beta(2) adrenoceptor. *Nature* **2011**, *469*, 175−180.
(67) Rasmussen, S. G. F.; Choi, H.-J.; Rosenbaum, D. M.; Kobilka, T. S.; Thian, F. S.; Edwards, P. C.; Burghammer, M.; Ratnala, V. R. P.; Sanishvili, R.; Fischetti, R. F.; Schertler, G. F. X.; Weis, W. I.; Kobilka, B. K. Crystal structure of the human β2 adrenergic G-protein-coupled receptor. *Nature* **2007**, *450*, 383−387.
(68) Warne, T.; Moukhametzianov, R.; Baker, J. G.; Nehme, R.; Edwards, P. C.; Leslie, A. G. W.; Schertler, G. F. X.; Tate, C. G. The structural basis for agonist and partial agonist action on a beta(1)-adrenergic receptor. *Nature* **2011**, *469*, 241−244.
(69) Ghorai, P.; Kraus, A.; Birnkammer, T.; Geyer, R.; Bernhardt, G.; Dove, S.; Seifert, R.; Elz, S.; Buschauer, A. Chiral NG-acylated hetarylpropylguanidine-type histamine H2 receptor agonists do not show significant stereoselectivity. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 3173−3176.
(70) Igel, P.; Schnell, D.; Bernhardt, G.; Seifert, R.; Buschauer, A. Tritium-labeled *N*(1)-[3-(1*H*-imidazol-4-yl)propyl]-*N*(2)-propionylguanidine ([(3)*H*]UR-PI294), a high-affinity histamine H(3) and H(4) receptor radioligand. *ChemMedChem* **2009**, *4*, 225−231.
(71) Iso, Y.; Shindo, H.; Hamana, H. Efficient synthesis of resin-bound a-TMSdiazoketones and their use in solid-phase organic synthesis. *Tetrahedron* **2000**, *56*, 5353−5361.
(72) Walseth, T. F.; Johnson, R. A. The enzymatic preparation of [alpha-(32)P]nucleoside triphosphates, cyclic [32P] AMP, and cyclic [32P] GMP. *Biochim. Biophys. Acta* **1979**, *562*, 11−31.
(73) Seifert, R. L., T. W.; Lam, V. T.; Kobilka, B. K. *Eur. J. Biochem.* **1998**, *255*, 369−382.
(74) Xie, S.-X.; Ghorai, P.; Ye, Q.-Z.; Buschauer, A.; Seifert, R. Probing ligand-specific histamine H_1- and H_2-receptor conformations with N^G-acylated imidazolylpropylguanidines. *J. Pharmacol. Exp. Ther.* **2006**, *317*, 139−146.
(75) Asano, T.; Pedersen, S. E.; Scott, C. W.; Ross, E. M. Reconstitution of catecholamine-stimulated binding of guanosine 5′-*O*-(3-thiotriphosphate) to the stimulatory GTP-binding protein of adenylate cyclase. *Biochemistry* **1984**, *23*, 5460−5467.
(76) Hilf, G.; Gierschik, P.; Jakobs, K. H. Muscarinic acetylcholine receptor-stimulated binding of guanosine 5′-*O*-(3-thiotriphosphate) to guanine-nucleotide-binding proteins in cardiac membranes. *Eur. J. Biochem.* **1989**, *186*, 725−731.