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Dimeric carbamoylguanidine-type histamine H_2 receptor ligands: A new class of potent and selective agonists

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ABSTRACT

The bioisosteric replacement of the acylguanidine moieties in dimeric histamine H_2 receptor (H_2R) agonists by carbamoylguanidine groups resulted in compounds with retained potencies and intrinsic activities, but considerably improved stability against hydrolytic cleavage. These compounds achieved up to 2500 times the potency of histamine when studied in [^{35}S]GTPγS assays on recombinant human and guinea pig H_2R. Unlike 3-(imidazol-4-yl)propyl substituted carbamoylguanidines, the corresponding 2-amino-4-methylthiazoles revealed selectivity over histamine receptor subtypes H_1R, H_3R and H_4R in radioligand competition binding studies. H_2R binding studies with three fluorescent compounds and one tritium-labeled ligand, synthesized from a chain-branched precursor, failed due to pronounced cellular accumulation and high non-specific binding. However, the dimeric H_2R agonists proved to be useful pharmacological tools for functional studies on native cells, as demonstrated for selected compounds by cAMP accumulation and inhibition of fMLP-stimulated generation of reactive oxygen species in human monocytes.

1. Introduction

Previously, N^G-acylated hetarylpropylguanidines were identified as potent histamine H_2 receptor (H_2R) agonists.[1–3] Surprisingly, an enormous increase in H_2R potency was achieved by linking two acylguanidine moieties. The most potent of those dimeric compounds showed up to 5000-fold potency of histamine (depending on the type of assay and the respective H_2R ortholog).[3] The first generation of N^G-acylated imidazolylpropylguanidines, showed a lack of selectivity, especially towards histamine H_3 receptor (H_3R) and histamine H_4 receptor (H_4R).[4] Selectivity for the H_2R was achieved by bioisosteric replacement of the imidazole ring by an amino(methyl)thiazole moiety.[2,3] Portoghese suggested that the distance between the orthosteric binding sites of two dimerising receptor protomers is about 22–27 Å.[5] In an approach to explore whether the bivalent H_2R agonists bind to a receptor monomer or a receptor dimer, Birnkammer et al. investigated compounds with a spacer length between 6 and 27 Å.[3] The highest potency resided in compounds with an octa- or hexamethylene chain.[3] Due to insufficient spacer length for interaction with the orthosteric binding pockets of dimeric H_2R protomers, the gain in potency seems to result from the interaction with an additional binding site at the same receptor molecule. However, so far the binding mode of bivalent H_2R agonists is not understood and appropriate pharmacological tools to study the mode and stoichiometry of binding are not available.

Acylguanidines turned out to undergo hydrolytic cleavage upon long-term storage in aqueous solution. Aiming at more stable dimeric H_2R agonists, we replaced the acylguanidine moieties by carbamoylguanidine groups according to a bioisosteric approach (Fig. 1). Moreover, a branched linker enabled the synthesis of radiolabeled and fluorescent derivatives. The compounds were characterized in functional and binding studies on recombinant histamine receptors. In addition, selected agonists were investigated on human monocytes.

2. Results and discussion

2.1. Chemistry

2.1.1. Synthesis of the dimeric carbamoylguanidine-type ligands

The building blocks 3-(1-trityl-1*H*-imidazol-4-yl)propan-1-amine (**1**) and *tert*-butyl [5-(3-aminopropyl)-4-methylthiazol-2-yl]carbamate (**2**) (Scheme 1) were synthesized as recently

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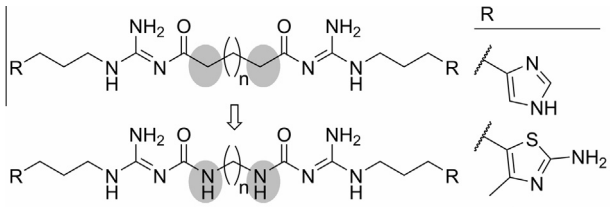


Figure 1. Bioisosteric replacement of the acylguanidine moieties in dimeric H_2R agonists by carbamoylguanidine groups.

reported.[1,2] The guanidinylating reagents (**3**–**7**) were obtained from diamines and diisocyanates in a one-pot reaction[6] (Scheme 1). The diamines were treated with triphosgene to give the corresponding diisocyanates, which were allowed to react with mono-Boc-protected *S*-methylisothiourea, a well-established guanidinylating reagent,[7] which was prepared as described previously.[2]

The dimeric ligands **8**–**17** were prepared by treating the building blocks **1**,**2** with the guanidinylating reagents **3**–**7** in the presence of $HgCl_2$ and base.[8] Treating the protected carbamoylguanidine-type intermediates with TFA gave compounds **8**–**17** (Scheme 1), which were purified by preparative HPLC.

2.1.2. Toward bivalent fluorescence- and radioligands

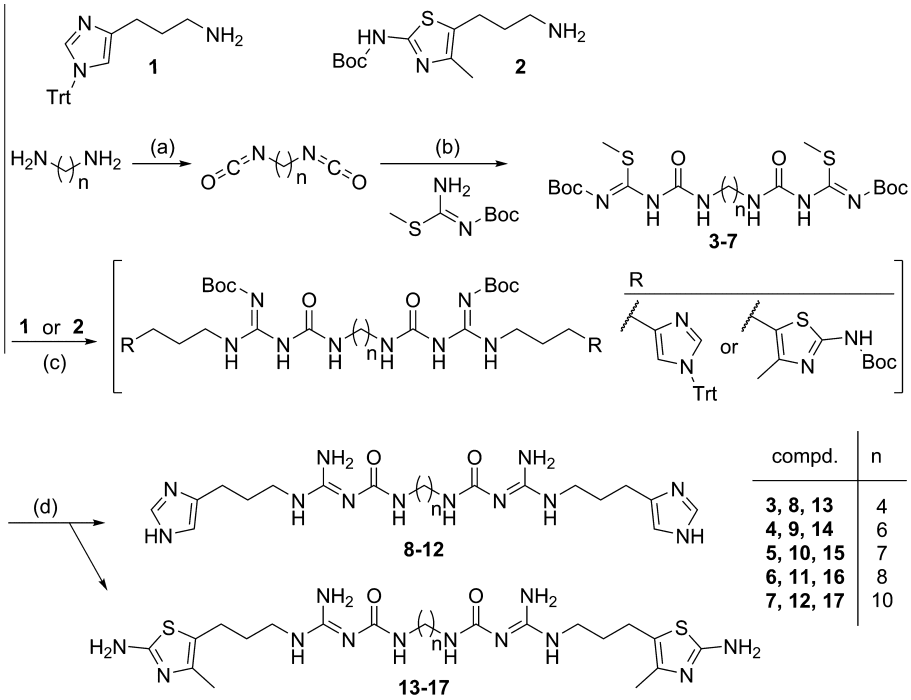
In view of radiolabeling and introduction of fluorophores, a branched linker comprising a primary amino group was synthesized. Starting from commercially available dicyclopropylketone, 1,7-dichloroheptan-4-one was obtained as recently described.[9] After nucleophilic displacement of chlorine by azides, the C=C bond was formed by a Wittig reaction[10] to yield 1-azido-4-(3-azidopropyl)-8-chlorooct-4-ene (**21**) (Scheme 2). Via the corresponding phthalimide (**22**), **21** was converted into the primary amine **23**. The amino group was Boc-protected (**24**) and the azide groups were reduced with $LiAlH_4$ to give the diamine **25**, which was converted to the diisocyanate **26**. The guanidinylating reagent **27** was prepared by analogy with the procedure for the preparation of **3**–**7**.

The precursor (**28**; Scheme 3) of the labeled compounds was synthesized from **27** according to the procedure described above for compounds **13**–**17** using **2** as building block. Compound **28** was coupled to three different fluorophores: the cyanine S2197 and the small pyrylium dyes Py-1 and Py-5 (Scheme 3).[11–13] S2197 was provided as succinimidyl ester and coupled to the bivalent precursor in the presence of triethylamine to give **29**. The pyrylium dyes reacted instantaneously in a ring transformation (color changes from blue into red) to give the positively charged N-substituted pyridinium compounds **30** and **31** (Scheme 3).[14]

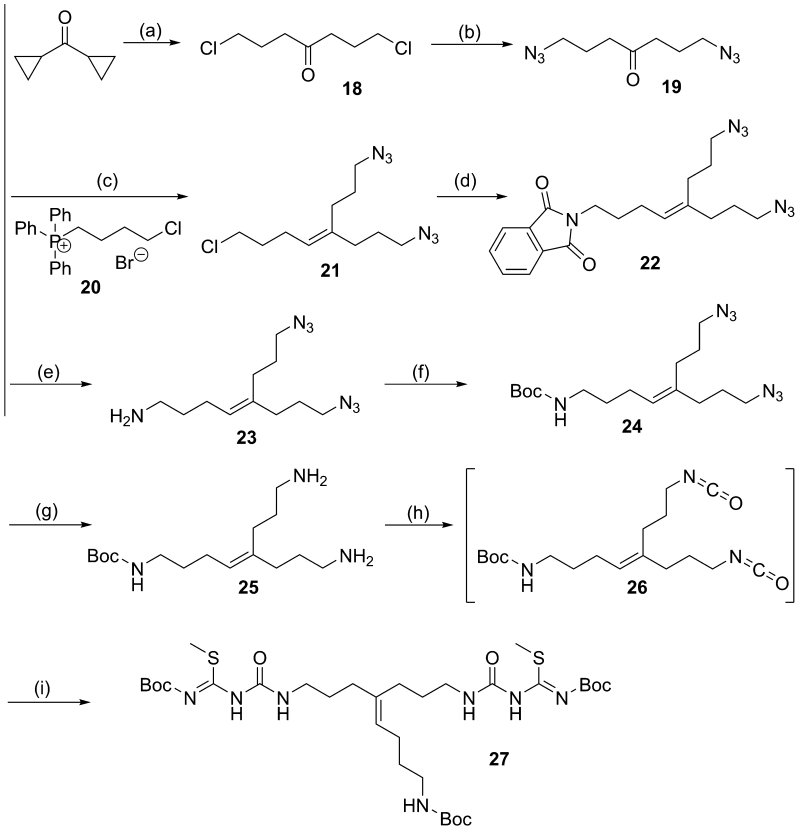
Compound **28** was acylated with 'cold' succinimidyl propionate (cf. **32a**) to optimize the reaction conditions for the propionylation with tritiated succinimidyl propionate (Scheme 4). This synthetic route afforded the radioligand **32b** in a yield of 90%. Purification by HPLC gave **32b** in a radiochemical purity of 99% with a specific activity of 70 Ci/mmol.

2.2. Stability of carbamoylguanidines versus acylguanidines

Acylguanidines are stable at acidic pH, but tend to decompose under alkaline conditions. The decomposition becomes extremely fast when an intramolecular nucleophilic attack is possible as previously demonstrated for aminoalkanoylguanidines with half-lives between 19 s and 13 h at pH 10.4.[15] Although the decomposition of acylguanidine-type H_2R agonists such as **33**[3] (Fig. 2A) turned out to occur at a much slower rate, hydrolytic cleavage has to be taken into account upon long-time storage in solution. For comparison, the stability of compounds **14** (Fig. 2B) and **33** as representative examples of carbamoylated and acylated guanidines, respectively, was investigated at a concentration of 100 μM in phosphate buffer (PBS, pH 7.4) at room temperature. After one week around 55% of compound **33** were decomposed.



Scheme 1. Synthesis of the bivalent carbamoylguanidine-type ligands **8**–**17**. Reagents and conditions: (a) triphosgene, DIPEA, DCM abs., 30 min, 0 °C; (b) *N*-*tert*-butoxycarbonyl-*S*-methylisothiourea, 2.5 h, rt, **3**: 83%, **4**: 74%, **5**: 75%, **6**: 74%, **7**: 76%; (c) **1** or **2**, $HgCl_2$, NEt_3, DMF or DCM abs., 12 h, rt; (d) 30% TFA, DCM abs., 12 h, rt, **8**: 7%, **9**: 12%, **10**: 8%, **11**: 20%, **12**: 27%, **13**: 14%, **14**: 30%, **15**: 29%, **16**: 9%, **17**: 9%.



Scheme 2. Synthesis of the branched building block **27**. Reagents and conditions: (a) HCl gas, rt, 100%; (b) NaN_3, DMF, 12 h, rt, 78%; (c) *n*-BuLi, THF abs, 15 h, −72 °C → rt, 19%; (d) phthalimide, Cs_2CO_3, KI (cat.), DMF, 60 °C, 12 h, 57%; (e) N_2H_2 x H_2O, EtOH, 1 h, reflux, 88%; (f) Boc_2O, NEt_3, DCM, rt, 24 h, 97%; (g) $LiAlH_4$, Et_2O/abs, 2 h, reflux, 66%; (h) triphosgene, DIPEA, DCM abs., 30 min, 0 °C; (i) *N*-*tert*-butoxycarbonyl-*S*-methylisothiourea, 2.5 h, rt, 44%.

2.3. Pharmacological results

The synthesized compounds were investigated on membrane preparations in the [^{35}S]GTPγS binding assay at the human (h) and at the guinea pig (gp) H_2R with regard to possible differences between species orthologs (Table 1, Fig. 3). The hH_2R as well as the gpH_2R were expressed as H_2R-$G_s\alpha_s$ fusion proteins in *Sf*9 insect cells. To test the selectivity of the compounds for the H_2R compared to hH_1R, hH_3R, and hH_4R, competition binding experiments were performed using membranes of *Sf*9 cells expressing the histamine receptor of interest (Table 2).

2.3.1. H_2R agonism in the [^{35}S]GTPγS binding assay

The prepared bivalent ligands **8**–**17**, **28** and **32a** were partial or full agonists at both the hH_2R and the gpH_2R. In agreement with previous studies on guanidine- and acylguanidine-type H_2R agonists,[2,3,16,17] higher potencies and intrinsic activities were achieved at the gpH_2R than at the hH_2R. Compound **11** was 2500 times more potent than histamine at the gpH_2R. Except for **14**, ligands bearing an imidazole ring were superior to the corresponding aminothiazoles at the hH_2R. Dimeric ligands with a spacer length of four methylene groups showed the lowest potency at the hH_2R, whereas the gpH_2R was less sensitive to variations in chain length. Interestingly, branching of the connecting chain hardly affected the potency (cf. **28**), though the maximal response decreased. Propionylation of the primary amino group in the side chain of **28** resulted in regaining potency and intrinsic activity at the hH_2R (**32a**) (Table 1, Fig. 3).

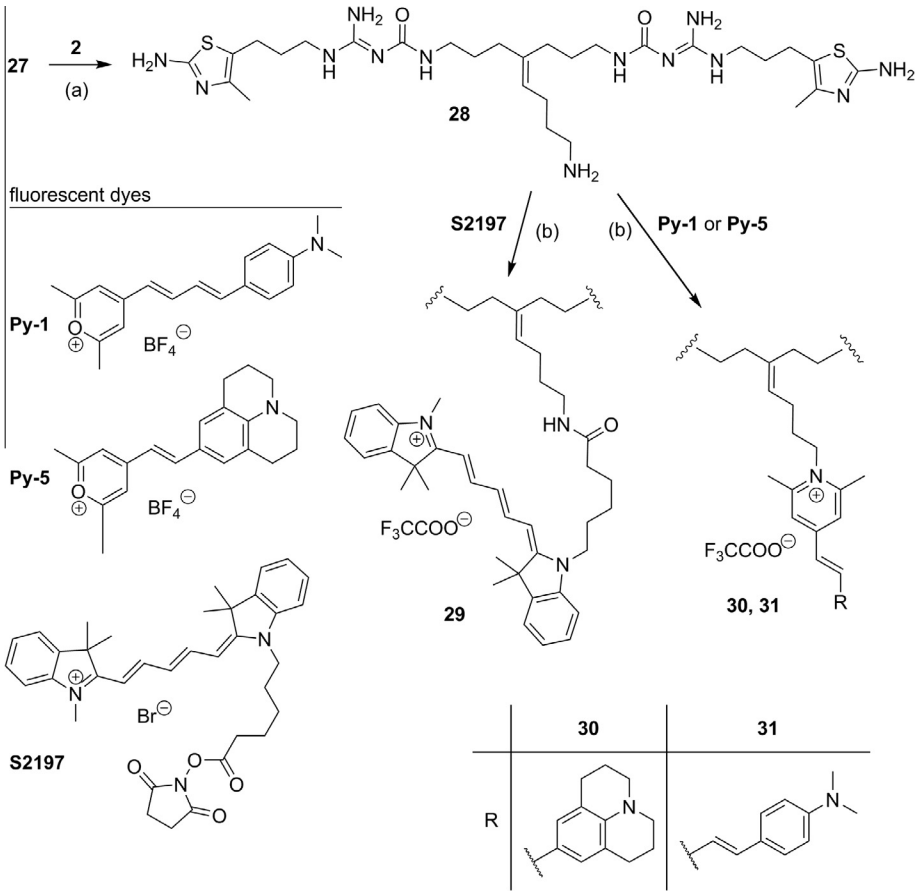
2.3.2. H_2R affinities and receptor subtype selectivities

The H_2R affinity of the compounds was determined in competition binding studies. The pK_i values were in good agreement with the pEC_{50} values, in particular when [^{3}H]UR-DE257[19] was used as radioligand. Minor differences between functional and binding data became obvious when [^{3}H]tiotidine was used for displacement. As suggested by Kelley et al.[17] [^{3}H]tiotidine is able to address only a subpopulation of the H_2R which might explain this phenomenon. As expected, carbamoylguanidine-type ligands bearing two imidazole moieties were not selective for the H_2R, but also showed high affinity for the H_3R and the H_4R. By contrast, the synthesized aminothiazoles preferred the H_2R (Table 2).

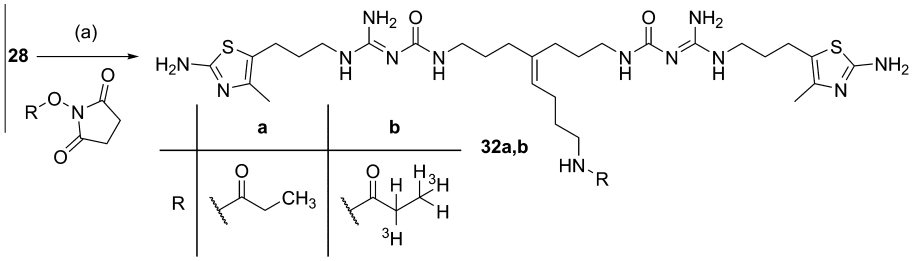
2.3.3. Investigation of the fluorescence ligands 29–31 and the radioligand 32b

The compounds **29**–**31** were investigated in a flow cytometric binding assay[25] at HEK293T cells transfected with the hH_2R (cf. Supplementary data, Fig. S1). None of these compounds bound in a saturable manner. Confocal microscopy revealed that the fluorescence ligands enter the cells in a receptor-independent manner (cf. Supplementary data, Fig. S2).

Similarly, it was impossible to perform saturation binding studies with the radioligand **32b** on *Sf*9 membranes (cf. Supplementary data, Fig. S3) and HEK293T cells because of high unspecific binding. Interestingly, lower unspecific binding was observed in the presence of competitors structurally similar to **32a**, hinting at reversible binding to non-H_2R sites at the cell membrane. Nevertheless, in case of HEK293T cells non-H_2R mediated internalization of **32b** cannot be excluded. Radioligand **32b** proved to be inappropriate for H_2R quantification. In spite of that, affinities determined for various H_2R ligands in competition binding experiments with **32b** (membranes of *Sf*9 cells expressing the hH_2R), were in the range of K_i values determined by displacement of [^{3}H]UR-DE257[19] (cf. Supplementary data,



Scheme 3. Synthesis of compound **28** and preparation of the fluorescence ligands **29**–**31**. Reagents and conditions: (a) (1) compd **2**, $HgCl_2$, NEt_3, DMF, 12 h, rt, (2) 30% TFA, DCM abs., 12 h, rt, 21%; (b) NEt_3, DMF, rt, 2 h, **29**: 45%, **30**: 36%, **31**: 38%.



Scheme 4. Synthesis of the unlabeled ('cold') propionamide **32a** and the corresponding radiolabeled version **32b**. Reagents and conditions: (a) (1) NEt_3, DMF, 2 h, 81%; (2) DIPEA, DMF, 3 h, 90%.

Table S1). However, it should be noted that, due to the low signal-to-noise ratio, the data determined with **32b** as radioligand are less reliable.

2.3.4. Investigation of H_2R agonists in human monocytes

Acute myeloid leukemia (AML) is an indication for post-consolidation therapy with histamine to prevent relapse.[26,27] It is assumed that histamine inhibits oxygen radical formation via H_2R mediated inhibition of NADPH oxidase, resulting in the protection of T cells and NK cells.[26]

Since histamine activates all four histamine receptor subtypes, the treatment is accompanied by serious side effects like itch, erythema, decrease in blood pressure and gastrointestinal problems.[26,27] Patients could benefit from selective H_2R stimulation due to reduction of side effects mediated by the other histamine receptor subtypes.

Compounds **9** and **14** were studied at the H_2R in human monocytes regarding functional responses such as stimulation of cAMP formation and inhibition of formyl peptide (fMLP)-induced production of reactive oxygen species (ROS) (Fig. 4, Table 3). To avoid cell lysis as observed for such cationic amphiphilic compounds depending on the chain length of the linker (for cytotoxicity of dimeric H_2R agonists cf. Supplementary data, Fig. S5), we selected compounds **9** and **14**, which are among the most potent H_2R agonists and proved to be nontoxic (cf. Supplementary data, Fig. S6) under the assay conditions.

Recently, we have shown that H_2R agonists increase cAMP levels in a concentration-dependent manner in human monocytes.[28] In accordance with these results, compounds **9** and **14** induced cAMP accumulation (Fig. 4A). Additionally, both ligands inhibited the fMLP-induced ROS production in a concentration-dependent manner via H_2R stimulation (Fig 4B).

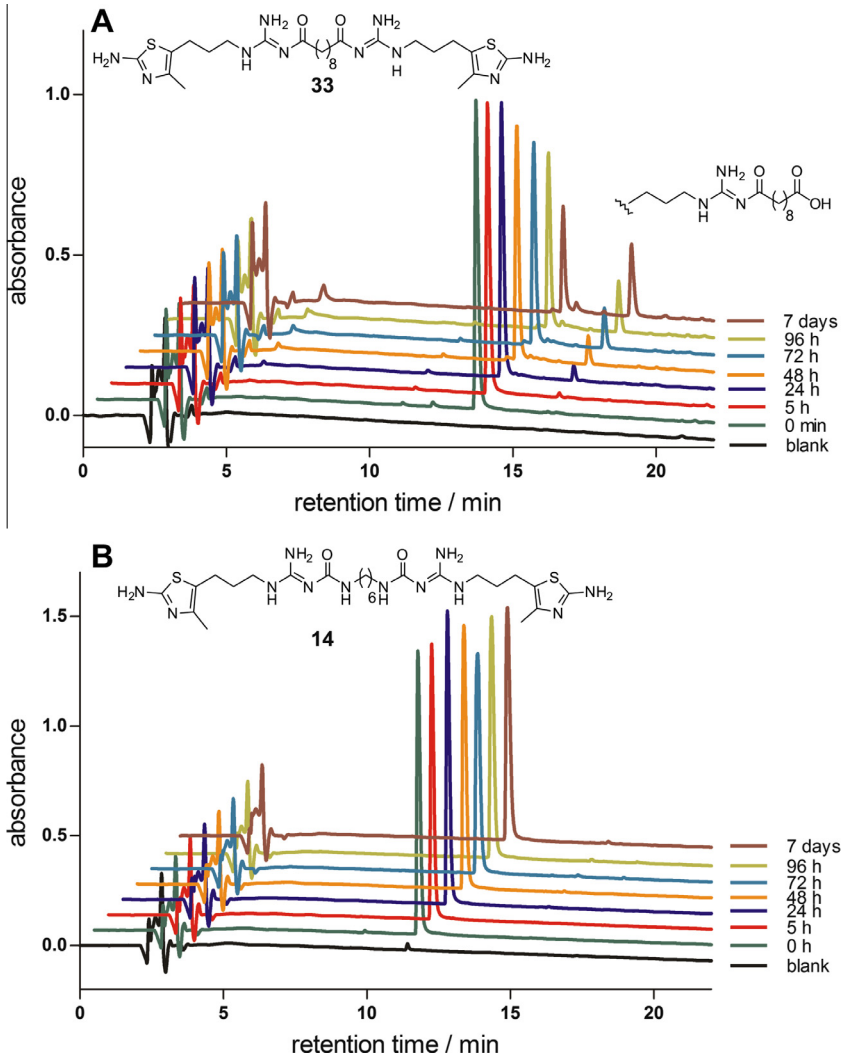


Figure 2. Chromatograms of the H_2R agonists **33** (**A**) and **14** (**B**) after different periods of incubation in PBS (pH 7.4) at rt.

Table 1
Histamine H_2 receptor agonism in the [^{35}S]GTPγS assay[a]

Compd	Human H_2R			Guinea pig H_2R		
	pEC_{50}	E_{max}	Pot_{rel}	pEC_{50}	E_{max}	Pot_{rel}
Histamine	5.85 ± 0.06	1.00	1.0	6.07 ± 0.14[18,b]	1.00	1.0
8	7.29 ± 0.08	0.97 ± 0.02	27.5	8.55 ± 0.04	0.99 ± 0.06	309.5
9	7.77 ± 0.02	0.88 ± 0.03	83.2	9.25 ± 0.14	0.96 ± 0.03	1519.9
10	8.31 ± 0.08	1.03 ± 0.06	288.4	8.45 ± 0.03	0.87 ± 0.08	239.7
11	8.35 ± 0.07	1.01 ± 0.07	316.0	9.47 ± 0.09	1.01 ± 0.06	2512.2
12	8.08 ± 0.09	0.70 ± 0.08	169.8	8.19 ± 0.07	0.95 ± 0.04	131.7
13	7.25 ± 0.11	0.96 ± 0.04	25.1	9.04 ± 0.13	1.04 ± 0.14	935.3
14	8.03 ± 0.02	0.92 ± 0.01	151.4	8.75 ± 0.15	1.09 ± 0.05	478.7
15	7.68 ± 0.03	0.90 ± 0.10	67.6	8.42 ± 0.19	0.97 ± 0.01	224.0
16	7.73 ± 0.15	0.80 ± 0.07	75.9	8.16 ± 0.07	0.80 ± 0.06	123.0
17	7.43 ± 0.12	0.64 ± 0.07	38.0	n.d.	n.d.	n.d.
28	7.49 ± 0.10	0.66 ± 0.06	43.7	8.11 ± 0.06	0.97 ± 0.06	109.7
32a	7.87 ± 0.12	0.73 ± 0.12	104.7	8.04 ± 0.05	0.89 ± 0.11	93.3

[a] [^{35}S]GTPγS binding assay on membranes of *Sf*9 cells expressing the hH_2R-$G_s\alpha_s$ and the gpH_2R-$G_s\alpha_s$. Data were analyzed by nonlinear regression and were best fit to three-parameter sigmoidal concentration-response curves. pEC_{50}: $-\log EC_{50}$; E_{max}: maximal response relative to histamine (E_{max} = 1.0); Pot_{rel}: relative potency represents the ratio of EC_{50} values of test compound and histamine. Data shown are means ± SEM of 2-6 independent experiments, each performed in triplicate.

[b] Determined in a steady-state [^{32}P]GTPase assay on *Sf*9 cells expressing the gpH_2R-$G_s\alpha_s$.

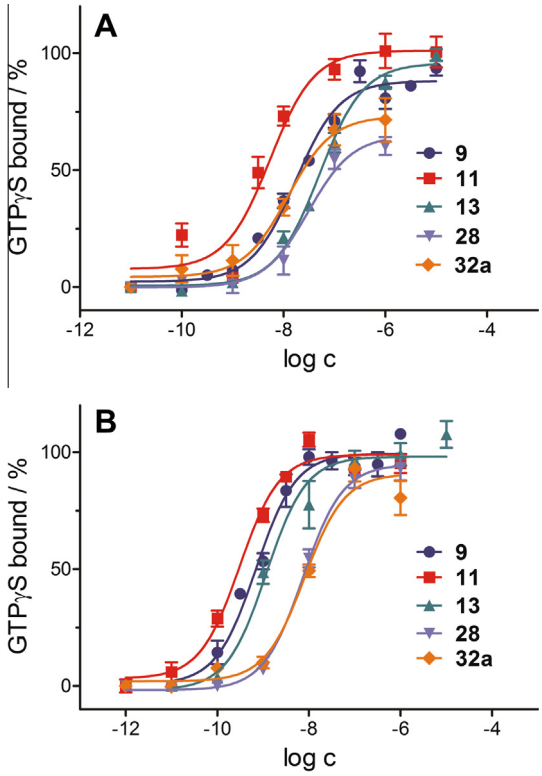


Figure 3. Concentration-response curves of selected agonists at hH_2R (**A**) and gpH_2R (**B**) in the [^{35}S]GTPγS assay. The increase in GTPγS binding is referred to the maximal response to histamine (HIS) = 100%. Data are means ± SEM of 2-6 independent experiments, each performed in triplicate.

3. Summary and conclusion

The exchange of the acylguanidine moieties in bivalent H_2R agonists by carbamoylguanidine groups resulted in compounds with retained potencies and intrinsic activities. Achieving up to 2500 times the potency of histamine these compounds are among the most potent H_2R agonists known so far. Unfortunately, H_2R binding studies with fluorescence and radiolabeled ligands derived from the chain-branched precursor **28** failed due to pronounced cellular accumulation and high non-specific binding, respectively. In this respect, the results underline the fact that, regardless of high affinity of the 'cold' form of a receptor ligand, applicability of the corresponding labeled version as a molecular tool cannot be taken for granted. Aminothiazoles were superior to the corresponding imidazoles in terms of histamine receptor subtype selectivity. Due to high potency and stability against hydrolytic cleavage, the new H_2R agonists are promising pharmacological tools for investigations on the biological role of the H_2R and are of potential value for in vivo studies on histamine receptors in the CNS. In human monocytes the investigated compounds revealed high H_2R agonist potency, suggesting this class of compounds to point a way to selective H_2R agonists as potential agents for the treatment of AML.

4. Experimental section

4.1. General experimental section

Unless otherwise indicated, chemicals and solvents were from commercial suppliers and were used as received. The fluorescence dye S2197 was from FEW Chemicals GmbH (Bitterfeld–Wolfen, Germany), the pyrylium dyes Py-1 and Py-5 were kindly provided by Prof. Dr. O. S. Wolfbeis (Institute of Analytical Chemistry, Chemo- and Biosensors, University of Regensburg, Germany). All of the solvents were of analytical grade or were distilled prior to use. THF and Et_2O were distilled over sodium, DCM was stored over $CaCl_2$ and was afterwards distilled from P_2O_5. For column chromatography silica gel 60 (Merck, 0.04–0.063 mm) was used. Reactions were monitored by thin layer chromatography (TLC) on Merck silica gel 60 F254 aluminium sheets, and spots were visualized with UV light at 254 nm, iodine vapor or ninhydrin spray. NMR spectra

Table 2
Binding data of the compounds **8**–**17**, **28**, **32a** on human histamine receptor subtypes[a]

Compd	hH_1R pK_i ± SEM[b]	hH_2R pK_i ± SEM[c,d]	hH_3R pK_i ± SEM[e,f]	hH_4R pK_i ± SEM[g]
Histamine	5.62 ± 0.03[20]	6.27 ± 0.04[c,15]	8.20 ± 0.04[21]	7.89 ± 0.01[22]
8	6.31 ± 0.10	7.57 ± 0.01[c]	8.56 ± 0.02[f]	7.02 ± 0.06
9	5.87 ± 0.04	7.87 ± 0.05[c]	8.16 ± 0.05[e]; 8.52[f]	7.12 ± 0.04
10	6.60 ± 0.12	7.52 ± 0.04[d]	8.77 ± 0.02[f]	7.81 ± 0.09
11	6.66 ± 0.03	8.47[c]; 8.52 ± 0.13[d]	8.82 ± 0.03[f]	8.27 ± 0.01
12	6.55 ± 0.02	7.56 ± 0.05[d]	8.21 ± 0.03[f]	7.58 ± 0.12
13	6.08 ± 0.03	7.76 ± 0.08[c]	6.17 ± 0.10[e]; 6.17[f]	5.29 ± 0.04
14	6.06 ± 0.05	8.07 ± 0.05[c]	5.94 ± 0.16[e]; 6.01[f]	5.69 ± 0.07
15	5.81 ± 0.09	7.55 ± 0.02[c]	5.76 ± 0.09[f]	5.55 ± 0.08
16	5.80 ± 0.06	7.17 ± 0.13[d]	6.00 ± 0.10[f]	6.26 ± 0.18
17	5.89 ± 0.01	7.21 ± 0.10[d]	5.81 ± 0.07[e]	5.97 ± 0.05
28	5.80 ± 0.08	7.59 ± 0.01[c]	6.56 ± 0.11[f]	6.08 ± 0.09
32a	6.36 ± 0.00	7.94 ± 0.09[c]	6.08 ± 0.08[f]	5.96 ± 0.09

[a] Competition binding assay on membranes of *Sf9* cells expressing the hH_1R plus RGS4, the hH_2R-$G_s\alpha_s$, co-expressing the hH_3R plus $G\alpha_{i2}$ plus $G\beta_1\gamma_2$ or co-expressing the hH_4R plus $G\alpha_{i2}$ plus $G\beta_1\gamma_2$. Incubation period was 60 min. Data were analyzed by nonlinear regression and were best fit to 3-parameter sigmoidal concentration–response curves. Data shown are means ± SEM of 2–6 independent experiments, each performed in triplicate.

[b] Displacement of 5 nM [^{3}H]mepyramine (K_d = 4.5 nM).

[c] Displacement of 30 nM [^{3}H]UR-DE257 (K_d = 31 nM).

[d] Displacement of 10 nM tiotidine (K_d = 19.7 nM).

[e] Displacement of 15 nM [^{3}H]histamine (K_d = 12.6 nM).

[f] Displacement of 3 nM [^{3}H]N^α-methylhistamine (K_d = 3 nM).

[g] Displacement of 10 nM histamine (K_d = 10 nM); Note: Representative compounds were investigated for functional activity at the hH_1R, hH_3R and hH_4R. In agreement with previous studies[2,3,23] on bivalent H_2R ligands, the carbamoylated imidazolylpropylguanidines behaved as agonists in the [^{35}S]GTPγS assay (Sf9 membranes) at the H_3R and the H_4R respectively, whereas the corresponding amino(methyl)thiazolylpropylguanidines proved to be H_3R and H_4R antagonists and were antagonists at the hH_1R (luciferase assay, HEK293 cells[24]). hH_1R, pK_b ± SEM: **10**: 6.48 ± 0.14; **11**: 6.29 ± 0.21; **14**: 5.71 ± 0.06; **16**: 5.69 ± 0.13; **32a**: 5.32 ± 0.13. hH_3R, pK_b ± SEM: **14**: 5.57 ± 0.05; **16**: 5.86 ± 0.01; **32a**: 5.87 ± 0.06. hH_4R: **10**: pEC_{50} = 8.00 ± 0.04, E_{max} = 0.65 ± 0.03; **11**: pEC_{50} = 8.04 ± 0.09, E_{max} = 0.86 ± 0.08; **14**: pK_b = 5.30 ± 0.02; **16**: pK_b = 6.12 ± 0.11; **32a**: pK_b = 5.09 ± 0.22.

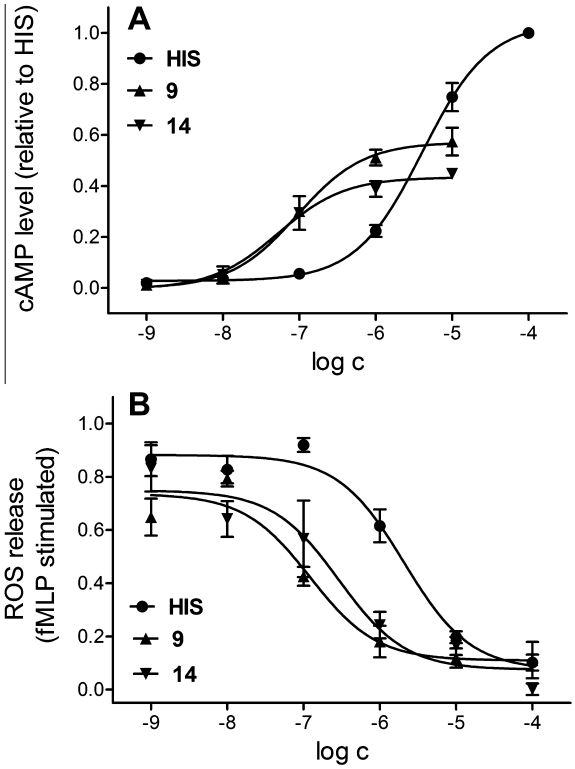


Figure 4. Effects of histamine (HIS), **9** and **14** on cAMP accumulation (**A**) and fMLP-induced ROS production (**B**) in human monocytes. Samples were analyzed by HPLC–MS/MS. A: Data were normalized to the maximal effect of HIS and represent means ± SEM from three independent experiments. B: Data were normalized to the ROS release induced by fMLP and are expressed as means ± SEM from four different experiments performed in triplicate. Note: The influence of H_2R agonists on ROS production had to be studied in an indirect way, that is, after stimulation of the cells with fMLP (1 μM). In accordance with previous studies,[28] we observed that the maximal effect of fMLP (set to 1.0 in Fig. 4B) was not fully restored in the presence of very low concentrations of histamine or H_2R agonists. So far, we have no explanation for this phenomenon, which only minimally affects the inflections points of the concentration-response curves. In Table 3 the maximal responses (E_{max}) of **9** and **14** are given relative to the amplitude of the concentration-response curve of histamine defined as E_{max} = 1.0.

Table 3
Potencies and efficacies in the cAMP assay and in the reactive oxygen species (ROS) assay in human monocytes[a]

Compd	cAMP assay		ROS assay	
	pEC_{50}	E_{max}	pIC_{50}	E_{max}
Histamine	5.39 ± 0.06	1.00	5.68 ± 0.13	1.00
9	7.03 ± 0.11	0.57 ± 0.03	6.91 ± 0.19	0.78 ± 0.07
14	7.27 ± 0.19	0.44 ± 0.04	6.54 ± 0.27	0.83 ± 0.10

[a] Data were analyzed by nonlinear regression and were best fit to 3-parameter sigmoidal concentration-response curves. The maximal responses (E_{max} values) of **9** and **14** are referred to the efficacy of histamine defined as E_{max} = 1.00. Data shown are means ± SEM of three to four independent experiments.

were recorded on a Bruker Avance 300 (^{1}H: 300 MHz, ^{13}C: 75.5 MHz) and a Bruker Avance 600 (^{1}H: 600 MHz, ^{13}C: 150.9 MHz) (Bruker, Karlsruhe, Germany) with deuterated solvents from Deutero (Kastellaun, Germany). LRMS was performed on a Finnigan ThermoQuest TSQ 7000 Instrument (Thermo Scientific, Waltham, MA) using an ESI source. HRMS was performed on an Agilent 6540 UHD Accurate-Mass Q-TOF LC/MS system (Agilent Technologies, Santa Clara, CA) using an ESI or an APCI source. Melting points (mp) were measured on a Büchi B-540 apparatus using an open capillary and are uncorrected. Preparative HPLC was performed with a system from Knauer (Berlin, Germany) consisting of two K-1800 pumps, a K-2001 detector and the column was either a Eurospher-100 C18 (250 × 32 mm, 5 μm), a Nucleodur 100-5 C18 (250 × 21 mm, 5 μm) (Macherey-Nagel, Düren, Germany) or in case of the fluorescence ligands a Phenomenex Kinetex (250 × 21 mm, 5 μm) (Phenomenex, Aschaffenburg, Germany). As mobile phase mixtures of acetonitrile and 0.1% aqueous TFA were used. The temperature was 30 °C and UV detection was carried out at 220 nm. Prior to lyophilisation (Christ alpha 2–4 LD freeze dryer (Osterode, Germany), equipped with a RZ 6 rotary vane vacuum pump (Vacuubrand, Wertheim, Germany), acetonitrile was removed under reduced pressure. Analytical HPLC was performed on a system from Thermo Separation Products equipped with an SN400 controller, a P4000 pump, an AS3000 autosampler, and a Spectra Focus UV/Vis detector. The column was either a Eurospher-100 C18 (250 × 4 mm, 5.0 μm; Knauer, Berlin, Germany) or a YMC C8 Column (250 × 4.6 mm, 5.0 μm); YMC Europe GmbH, Dinslaken, Germany), thermostated at 30 °C. As mobile phase, mixtures of MeCN/aqueous TFA were used. Gradient mode (unless otherwise indicated): MeCN/TFA (0.05%) (v/v) 0 min: 5:95, 30 min: 40:60, 31–41 min: 90:10; flow rate = 0.75 mL/min, t_0 = 2.95 min (Eurospher), t_0 = 2.55 min (YMC); capacity factor $k = (t_R - t_0)/t_0$. Absorbance was detected at 210 nm. Compound purities were calculated as the percentage peak area of the analyzed compound by UV detection at 210 nm. UV Spectra were recorded with a Cary Eclipse spectrofluorimeter (Varian, Mulgrave, Victoria, Australia).

4.2. Chemistry: Protocols and analytical data

4.2.1. N^1,N^4-Bis{[(*tert*-butoxycarbonylamino)(methylsulfanyl) methylene]aminocarbonyl}butane-1,4-diamine (3)

To a solution of *N*-*tert*-butoxycarbonyl-*S*-methylisothiourea (855 mg, 4.50 mmol) and NEt_3 (30 μL, 0.22 mmol) in DCM was added dropwise a solution of 1,4-diisocyanatobutane (300 mg, 2.14 mmol) in DCM. The solution was stirred for 2.5 h at room temperature. The solvent was removed under reduced pressure and the crude product was purified by column chromatography (eluent: DCM/EA 15:1) (R_f = 0.19 in Hex/EA 3:2). The product was obtained as a white solid (928 mg, 83%). mp = 144.9–146.1 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 1.47 (s, 18H), 1.55–1.65 (m, 4H), 2.29 (s, 6H), 3.22–3.26 (m, 4H), 5.62 (t, 2H, *J* 6.0 Hz), 12.27 (s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 14.4, 27.3, 28.1, 39.8, 82.7, 151.2, 162.1, 167.6. HRMS (ESI) *m*/*z* (%) 521 (100) $[M+H]^+$; *m*/*z* $[M+H]^+$ calculated for $C_{20}H_{37}N_6O_6S_2^+$: 521.2211, found 521.2214; $C_{20}H_{36}N_6O_6S_2$ (520.67).

4.2.2. N^1,N^6-Bis{[(*tert*-butoxycarbonylamino)(methylsulfanyl) methylene]aminocarbonyl}hexane-1,6-diamine (4)

To a solution of *N*-*tert*-butoxycarbonyl-*S*-methylisothiourea (700 mg, 3.68 mmol) and NEt_3 (30 μL, 0.22 mmol) in DCM was added dropwise a solution of 1,6-diisocyanatohexane (300 mg, 2.02 mmol) in DCM. The solution was stirred for 2.5 h at room temperature. The solvent was removed under reduced pressure and the crude product was purified by column chromatography (eluent: DCM/EA 15:1) (R_f = 0.29 in Hex/EA 3:2). The product was obtained as a white solid (820 mg, 74%). mp = 128.8–133.6 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 1.34–1.39 (m, 4H), 1.46 (s, 18H), 1.50–1.56 (m, 4H), 2.29 (s, 6H), 3.17–3.24 (m, 4H), 5.55 (t, 2H, *J* 5.8 Hz), 12.30 (s, 2H); ^{13}C NMR (75 MHz, $[D_4]$MeOH) δ (ppm) 14.4, 26.7, 28.1, 29.7, 40.1, 82.7, 151.2, 162.1, 167.4. HRMS (ESI) *m*/*z* (%) 549 (100) $[M+H]^+$; *m*/*z* $[M+H]^+$ calculated for $C_{22}H_{41}N_6O_6S_2^+$: 549.2524, found 549.2525; $C_{22}H_{40}N_6O_6S_2$ (548.72).

4.2.3. General procedure for the synthesis of the guanidinylating reagents (5–7)

The respective diamine was dried overnight in vacuo and the reactions were carried out in an argon purged two necked round bottom flask. The diamines (1 equiv) were dissolved in freshly distilled DCM (8 mL) and DIPEA (5.6 equiv) was added. This mixture was added dropwise under external cooling to a solution of triphosgene (1 equiv) in anhydrous DCM over a period of 30 min. *N*-*tert*-Butoxycarbonyl-*S*-methylisothiourea (4 equiv) was added and stirring was continued for 2.5 h. The solvent was removed under reduced pressure and the crude product was purified by column chromatography (eluent: DCM/EA 15:1).

4.2.3.1. N^1,N^7-Bis{[(tert-butoxycarbonylamino)(methylsulfanyl) methylene]aminocarbonyl}heptane-1,7-diamine (5). The title compound was prepared from heptane-1,7-diamine (262 mg, 2.01 mmol), DIPEA (1963 μL, 11.27 mmol), triphosgene (596 mg, 2.01 mmol) in anhydrous DCM and *N*-*tert*-Butoxycarbonyl-*S*-methylisothiourea (1530 mg, 8.08 mmol) according to the general procedure (R_f = 0.29 in Hex/EA 3:2). The product was obtained as colorless glassy solid (855 mg, 75%). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 1.33 (m, 6H), 1.46 (s, 18H), 1.49 (overlap with *tert*-Bu, 4H), 2.29 (s, 6H), 3.16–3.23 (m, 4H), 5.55 (t, 2H, *J* 5.0 Hz), 12.31 (s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 14.4, 26.9, 28.1, 29.1, 29.7, 40.2, 82.6, 151.2, 162.0, 167.3. HRMS (ESI) *m*/*z* (%) 563 (100) $[M+H]^+$; *m*/*z* $[M+H]^+$ calculated for $C_{23}H_{43}N_6O_6S_2^+$: 563.2680, found 563.2690; $C_{23}H_{42}N_6O_6S_2$ (562.75).

4.2.3.2. N^1,N^8-Bis{[(tert-butoxycarbonylamino)(methylsulfanyl) methylene]aminocarbonyl}octane-1,8-diamine (6). The title compound was prepared from octane-1,8-diamine (400 mg, 2.77 mmol), DIPEA (2701 μL, 15.50 mmol), triphosgene (823 mg, 2.77 mmol) and *N*-*tert*-Butoxycarbonyl-*S*-methylisothiourea (2108 mg, 11.08 mmol) according to the general procedure (R_f = 0.34 in Hex/EA 3:2). The product was obtained as a white solid (1183 mg, 74%). mp = 116.4–119.2 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 1.32 (m, 8H), 1.47 (s, 18H), 1.50 (m, 4H), 2.29 (s, 6H), 3.17–3.24 (m, 4H), 5.53 (t, 2H, *J* 5.6 Hz), 12.32 (s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 14.4, 27.0, 28.2, 29.3, 29.8, 40.3, 82.7, 151.3, 162.1, 167.4. HRMS (ESI) *m*/*z* (%) 577 (100) $[M+H]^+$; *m*/*z* $[M+H]^+$ calculated for $C_{24}H_{45}N_6O_6S_2^+$: 577.2837, found 577.2845; $C_{24}H_{44}N_6O_6S_2$ (576.77).

4.2.3.3. N^1,N^{10}-Bis{[(tert-butoxycarbonylamino)(methylsulfanyl) methylene]aminocarbonyl}decane-1,10-diamine (7). The title compound was prepared from decane-1,10-diamine (300 mg, 1.74 mmol), DIPEA (1697 μL, 9.74 mmol), triphosgene (517 mg, 1.74 mmol) and *N*-*tert*-Butoxycarbonyl-*S*-methylisothiourea (1325 mg, 6.97 mmol) according to the general procedure (R_f = 0.35 in Hex/EA 3:2). The product was obtained as a colorless glassy solid (798 mg, 76%). ^{1}H NMR (300 MHz, $[D_4]$MeOH) δ (ppm) 1.33 (m, 12H), 1.48–1.54 (m, 22H), 2.32 (s, 6H), 3.14 (t, 4H, *J* 7.0 Hz); ^{13}C NMR (75 MHz, $[D_4]$MeOH) δ (ppm) 14.4, 28.0, 28.2, 30.4, 30.6, 41.0, 83.6, 152.1, 163.7, 167.2. HRMS (ESI) *m*/*z* (%) 605 (100) $[M+H]^+$; *m*/*z* $[M+H]^+$ calculated for $C_{26}H_{49}N_6O_6S_2^+$: 605.3150, found 605.3154; $C_{26}H_{48}N_6O_6S_2$ (604.83).

4.2.4. General procedure for the synthesis of the bivalent carbamoylguanidine-type ligands (8–17)

The guanidinylating reagents (**8**–**17**) (1 equiv) and 2.1 eq of 3-(1-trityl-1*H*-imidazol-4-yl)propan-1-amine (**1**) or *tert*-butyl 5-(3-aminopropyl)-4-methylthiazol-2-ylcarbamate (**2**) were dissolved in DMF (unless indicated otherwise). NEt_3 (5 equiv) and $HgCl_2$ (4 equiv) were added to the mixture and stirring was continued for 12 h. The precipitate was centrifuged, washed with DMF (unless otherwise indicated) and centrifuged for a second time. The bis(Boc)-bis(trityl)-(cf. imidazoles) or tetrakis(Boc)-protected intermediate (cf. aminothiazoles) was extracted with EA (unless indicated otherwise), purified by column chromatography (eluent: DCM/MeOH 100:1 to 50:1), and dried in vacuo. Subsequently, deprotection was performed by stirring with 30% TFA in DCM for 12 h. The obtained carbamoylguanidine (cf. **8**–**17**) was purified by preparative HPLC.

4.2.4.1. 1-(Amino{[3-(1H-imidazol-4-yl)propyl]amino}methylene)-3-{4-[3-(amino{[3-(1H-imidazol-4-yl)propyl]amino}methylene) ureido]butyl}urea (8). The title compound was prepared from **3** (175 mg, 0.336 mmol), amine **1** (260 mg, 0.708), NEt_3 (233 μL, 1.67 mmol) and $HgCl_2$ (365 mg, 1.34 mmol) according to the general procedure, yielding **8** as a white fluffy, hygroscopic solid (21.88 mg, 7%): RP-HPLC: 99%, (t_R = 11.75 min, k = 2.99); UV_{max} 204 nm. ^{1}H NMR (600 MHz, $[D_4]$MeOH) δ (ppm) 1.57 (m, 4H), 2.03 (tt, 4H, ^2J 7.0 Hz, ^3J 7.6 Hz), 2.84 (t, 4H, *J* 7.7 Hz), 3.23 (m, 4H), 3.36 (t, 4H, *J* 6.9 Hz), 7.36 (s, 2H), 8.80 (d, 2H, *J* 1.2 Hz); ^{13}C NMR (151 MHz, $[D_4]$MeOH, trifluoroacetate) δ (ppm) 22.6, 27.7, 28.2, 40.3, 41.4, 117.1, 134.4, 135.0, 155.6, 156.0, 163.3, 163.5. HRMS (ESI) *m*/*z* (%) 475 (29) $[M+H]^+$, 238 (75) $[M+2H]^{2+}$; *m*/*z* $[M+H]^+$ calculated for $C_{20}H_{35}N_{12}O_2^+$: 475.3000, found 475.3003; $C_{20}H_{34}N_{12}O_2 \times 4$ TFA (930.64).

4.2.4.2. 1-(Amino{[3-(1H-imidazol-4-yl)propyl]amino}methylene)-3-{6-[3-(amino{[3-(1H-imidazol-4-yl)propyl]amino}methylene) ureido]hexyl}urea (9). The title compound was prepared from **4** (175 mg, 0.319 mmol), amine **1** (250 mg, 0.680 mmol), NEt_3 (222 μL, 1.60 mmol) and $HgCl_2$ (346 mg, 1.27 mmol) according to the general procedure, yielding **9** as a white fluffy hygroscopic solid (36.00 mg, 12%): RP-HPLC: 98%, (t_R = 15.18 min, k = 4.15); UV_{max} 205 nm. ^{1}H NMR (600 MHz, $[D_4]$MeOH) δ (ppm) 1.36–1.38 (m, 4H), 1.52–1.58 (m, 4H), 2.03 (tt, 4H, ^2J 7.1 Hz, ^3J 7.5 Hz), 2.84 (t, 4H, *J* 7.7 Hz), 3.20 (t, 4H, *J* 7.1 Hz,), 3.37 (t, 4H, *J*= 6.9 Hz), 7.37 (s, 2H), 8.81 (d, 2H, *J* 1.3 Hz); ^{13}C NMR (151 MHz, $[D_4]$MeOH, trifluoroacetate) δ (ppm) 22.5, 27.4, 28.2, 30.3, 40.7, 41.4, 117.1, 134.3, 135.0, 155.5, 156.0. HRMS (ESI) *m*/*z* (%) 617 (4) $[M+H+TFA]^+$, 503 (22) $[M+H]^+$, 252 (84) $[M+2H]^{2+}$; *m*/*z* $[M+H]^+$ calculated for $C_{22}H_{39}N_{12}O_2^+$: 503.3313, found 503.3314; $C_{22}H_{38}N_{12}O_2 \times 4$ TFA (958.70).

4.2.4.3. 1-(Amino{[3-(1H-imidazol-4-yl)propyl]amino}methylene)-3-{7-[3-(amino{[3-(1H-imidazol-4-yl)propyl]amino}methylene) ureido]heptyl}urea (10). The title compound was prepared from **5** (106 mg, 0.188 mmol), amine **1** (145 mg, 0.395 mmol), NEt_3 (128 μL, 0.92 mmol) and $HgCl_2$ (201 mg, 0.74 mmol) according to the general procedure, yielding **10** as a white fluffy hygroscopic solid (15.30 mg, 8%): RP-HPLC: 97%, (t_R = 16.65 min, k = 4.65); UV_{max} 205 nm. ^{1}H NMR (600 MHz, $[D_4]$MeOH) δ (ppm) 1.36 (m, 6H), 1.54 (m, 4H), 2.03 (tt, 4H, ^2J 7.0 Hz, ^3J 7.5 Hz), 2.84 (t, 4H, *J* 7.7 Hz), 3.19 (t, 4H, *J* 7.1 Hz), 3.36 (t, 4H, *J* 6.9 Hz), 7.36 (s, 2H), 8.80 (d, 2H, *J* 1.1 Hz); ^{13}C NMR (151 MHz, $[D_4]$MeOH, trifluoroacetate) δ (ppm); 22.5, 27.7, 28.2, 29.9, 30.4, 40.7, 41.4, 117.1, 134.3, 134.9, 155.5, 156.1. HRMS (ESI) *m*/*z* (%) 517 (13) $[M+H]^+$, 259 (41) $[M+2H]^{2+}$; *m*/*z* $[M+H]^+$ calculated for $C_{23}H_{41}N_{12}O_2^+$: 517.3473, found 517.3473; $C_{23}H_{40}N_{12}O_2 \times 4$ TFA (972.72).

4.2.4.4. 1-(Amino{[3-(1H-imidazol-4-yl)propyl]amino}methylene)-3-{8-[3-(amino{[3-(1H-imidazol-4-yl)propyl]amino}methylene) ureido]octyl}urea (11). Compound **6** (175 mg, 0.303 mmol) and amine **1** (234 mg, 0.637 mmol) were dissolved in DCM. NEt_3 (210 μL, 1.51 mmol) and $HgCl_2$ (330 mg, 1.21 mmol) were added to the mixture. The mixture was centrifuged and the precipitate was washed with DCM and centrifuged for a second time. The sol-

vent was removed under reduced pressure. Purification by column chromatography (eluent: DCM/MeOH 100:1 to 50:1) yielded **11** as a white fluffy hygroscopic solid (58.62 mg, 20%): RP-HPLC: 96%, (t_R = 18.80 min, k = 5.38); UV_{max} 205 nm. ^{1}H NMR (600 MHz, [D$_4$]MeOH) δ (ppm) 1.35 (m, 8H), 1.46–1.66 (m, 4H), 2.03 (tt, 4H, ^2J 7.0 Hz, ^3J 7.5 Hz), 2.86 (t, 4H, J 7.7 Hz), 3.19 (t, 4H, J 7.0 Hz), 3.39 (t, 4H, J 6.4 Hz), 7.39 (s, 2H), 8.83 (d, 2H, J 0.9 Hz); ^{13}C NMR (151 MHz, [D$_4$]MeOH, trifluoroacetate) δ (ppm) 22.5, 27.8, 28.2, 30.2, 30.4, 40.8, 41.5, 117.1, 134.3, 134.9, 155.3, 155.9. HRMS (ESI) m/z (%) 531 (15) [M+H]$^+$, 266 (47) [M+2H]$^{2+}$; m/z [M+H]$^+$ calculated for $C_{24}H_{43}N_{12}O_2^+$: 531.3626, found 531.3625; $C_{24}H_{42}N_{12}O_2 \times 4$ TFA (986.75).

4.2.4.5. 1-(Amino{[3-(1H-imidazol-4-yl)propyl]amino}methylene)-3-{10-[3-(amino{[3-(1H-imidazol-4-yl)propyl]amino}methylene)ureido]decyl}urea (12). The title compound was prepared from **7** (150 mg, 0.248 mmol), amine **1** (191 mg, 0.520 mmol), NEt_3 (172 μL, 1.24 mmol) and $HgCl_2$ (270 mg, 0.99 mmol) according to the general procedure, yielding **12** as a white fluffy hygroscopic solid (68.83 mg, 27%): RP-HPLC: 91%, (t_R = 22.73 min, k = 6.71); UV_{max} 205 nm. ^{1}H NMR (600 MHz, [D$_4$]MeOH) δ (ppm) 1.32 (m, 12H), 1.49–1.57 (m, 4H), 2.02 (tt, 4H, ^2J 7.0 Hz, ^3J 7.6 Hz), 2.83 (t, 4H, J 7.7 Hz), 3.18 (t, 4H, J 7.0 Hz), 4H), 3.36 (t, 4H, J 6.9 Hz), 7.36 (s, 2H), 8.80 (d, 2H, J 0.7 Hz); ^{13}C NMR (151 MHz, [D$_4$]MeOH, trifluoroacetate) δ (ppm) 22.5, 27.8, 28.1, 30.3, 30.4, 30.5, 40.8, 41.4, 117.0, 134.3, 134.9, 155.4, 156.0. HRMS (ESI) m/z (%) 559 (10) [M+H]$^+$, 280 (27) [M+2H]$^{2+}$; m/z [M+H]$^+$ calculated for $C_{26}H_{47}N_{12}O_2^+$: 559.3939, found 559.3935; $C_{26}H_{46}N_{12}O_2 \times 4$ TFA (1014.80).

4.2.4.6. 1-(Amino{[3-(2-amino-4-methyl-thiazol-5-yl)propyl]amino}methylene)-3-{4-[3-(amino{[3-(2-amino-4-methyl-thiazol-5-yl)propyl]amino}methylene)ureido]butyl}urea (13). Compound **3** (200 mg, 0.384 mmol) and amine **2** (216 mg, 0.796 mmol) were dissolved in DCM. NEt_3 (265 μL, 1.80 mmol) and $HgCl_2$ (412 mg, 1.52 mmol) were added to the mixture. The mixture was centrifuged and the precipitate was washed with DCM and centrifuged for a second time. The solvent was removed under reduced pressure and the product was purified by column chromatography (eluent: DCM/MeOH 100:1 to 50:1). Yielding **13** as a white fluffy hygroscopic solid (53.34 mg, 14%): RP-HPLC: 99%, (t_R = 15.61 min, k = 4.29); UV_{max} 205 nm and 265 nm. ^{1}H NMR (600 MHz, [D$_4$]MeOH) δ (ppm) 1.57 (m, 4H), 1.90 (tt, 4H, ^2J 7.0 Hz, ^3J 7.3 Hz), 2.18 (s, 6H), 2.72 (t, 4H, J 7.6 Hz), 3.23 (m, 4H), 3.33 (t, 4H, J 6.9 Hz); ^{13}C NMR (151 MHz, [D$_4$]MeOH, trifluoroacetate) δ (ppm) 11.4, 23.6, 27.7, 29.9, 40.34, 41.4, 118.4, 132.6, 155.6, 156.0, 170.3. HRMS (ESI) m/z (%) 567 (34) [M+H]$^+$, 284 (58) [M+2H]$^{2+}$; m/z [M+H]$^+$ calculated for $C_{22}H_{39}N_{12}O_2S_2^+$: 567.2755, found 567.2760; $C_{22}H_{38}N_{12}O_2S_2 \times 4$ TFA (1022.83).

4.2.4.7. 1-(Amino{[3-(2-amino-4-methyl-thiazol-5-yl)propyl]amino}methylene)-3-{6-[3-(amino{[3-(2-amino-4-methyl-thiazol-5-yl)propyl]amino}methylene)ureido]hexyl}urea (14). Compound **4** (175 mg, 0.319 mmol) and amine **2** (177 mg, 0.652 mmol) were dissolved in DCM. NEt_3 (215 μL, 1.55 mmol) and $HgCl_2$ (378 mg, 1.24 mmol) were added to the mixture. The mixture was centrifuged and the precipitate was washed with DCM and centrifuged for a second time The solvent was removed under reduced pressure and the product was purified by column chromatography (eluent: DCM/MeOH 100:1 to 50:1). Yielding **14** as a white fluffy hygroscopic solid (99.34 mg, 30%): RP-HPLC: 99%, (t_R = 18.12 min, k = 5.15); UV_{max} 203 nm and 265 nm. ^{1}H NMR (600 MHz, [D$_4$]MeOH) δ (ppm) 1.37 (m, 4H), 1.48–1.62 (m, 4H), 1.89 (tt, 4H, ^2J 7.0 Hz, ^3J 7.4 Hz), 2.18 (s, 6H), 2.72 (t, 4H, J 7.5 Hz), 3.20 (t, 4H, J 6.9 Hz), 3.33 (t, 4H, J 6.8 Hz, overlap with solvent). ^{13}C NMR (75 MHz, [D$_4$]MeOH, trifluoroacetate) δ (ppm) 11.4, 23.6, 27.4, 29.9, 30.3, 40.6, 41.4, 118.4, 132.6, 155.5, 156.0, 170.3. HRMS (ESI) m/z (%) 595 (23) [M+H]$^+$, 298 (38) [M+2H]$^{2+}$; m/z [M+H]$^+$ calculated for $C_{24}H_{43}N_{12}O_2S_2^+$: 595.3068, found 595.3068; $C_{24}H_{42}N_{12}O_2S_2 \times 4$ TFA (1050.88).

4.2.4.8. 1-(Amino{[3-(2-amino-4-methyl-thiazol-5-yl)propyl]amino}methylene)-3-{7-[3-(amino{[3-(2-amino-4-methyl-thiazol-5-yl)propyl]amino}methylene)ureido]heptyl}urea (15). The title compound was prepared from **5** (175 mg, 0.311 mmol), **2** (177 mg, 0.652 mmol), NEt_3 (215 μL, 1.55 mmol) and $HgCl_2$ (338 mg, 1.24 mmol) according to the general procedure, yielding **15** as a white fluffy hygroscopic solid (95.46 mg, 29%): RP-HPLC: 99%, (t_R = 19.85 min, k = 5.74); UV_{max} 204 nm and 264 nm. ^{1}H NMR (600 MHz, [D$_4$]MeOH) δ (ppm) 1.36 (s, 6H), 1.51–1.57 (m, 4H), 1.90 (tt, 4H, ^2J 7.2 Hz, ^3J 7.4 Hz), 2.18 (s, 6H), 2.72 (t, 4H, J 7.6 Hz), 3.19 (t, 4H, J 7.1 Hz), 3.33 (t, 4H, J 6.9 Hz); ^{13}C NMR (151 MHz, [D$_4$]MeOH, trifluoroacetate) δ (ppm) 11.4, 23.6, 27.7, 29.9, 30.0, 30.4, 40.8, 41.4, 118.4, 132.7, 155.5, 156.0, 170.3. HRMS (ESI) m/z (%) 609 (23) [M+H]$^+$, 305 (50) [M+2H]$^{2+}$; m/z [M+H]$^+$ calculated for $C_{25}H_{45}N_{12}O_2S_2^+$: 609.3224, found 609.3226; $C_{25}H_{44}N_{12}O_2S_2 \times 4$ TFA (1064.91).

4.2.4.9. 1-(Amino{[3-(2-amino-4-methyl-thiazol-5-yl)propyl]amino}methylene)-3-{8-[3-(amino{[3-(2-amino-4-methyl-thiazol-5-yl)propyl]amino}methylene)ureido]octyl}urea (16). The title compound was prepared from **6** (130 mg, 0.225 mmol), **2** (128 mg, 0.472 mmol), NEt_3 (156 μL, 1.12 mmol) and $HgCl_2$ (244 mg, 0.90 mmol) according to the general procedure, yielding **6** as a white fluffy hygroscopic solid (21.77 mg, 9%): RP-HPLC: 98%, (t_R = 21.69 min, k = 6.36); UV_{max} 204 nm and 264 nm. ^{1}H NMR (600 MHz, [D$_4$]MeOH) δ (ppm) 1.34 (m, 8H), 1.50–1.56 (m, 4H), 1.90 (tt, 4H, ^2J 7.0 Hz, ^3J 7.4 Hz), 2.18 (s, 6H), 2.72 (t, 4H, J 7.6 Hz), 3.19 (t, 4H, J 7.1 Hz), 3.33 (t, 4H, J 6.9 Hz). ^{13}C NMR (151 MHz, [D$_4$]MeOH, trifluoroacetate) δ (ppm) 11.4, 23.6, 27.8, 29.9, 30.3, 30.4, 40.8, 41.4, 118.4, 132.6, 155.5, 156.0, 170.3. HRMS (ESI) m/z (%) 623 (11) [M+H]$^+$, 312 (21) [M+2H]$^{2+}$; m/z [M+H]$^+$ calculated for $C_{26}H_{47}N_{12}O_2S_2^+$: 623.3381, found 623.3375; $C_{26}H_{46}N_{12}O_2S_2 \times 4$ TFA (1078.94).

4.2.4.10. 1-(Amino{[3-(2-amino-4-methyl-thiazol-5-yl)propyl]amino}methylene)-3-{10-[3-(amino{[3-(2-amino-4-methyl-thiazol-5-yl)propyl]amino}methylene)ureido]decyl}urea (17). The title compound was prepared from **7** (150 mg, 0.248 mmol), **2** (141 mg, 0.519 mmol), NEt_3 (172 μL, 1.24 mmol) and $HgCl_2$ (269 mg, 0.99 mmol) according to the general procedure, yielding **7** as a white fluffy hygroscopic solid (24.25 mg, 9%): RP-HPLC: 99%, (t_R = 25.49 min, k = 7.65); UV_{max} 205 nm and 263 nm. ^{1}H NMR (600 MHz, [D$_4$]MeOH) δ (ppm) 1.32 (m, 12H), 1.50–1.56 (m, 4H), 1.90 (tt, 4H, ^2J 7.0 Hz, ^3J 7.3 Hz), 2.18 (s, 6H), 2.72 (t, 4H, J 7.1 Hz), 3.19 (t, 4H, J 7.1 Hz), 3.33 (t, 4H, J 6.9 Hz). ^{13}C NMR (151 MHz, [D$_4$]MeOH, trifluoroacetate) δ (ppm) 11.5, 23.6, 27.8, 29.9, 30.3, 30.4, 30.6, 40.8, 41.4, 118.4, 132.7, 155.5, 156.0, 170.3. HRMS (ESI) m/z (%) 651 (4) [M+H]$^+$, 326 (17) [M+2H]$^{2+}$, 218 (27) [M+3H]$^{3+}$, 163 (42) [M+4H]$^{4+}$; m/z [M+H]$^+$ calculated for $C_{28}H_{51}N_{12}O_2S_2^+$: 651.3694, found 651.3684; $C_{28}H_{50}N_{12}O_2S_2 \times 4$ TFA (1106.99).

4.2.5. 1,7-Dichloroheptan-4-one (18)[9]

HCl gas (10 fold excess) was passed through dicyclopropyl ketone (15.00 g, 136.16 mmol) for 30 min. After stirring for 3 h, HCl gas was passed through the mixture for another 30 min. The crude product was obtained as a brown oil (24.93 g, 100%) and was used in the next step without any further purification. ^{1}H

NMR (300 MHz, $CDCl_3$): δ (ppm) 2.02 (m, 4H), 2.61 (t, 4H, *J* 7.0 Hz), 3.55 (t, 4H, *J* 6.3 Hz). ^{13}C NMR (75 MHz, [D_4]MeOH) δ (ppm) 27.6, 40.3, 45.2, 210.7. HRMS (APCI; MeOH) *m*/*z* (%) 183 (100); *m*/*z* $[M+H]^+$ calculated for $C_7H_{13}Cl_2O^+$: 183.0337, found 183.0337. $C_7H_{12}Cl_2O$ (183.08).

4.2.6. 1,7-Diazidoheptan-4-one (19)

To a solution of 1,7-dichloroheptan-4-one (**18**) (3.40 g, 18.57 mmol) in 50 ml DMF, sodium azide (3.62 g, 55.68 mmol) was added and stirred overnight at 60 °C. After cooling and addition of water (500 mL), the mixture was extracted with EA (3 × 100 mL). The combined organic phases were dried over sodium sulfate. Filtration and evaporation of the solvent yielded a yellow oil (2.87 g, 79%). The crude product **19** was used in the next step without any further purification. ^1H NMR (300 MHz, $CDCl_3$) δ (ppm) 1.86 (m, 4H), 2.52 (t, 4H, *J* 7.1 Hz), 3.31 (t, 4H, *J* 6.6 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 22.6, 39.0, 50.4, 208.2. HRMS (ESI; MeOH) *m*/*z* (%) 219 (46) $[M+Na^+]$, 197 (2) $[M+H]^+$, 169 (100) $[M+H-N_2]^+$; *m*/*z* $[M+H]^+$ calculated for $C_7H_{13}N_6^+$: 197.1145, found 197.1138. $C_7H_{12}N_6$(196.21).

4.2.7. 1-Azido-4-(3-azidopropyl)-8-chlorooct-4-ene (21)

(4-Chlorobutyl)triphenylphosphonium bromide (**20**) (2.50 g, 5.76 mmol) was suspended in anhydrous THF (20 mL) under an atmosphere of argon and the mixture was cooled to −72 °C. *n*-Butyllithium (1.6 M in *n*-hexane, 3.60 mL, 5.76 mmol) was added to the suspension and the mixture was allowed to warm up to −10 °C over a period of 45 min. The temperature was kept at −10 °C for 1.5 h and then cooled again to −72 °C. 1,7-diazidoheptan-4-one (1.13 g, 5.76 mmol) was added and the mixture was allowed to warm up to room temperature over a period of 60 min, stirring was continued overnight. After filtration of solid material and evaporation of the solvent, the product was purified by column chromatography using mixtures of Hex and EA as eluent (R_f = 0.45 for Hex/EA 4:1), affording **21** as yellow oil (300 mg, 19%). ^1H NMR (300 MHz, [D_4]MeOH) δ (ppm) 1.61–1.74 (m, 4H), 1.76–1.86 (m, 2H), 2.07–2.26 (bm, 6H), 3.26–3.31 (overlap with solvent, m, 4H), 3.56 (t, 2H, *J* 6.5 Hz), 5.23 (t, 1H, *J* 7.3 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 25.0, 27.0, 27.4, 27.6, 32.7, 33.6, 44.6, 51.0, 51.2, 125.3, 138.0. HRMS (ESI) *m*/*z* (%) 243 (100) $[M+H-N_2]^+$; *m*/*z* $[M+H-N_2]^+$ calculated for $C_{11}H_{20}ClN_4^+$: 243.1376, found 243.1374; $C_{11}H_{19}ClN_6$ (270.76)

4.2.8. 2-[8-Azido-5-(3-azidopropyl)oct-4-en-1-yl]isoindoline-1,3-dione (22)

To a solution of **21** (1.89 g, 6.98 mmol) and phthalimide (1.54 g, 10.47 mmol) in 40 mL DMF, Cs_2CO_3 (5.00 g, 15.34 mmol) and catalytic traces of KI were added. The mixture was stirred at 60 °C overnight. After cooling 400 mL 5% NaOH were added and the product was extracted with EA. The combined organic phases were dried over Na_2SO_4. After filtration and evaporation, the crude product was purified by column chromatography using mixtures of Hex and EA as eluent (R_f = 0.45 for Hex/EA 2:1) affording **22** as yellow oil (1.51 g, 57%). ^1H NMR (300 MHz, $CDCl_3$) δ (ppm) 1.56–1.58 (m, 4H), 1.69–1.78 (m, 2H), 1.96–2.15 (m, 6H), 3.24 (td, 4H, ^2J 6.8, ^3J 2.9), 3.65–3.73 (m, 2H), 5.21 (t, 1H, *J* 7.1 Hz), 7.75–7.67 (m, 2H), 7.80–7.87 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 25.1, 26.9, 27.2, 27.3, 28.7, 33.4, 37.7, 50.9, 51.1, 123.2, 125.7, 132.1, 133.9, 137.3, 168.4. HRMS (ESI) *m*/*z* (%) 404 (31) $[M+Na^+]$, 354 (100) $[M+H-N_2]^+$; *m*/*z* $[M+H-N_2]^+$ calculated for $C_{19}H_{24}N_5O_2^+$: 354.1925, found 354.1925; $C_{19}H_{23}N_7O_2$ (381.44).

4.2.9. 8-Azido-5-(3-azidopropyl)oct-4-en-1amine (23)

A mixture of **22** (1.00 g, 2.62 mmol) and hydrazine (0.50 g, 15.60 mmol) in ethanol was refluxed for 1 h. Solid material was removed by filtration. After removing the solvent in vacuo, the product was purified by column chromatography (eluent: EA/MeOH/NH_3 9:0.8:0.2 to EA/MeOH/NH_3 8:1.5:0.5), (R_f = 0.14 for DCM/7 N NH_3 in MeOH 4:1). Compound **23** was obtained as yellow oil (580 mg, 88%). ^1H NMR (300 MHz, $CDCl_3$) δ (ppm) 1.45–1.54 (m, 2H), 1.58–1.85 (m, 6H), 1.99–2.13 (m, 6H), 2.70 (t, 2H, *J* 7.1 Hz), 3.26 (td, 4H, ^2J 6.8, ^3J 2.4) 5.20 (t, 1H, *J* 7.1). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 25.2, 27.3, 27.5, 33.3, 33.6, 39.5, 41.6, 51.0, 51.2, 126.6, 136.7. HRMS (ESI) *m*/*z* (%) 252 (100) $[M+H]^+$; *m*/*z* $[M+H^+]^+$ calculated for $C_{11}H_{22}N_7^+$: 252.1931, found 252.1925; $C_{11}H_{21}N_7$ (251.34).

4.2.10. *tert*-Butyl [8-azido-5-(3-azidopropyl)oct-4-en-1-yl] carbamate (24)

A solution of di-*tert*-butyldicarbonat (756 mg, 3.46 mmol) in DCM was added dropwise to an external cooled solution of number 8-azido-5-(3-azidopropyl)oct-4-en-1amine (**23**) (870 mg, 3.46 mmol) and NEt_3 (480 µL, 3.46 mmol) in DCM over a period of 1 h. The mixture was stirred at room temperature for 24 h. The solvent was removed in vacuo. The product was purified by column chromatography (eluent: Hex/EA 10:90 to Hex/EA 50:50), (R_f = 0.23 for Hex/EA 3:2) and obtained as pale yellowish oil (1.18 g, 97%). ^1H NMR (300 MHz, $CDCl_3$) δ (ppm) 1.44 (s, 9H), 1.47–1.59 (m, 2H), 1.59–1.73 (m, 4H), 2.00–2.12 (m, 6H), 3.11 (t, 2H, *J* 6.9 Hz), 3.23–3.30 (m, 4H), 4.54 (s, 1H), 5.19 (t, 1H, *J* 7.1 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 25.1, 26.8, 27.3, 27.4, 28.5, 30.3, 33.5, 40.3, 51.0, 51.1, 79.1, 126.3, 137.0, 156.0. HRMS (ESI) *m*/*z* (%) 374 (26) $[M+Na^+]$, 324 (25) $[M+H-N_2]^+$, 268 (100) $(M+H-C_4H_8]^+$; *m*/*z* $[M+Na^+]$ calculated for $C_{16}H_{29}N_7O_2Na^+$: 374.2275, found 374.2282; $C_{16}H_{29}N_7O_2$ (351.45).

4.2.11. *tert*-Butyl [8-amino-5-(3-aminopropyl)oct-4-en-1-yl] carbamate (25)

A solution of **24** (200 mg, 0.57 mmol) in anhydrous diethyl ether was dropped to a stirred suspension of $LiAlH_4$ (100 mg, 2.63 mmol) in anhydrous diethyl ether over 1 h. The mixture was heated to reflux for an additional hour. After cooling and quenching with a solution of 2 g Seignette salt in 12 g water (pH = 9–10), the organic phase was washed twice with 0.1 M KOH and the solvent was evaporated in vacuo. The crude product was used in the next step without any further purification (113 mg, 66%). ^1H NMR (300 MHz, [D_4]MeOH) δ (ppm) 1.42 (s, 9H), 1.47–1.57 (m, 6H), 1.63 (s, 4H), 1.98–2.06 (m, 6H), 2.66 (td, 4H, ^2J 7.1, ^3J 2.5), 3.05–3.12 (m, 2H), 4.67 (s, 1H), 5.11 (t, 1H *J* 7.0 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 25.1, 27.4, 28.5, 30.4, 32.2, 32.4, 34.2, 40.4, 42.0, 42.3, 79.1, 124.4, 139.3, 156.1. HRMS (ESI) *m*/*z* (%) 300 (40) $[M+H]^+$, 244 (51) $[M+H-C_4H_8]^+$, 200 (100) $[M+H-Boc]^+$; *m*/*z* $[M+H^+]^+$ calculated for $C_{16}H_{34}N_3O_2^+$: 300.2646, found 300.2649; $C_{16}H_{33}N_3O_2$ (299.45).

4.2.12. *tert*-Butyl ({3-[4-({3-[(*tert*-butoxycarbonylimino)(methylsulfanyl)methyl]ureido}propyl)-8-(*tert*-butoxycarbonylamino)oct-4-en-1-yl]ureido}(methylsulfanyl)methylene)carbamate (27)

The title compound was prepared from **25** (122 mg, 0.41 mmol), DIEA (393 µL, 2.3 mmol) and triphosgene (122 mg, 0.41 mmol), giving *tert*-butyl [8-isocyanato-5-(3-isocyanatopropyl)oct-4-en-1-yl]carbamate (**26**) as intermediate. Compound **26** was not isolated but allowed to react with *N*-Boc-*S*-methylisothiourea (310 mg, 1.63 mmol) according to the general procedure for the synthesis of the guanidinylating reagents (**5-7**). **27** was obtained as a yellowish oil (R_f = 0.37 in DCM) (131 mg, 44%). ^1H NMR (300 MHz, [D_4]MeOH) δ (ppm) 1.43 (s, 9H), 1.48–1.54 (m, 20H), 1.59–1.66 (m, 4H), 2.03–2.07 (m, 4H), 2.08–2.12 (m, 2H), 2.33 (d, 6H, *J* 2.1 Hz), 3.03 (t, 2H, *J* 7.0 Hz), 3.14 (m, 4H), 5.22 (t, 1H, *J* 7.1 Hz).

^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 14.4, 26.1, 28.2, 28.8, 29.1, 29.3, 31.2, 35.1, 40.8, 41.1, 83.6, 126.3, 139.8, 152.2, 163.7, 163.72, 167.2, 167.3. HRMS (ESI) *m*/*z* (%) 754 (14) $[M+Na^+]$, 732 (100) $[M+H]^+$, 266 (69) $[M+2H-2Boc]^{2+}$; *m*/*z* $[M+H^+]^+$ calculated for $C_{32}H_{58}N_7O_8S_2^+$: 732.3794, found 732.3794; $C_{32}H_{57}N_7O_8S_2$ (731.97).

4.2.13. 1-(8-Amino-4-{[3-(amino{[3-(2-amino-4-methylthiazol-5-yl)propyl]amino}methylene)ureido]propyl}oct-4-enyl)-3-[(amino{[3-(2-amino-4-methyl-thiazol-5-yl)propyl]amino}methylene)]urea (28)

The title compound was prepared from **27** (111 mg, 0.15 mmol), **2** (86 mg, 0.317 mmol), NEt_3 (105 μL, 0.76 mmol) and $HgCl_2$ (165 mg, 0.60 mmol) according to the general procedure for the synthesis of the bivalent carbamoylguanine-type ligands (**8**–**17**), yielding **28** as a white fluffy hygroscopic solid (34.80 mg, 18%). RP-HPLC: 96%, (t_R = 17.19 min, k = 4.83); UV_{max} 204 nm and 263 nm. ^{1}H NMR (600 MHz, [D$_4$]MeOH) δ (ppm) 1.59–1.67 (m, 4H), 1.70 (m, 2H), 1.90 (tt, 4H, ^2J 7.0 Hz, ^3J 7.3 Hz), 2.05–2.17 (bm, 6H), 2.18 (s, 6H), 2.72 (t, 4H, *J* 7.6 Hz), 2.90–2.94 (m, 2H), 3.19 (m, 4H), 3.34 (t, 4H, *J* 6.8 Hz, overlap with solvent), 5.23 (t, 1H, *J* 7.1 Hz). ^{13}C NMR (151 MHz, [D$_4$]MeOH, trifluoroacetate) δ (ppm) 11.4, 23.6, 25.6, 28.1, 28.8, 28.9, 29.1, 29.9, 34.7, 40.41, 40.44, 40.8, 41.4, 118.4, 125.3, 132.6, 140.6, 155.6, 156.0, 170.3. HRMS (ESI) *m*/*z* (%) 678 (10) $[M+H]^+$, 340 (23) $[M+2H]^{2+}$; *m*/*z* $[M+H]^+$ calculated for $C_{29}H_{52}N_{13}O_2S_2^+$: 678.3803, found 678.3806. $C_{29}H_{51}N_{13}O_2S_2 \times 5$ TFA (1248.03).

4.2.14. General procedure for the synthesis of the bivalent fluorescence ligands (29–31)

The reactions were carried out in a 1.5-mL Eppendorf reaction vessel. The amine precursor **28** (2 equiv) was dissolved in 30 μL DMF and NEt_3 (15 equiv) was added. The fluorescence dyes (1 equiv) were dissolved in 20 μL DMF and this solution was added to the mixture, the cup was rinsed two times with DMF (20 μL and 10 μL). The mixture was stirred for 2 h at room temperature and then the reaction was stopped by adding 20 μL of 10% TFA. The crude products were purified by preparative HPLC.

4.2.14.1. 2-{(1E,3E)-5-[(Z)-1-(5-Amino-12-{3-[3-(amino{[3-(2-amino-4-methylthiazol-5-yl)propyl]amino}methylene)ureido]propyl}-1-(2-amino-4-methylthiazol-5-yl)-7,18-dioxo-4,6,8,17-tetraazatricosa-5,12-dien-23-yl)-3,3-dimethylindolin-2-ylidene]penta-1,3-dien-1-yl}-1,3,3-trimethyl-3H-indol-1-ium trifluoroacetate (29).

The title compound was prepared from **28** (1.60 mg, 1.28 μmol), S2197 (422 μg, 0.64 μmol) and NEt_3 (1.33 μL, 9.60 μmol) according to the general procedure, yielding **29** as a dark blue solid (490 μg, 45%): RP-HPLC: 99%, (t_R = 24.83 min, k = 7.42) (eluent: MeCN/TFA (0.05% aq) 0 min: 10:90, 30 min: 60:40, 31–41 min: 80:20; flow rate = 0.75 mL/min). MS (ESI) *m*/*z* (%) 1143.8 (1) $[M]^+$, 571.9 (34) $[M+H]^{2+}$, 381.6 (100) $[M+2H]^{3+}$, 296.6 (24) $[M+4H+MeOH]^{4+}$. $C_{63}H_{88}F_3N_{15}O_5S_2 \times 4$ TFA (1713.70).

4.2.14.2. 1-{8-[3-(Amino{[3-(2-amino-4-methylthiazol-5-yl)propyl]amino}methylene)ureido]-5-{3-[3-(amino{[3-(2-amino-4-methylthiazol-5-yl)propyl]amino}methylene)ureido]propyl}oct-4-en-1-yl}-2,6-dimethyl-4-[(E)-2-(2,3,6,7-tetrahydro-1H,5H-pyrido[3,2,1-ij]quinolin-9-yl)vinyl]pyridin-1-ium trifluoroacetate (30).

The title compound was prepared from **28** (1.50 mg, 1.20 μmol), Py-1 (236 μg, 0.60 μmol) and NEt_3 (1.25 μL, 9.00 μmol) according to the general procedure, yielding **30** as a red solid (332 μg, 36%): RP-HPLC: 93%, (t_R = 23.11 min, k = 6.84) (eluent: MeCN/TFA (0.05% aq) 0 min: 10:90, 30 min: 60:40, 31–41 min: 80:20; flow rate = 0.75 mL/min). MS (ESI) *m*/*z* (%) 965.7 (1) $[M]^+$, 483.2 (82) $[M+H]^{2+}$, 336.1 (100) $[M+2H+MeCN]^{3+}$, 322.4 (15) $[M+2H]^{3+}$. $C_{52}H_{73}F_3N_{14}O_4S_2 \times 4$ TFA (1536.45).

4.2.14.3. 1-{8-[3-(amino{[3-(2-amino-4-methylthiazol-5-yl)propyl]amino}methylene)ureido]-5-{3-[3-(amino{[3-(2-amino-4-methylthiazol-5-yl)propyl]amino}methylene)ureido]propyl}oct-4-en-1-yl}-4-{(1E,3E)-4-[4-(dimethylamino)phenyl]buta-1,3-dien-1-yl}-2,6-dimethylpyridin-1-ium trifluoroacetate (31).

The title compound was prepared from **28** (2.00 mg, 1.60 μmol), Py-5 (294 μg, 0.80 μmol) and NEt_3 (1.66 μL, 12 μmol) according to the general procedure, yielding **31** as a red solid (462 μg, 38%): RP-HPLC: 96%, (t_R = 19.68 min, k = 5.68) (eluent: MeCN/TFA (0.05% aq) 0 min: 10:90, 30 min: 60:40, 31–41 min: 80:20; flow rate = 0.75 mL/min). MS (ESI) *m*/*z* (%) 939.6 (1) $[M]^+$, 470 (90) $[M+H]^{2+}$, 327 (100) $[M+2H+MeCN]^{3+}$, 313 (11) $[M+2H]^{3+}$. $C_{50}H_{71}F_3N_{14}O_4S_2 \times 4$ TFA (1510.41).

4.2.15. *N*-{8-[3-(Amino{[3-(2-amino-4-methylthiazol-5-yl)propyl]amino}methylene)ureido]-5-{3-[3-(amino{[3-(2-amino-4-methylthiazol-5-yl)propyl]amino}methylene)ureido]propyl}oct-4-en-1-yl}propionamide (32a)

The reaction was carried out in a 1.5-mL Eppendorf reaction vessel. Compound **28** (13.62 μmol, 17.00 mg) was dissolved in 30 μL DMF and NEt_3 (102.20 μmol, 14.14 μL) was added. Succinimidyl propionate (16.36 μmol, 2.80 mg) was dissolved in 20 μL DMF and this solution was added to the mixture. The cup was rinsed a second time with 10 μL DMF and the reaction mixture was stirred for 2 h at room temperature. The reaction was stopped by adding 20 μL of 10% TFA. The crude product was purified by preparative HPLC, yielding **32a** as a white fluffy hygroscopic solid (13.18 mg, 81%): RP-HPLC: 94%, (t_R = 21.77 min, k = 7.54) (YMC C8 column); UV_{max} 204 nm and 264 nm. ^{1}H NMR (600 MHz, [D$_4$]MeOH) δ (ppm) 1.12 (t, 3H, *J* 7.6 Hz), 1.54 (tt, 2H, ^2J 7.0 Hz, ^3J 7.4 Hz), 1.62 (m, 4H), 1.90 (tt, 4H, ^2J 7.3 Hz, ^3J 7.4 Hz), 2.03–2.11 (bm, 6H), 2.16–2.21 (m, 8H), 2.72 (t, 4H, *J* 7.6 Hz), 3.14–3.20 (m, 6H), 3.33 (t, 4H, *J* 6.9 Hz), 5.23 (t, 1H, *J* 7.1 Hz). ^{13}C NMR (151 MHz, [D$_4$]MeOH, trifluoroacetate) δ (ppm) 10.6, 11.4, 23.6, 26.1, 27.9, 28.8, 29.1, 29.2, 30.2, 30.7, 34.7, 40.1, 40.4, 40.8, 41.4, 118.4, 126.6, 132.6, 139.3, 155.5, 156.0, 170.3, 177.0. HRMS (ESI) *m*/*z* (%) 734.4 (10) $[M+H]^+$, 367.7 (23) $[M+2H]^{2+}$, 245.5 (77) $[M+3H]^{3+}$; *m*/*z* $[M+H]^+$ calculated for $C_{32}H_{56}N_{13}O_3S_2^+$: 734.4065, found 734.4068. $C_{32}H_{55}N_{13}O_3S_2 \times 4$ TFA (1190.08).

4.2.16. *N*-{8-[3-(Amino{[3-(2-amino-4-methylthiazol-5-yl)propyl]amino}methylene)ureido]-5-{3-[3-(amino{[3-(2-amino-4-methylthiazol-5-yl)propyl]amino}methylene)ureido]propyl}oct-4-en-1-yl}-2,3-ditritiopropionamide (32b)

Succinimidyl [2,3-$^3H_2$]propionate was from Hartmann Analytic (Braunschweig, Germany), provided in EA (a_s = 2.96 TBq/mmol, 80 Ci/mmol; a_v = 18.5 GBq/mL, 5 mCi/mL). The reaction was carried out in a 1.5 mL Eppendorf reaction vessel. The precursor **28** (0.375 μmol, 468 μg) was dissolved in a mixture of 50 μL DMF and DIPEA (5.625 μmol, 0.96 μL). This mixture was added to the solved succinimidyl [2,3-$^3H_2$]propionate (41.46 nmol, 3.32 mCi). The EA was removed in a vacuum concentrator for about 45 min. Afterwards 9 μL EA and 1 μL DIPEA were added, and the mixture was stirred for 3 h with a magnetic stirrer at room temperature. 80 μL of 2% aq TFA and 280 μL Millipore water were added. The product was isolated with a Waters HPLC system (column: YMC–Triart C18 (250 × 6.0 mm, 5 μm). Eluent: mixtures of MeCN and 0.04% TFA (A) and 0.05% aq TFA (B), gradient 0–20 min A/B 5:95 to 30:70. The retention time of the radioligand was 18.4 min and the eluates were collected in a 1.5 mL reaction vessel. The combined fractions were concentrated in a vacuum concentrator to a volume of around 600 μL.

Quantification: The concentration of the radioligand was determined by a 4 point calibration with **32a**. The solutions for the calibration were prepared freshly prior to the injection. All of the solutions were prepared separately in mobile phase from a

20 μM solution of **32a**. Eluent: mixtures of MeCN and 0.04% TFA (A) and 0.05% aq TFA (B), gradient: 0–20 min A/B 5:95 to 26:74 (column: Phenomenex Luna C18 (150 × 4.6 mm, 3 μm). The molarity of the solution of **32b** was calculated from the peak areas of the standards.

Determination of the specific activity: 10 aliquots of the diluted radioligand in mobile phase were counted in 3 mL of Rotiszint eco plus (Roth, Karlsruhe, Germany) in a LS 6500 liquid scintillation counter (Beckmann-Coulter, München). The calculated specific activity was 2.60 tBq/mmol (70.15 Ci/mmol). The concentration was adjusted to 1 mCi/ml by adding 1291 μL of a mixture of Millipore water and EtOH (60:40). This yielded in a concentration of 14.25 μM.

The radioligand was obtained in a yield of 90% (37.34 nmol, 44.41 μg) with a radiochemical purity of 99%. $C_{32}H_{55}N_{13}O_3S_2 \times 4$ TFA (1190.08).

4.3. Pharmacological protocols

4.3.1. [^{35}S]GTPγS binding assay

[^{35}S]GTPγS was from PerkinElmer Life Science (Boston, USA) or Hartmann Analytic (Braunschweig, Germany). *Sf9* membranes expressing the hH_2R-$G_s\alpha_s$ or the gpH_2R-$G_s\alpha_s$ were thawed and sedimented by centrifugation at 13,000 *g* (4 °C) for 10 min. The membranes were resuspended in cold (4 °C) binding buffer BB (12.5 mM $MgCl_2$, 1 mM EDTA and 75 mM Tris/HCl, pH 7.4) so that the final concentration was 1 μg of protein per 1 μL BB. Experiments were performed in 96-well plates (PP microplates 96 well, Greiner Bio-One, Frickenhausen, Germany) in a total volume of 100 μL, containing 10 μg of membrane, 1 μM GDP, 0.05% BSA, 20 nCi [^{35}S]GTPγS and the investigated ligands at various concentration (dissolved in water). During the incubation period of 90 min, at room temperature, the plates were shaken at 250 rpm. Afterwards, bound [^{35}S]GTPγS was separated from free [^{35}S]GTPγS by filtration through GF/C filters using a 96-well Brandel harvester (Brandel Inc., Unterföhring, Germany). Filter bound radioactivity was either quantified by liquid scintillation counting or the filters were dried overnight and MeltiLex solid scintillator was melted onto the filtermats. Scintillation counting was performed with the MicroBeta21450 PlateCounter (Perkin Elmer, Rodgau, Germany).

4.3.2. Competition binding experiments

[^{3}H]mepyramine, [^{3}H]tiotidine, [^{3}H]histamine and [^{3}H]N^{α}-Methylhistamine were from Hartmann analytic (Braunschweig, Germany). *Sf9* membranes (general procedures for the generation of recombinant baculoviruses, culture of *Sf9* cells and membrane preparation have been described elsewhere[29]) were thawed and sedimented by centrifugation at 13,000 *g* and 4 °C for 10 min. The membranes were resuspended in cold (4 °C) binding buffer (BB: 12.5 mM $MgCl_2$, 1 mM EDTA and 75 mM Tris/HCl, pH 7.4) so that the final concentration was 2-6 μg of protein per 1 μL BB. Experiments were performed in 96-well plates (PP microplates 96 well, Greiner Bio-One, Frickenhausen, Germany) in a total volume of 100 μL containing 20–60 μg of membrane, 0.05% BSA, the respective radioligand and the investigated ligands at various concentrations (dissolved in water). During the incubation period of 60 min at room temperature, the plates were shaken at 250 rpm. Afterwards, bound radioligand was separated from free radioligand by filtration through, PEI covered, GF/C filters using a 96-well Brandel harvester (Brandel Inc., Unterföhring, Germany). After two washing steps with binding buffer, filter pieces were punched and transferred into untapped 96-well sample plates 1450–401 (Perkin Elmer, Rodgau, Germany). Each well was supplemented with 200 μL of scintillation cocktail (Rotiscint Eco plus, Roth, Karlsruhe, Germany) and incubated in the dark for 12 h. Radioactivity was measured with a MicroBeta2 1450 scintillation counter (Perkin Elmer, Rodgau, Germany).

4.3.3. Studies on human monocytes

The present study was approved by the Ethics Committee of the Hannover Medical School. Monocytes were isolated from peripheral blood of healthy human volunteers using density gradient centrifugation and magnetic activated cell sorting. To study the H_2R mediated responses in human monocytes, increases in cAMP levels were analyzed via reversed phase HPLC coupled to mass spectrometry (HPLC–MS/MS). For this purpose, 5×10^5 cells were treated with the ligands (1 nM–100 μM) in presence of 100 μM IBMX at 37 °C for 10 min. Inhibition of fMLP-induced production of ROS was investigated using the lucigenin chemiluminescence assay: 1×10^5 cells loaded with the chemiluminescence dye lucigenin were pre-incubated with the ligands (1 nM–100 μM) at 37 °C for 5 min. After adding fMLP (1 μM), the chemiluminescence was recorded at 37 °C for 60 min. These methods have been described in detail in Werner et al.[28]

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Supplementary data

Supplementary data (Preparation of **20**, ^{1}H NMR and ^{13}C NMR spectra of compounds **8**–**17**, **27** and **32a**, RP-HPLC chromatograms of compounds **8**–**17**, **28**–**31**, **32a**,**b**, binding experiments with **32b** on membranes of *Sf9* cells expressing the hH_2R-$G_s\alpha_s$ fusion protein, confocal microscope images of HEK293T cells upon incubation with **31**, cytotoxicity in the crystal violet based chemosensitivity assay, ethidium bromide/acridine orange staining of monocytes after incubation with compounds **9** and **14**, concentration–response curve of histamine in the [^{35}S]GTPγS binding assay.) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2015.01.012.

References and notes

1. Ghorai, P.; Kraus, A.; Keller, M.; Götte, C.; Igel, P.; Schneider, E.; Schnell, D.; Bernhardt, G.; Dove, S.; Zabel, M.; Seifert, R.; Buschauer, A. *J. Med. Chem.* **2008**, *51*, 7193.
2. Kraus, A.; Ghorai, P.; Birnkammer, T.; Schnell, D.; Elz, S.; Seifert, R.; Dove, S.; Bernhardt, G.; Buschauer, A. *ChemMedChem* **2009**, *4*, 232.
3. Birnkammer, T.; Spickenreither, A.; Brunskole, I.; Lopuch, M.; Kagermeier, N.; Bernhardt, G.; Dove, S.; Seifert, R.; Elz, S.; Buschauer, A. *J. Med. Chem.* **2012**, *55*, 1147.
4. Ghorai, P.; Kraus, A.; Birnkammer, T.; Geyer, R.; Bernhardt, G.; Dove, S.; Seifert, R.; Elz, S.; Buschauer, A. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 3173.
5. Portoghese, P. S. *J. Med. Chem.* **2001**, *44*, 2259.
6. Keller, M.; Erdmann, D.; Pop, N.; Pluym, N.; Teng, S.; Bernhardt, G.; Buschauer, A. *Bioorg. Med. Chem.* **2011**, *19*, 2859.
7. Gers, T.; Kunce, D.; Markowski, P.; Izdebski, J. *Synthesis* **2004**, 37.
8. Kim, K. S.; Qian, L. G. *Tetrahedron Lett.* **1993**, *34*, 7677.
9. Garcia-Delgado, N.; Bertrand, S.; Nguyen, K. T.; van Deursen, R.; Bertrand, D.; Reymond, J. L. *ACS Med. Chem. Lett.* **2010**, *1*, 422.
10. Wittig, G.; Schöllkopf, U. *Chem. Ber.* **1954**, *87*, 1318.
11. Craig, D. B.; Wetzl, B. K.; Duerkop, A.; Wolfbeis, O. S. *Electrophoresis* **2005**, *26*, 2208.
12. Wetzl, B. K.; Yarmoluk, S. M.; Craig, D. B.; Wolfbeis, O. S. *Angew. Chem., Int. Ed.* **2004**, *43*, 5400.
13. Hoefelschweiger, B. K.; Duerkop, A.; Wolfbeis, O. S. *Anal. Biochem.* **2005**, *344*, 122.
14. Caro, B.; Le Guen-Robin, F.; Salmain, M.; Jaouen, G. *Tetrahedron* **2000**, *56*, 257.
15. Brennauer, A.; Keller, M.; Freund, M.; Bernhardt, G.; Buschauer, A. *Tetrahedron Lett.* **2007**, *48*, 6996.
16. Xie, S. X.; Ghorai, P.; Ye, Q. Z.; Buschauer, A.; Seifert, R. *J. Pharmacol. Exp. Ther.* **2006**, *317*, 139.

17. Kelley, M. T.; Burckstummer, T.; Wenzel-Seifert, K.; Dove, S.; Buschauer, A.; Seifert, R. *Mol. Pharmacol.* **2001**, *60*, 1210.
18. Preuss, H.; Ghorai, P.; Kraus, A.; Dove, S.; Buschauer, A.; Seifert, R. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **2007**, *376*, 253.
19. Baumeister, P.; Erdmann, D.; Biselli, S.; Kagermeier, N.; Elz, S.; Bernhardt, G.; Buschauer, A. *ChemMedChem* **2015**, *10*, 83.
20. Pertz, H. H.; Gornemann, T.; Schurad, B.; Seifert, R.; Strasser, A. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **2006**, *374*, 215.
21. Schnell, D.; Strasser, A.; Seifert, R. *Biochem. Pharmacol.* **2010**, *80*, 1437.
22. Brunskole, I.; Strasser, A.; Seifert, R.; Buschauer, A. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **2011**, *384*, 301.
23. Igel, P.; Schneider, E.; Schnell, D.; Elz, S.; Seifert, R.; Buschauer, A. *J. Med. Chem.* **2009**, *52*, 2623.
24. Nordemann, U.; Wifling, D.; Schnell, D.; Bernhardt, G.; Stark, H.; Seifert, R.; Buschauer, A. *PLoS ONE* **2013**, *8*, e73961.
25. Schneider, E.; Mayer, M.; Ziemek, R.; Li, L.; Hutzler, C.; Bernhardt, G.; Buschauer, A. *ChemBioChem* **2006**, *7*, 1400.
26. Romero, A. I.; Thoren, F. B.; Aurelius, J.; Askarieh, G.; Brune, M.; Hellstrand, K. *Scand. J. Immunol.* **2009**, *70*, 194.
27. Martner, A.; Thoren, F. B.; Aurelius, J.; Soderholm, J.; Brune, M.; Hellstrand, K. *Expert Rev. Hematol.* **2010**, *3*, 381.
28. Werner, K.; Neumann, D.; Seifert, R. *Biochem. Pharmacol.* **2014**, *92*, 369.
29. Pop, N.; Igel, P.; Brennauer, A.; Cabrele, C.; Bernhardt, G.; Seifert, R.; Buschauer, A. *J. Receptors Signal Transduct.* **2011**, *31*, 271.