Highly Potent, Stable, and Selective Dimeric Hetarylpropylguanidine-Type Histamine H2 Receptor Agonists†

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Supporting Information

ABSTRACT: On the basis of the long-known prototypic pharmacophore 3-(1H-imidazol-4-yl)propylguanidine (SK&F 91486, 2), monomeric, homodimeric, and heterodimeric bisalkylguanidine-type histamine H2 receptor (H2R) agonists with various alkyl spacers were synthesized. Aiming at increased H2R selectivity of the ligands, the imidazol-4-yl moiety was replaced by imidazol-1-yl, 2-aminothiazol-5-yl or 2-amino-4-methylthiazol-5-yl according to a bioisosteric approach. All compounds turned out to be partial or full agonists at the hH2R. The most potent analogue, the thiazole-type heterodimeric ligand 63 (UR-Po461), was a partial agonist (Emax = 88%) and 250 times more potent than histamine (pEC50: 8.56 vs 6.16, gpH2R, atrium). The homodimeric structures 56 (UR-Po395) and 58 (UR-Po448) exhibited the highest hH2R affinities (pKi: 7.47, 7.33) in binding studies. Dimeric amino(methyl)thiazole derivatives, such as 58, generated an increased hH4R selectivity compared to the monomeric analogues, e.g., 139 (UR-Po444). Although monomeric ligands showed up lower affinities and potencies at the H2R, compounds with a short alkyl side chain like 129 (UR-Po194) proved to be highly affine hH1R ligands.

1. INTRODUCTION

In humans, the histamine receptor family comprises four subtypes, namely, H1, H2, H3, and H4 receptors. They are activated by the biogenic amine histamine (1, Figure 1) and belong to the superfamily of G-protein-coupled receptors (GPCRs). For more than three decades, 3-(1H-imidazol-4-yl)propylguanidine (SK&F 91486, 2, Figure 1) has been used as prototypic pharmacophore for the synthesis of highly potent histamine H2 receptor (H2R) agonists of the guanidine class, e.g., compounds such as arpromidine (3, Figure 1). The application of the bivalent ligand approach to acylguanidine-type H2R agonists by Birnkammer et al. led to highly potent and selective H2R agonists, e.g., UR-AK 381 (4, Figure 1), raising questions about the binding mode and usability of such dimeric ligands as pharmacological tools. Insufficient chemical stability of these acylguanidines, due to hydrolytic cleavage, led to carbamoylguanidine-type H2R agonists, which proved to be stable. As many class A GPCRs were reported to form homodimers, bivalent ligands can potentially be used as pharmacological tools to investigate the binding mode. Using different spacer lengths, Birnkammer et al. showed that an interaction of the second pharmacophore with an allosteric binding site at the same receptor protomer is more likely than binding to the second orthosteric binding site of an H2R homodimer.

For a better understanding of the structure–activity relationship of bisalkylguanidine-type dimeric H2R ligands, we prepared and pharmacologically characterized several monomeric and dimeric compounds derived from the recently reported homodimeric H2R agonist SK&F 93082 (5, Figure 1). In particular, the influence of different heteroaromatic ring systems and different spacer lengths on histamine receptor subtype selectivity was studied, using radioligand binding assays to investigate the affinities to the respective receptors. Investigations on H2R species selectivity were performed involving recombinant human, guinea pig, and rat H2Rs ([35S]GTPγS binding assay), which gave information about the type, affinity/potency, and efficacy of the receptor ligand. Organ pharmacological studies (gpH2R (ileum), gpH2R (right atrium)) afforded agonistic (H2R) and antagonistic (H1R) activities under more physiological conditions. Functional activities on the guinea pig ileum and right atrium were measured via the contractility of the tissue and the increase of the heart frequency, respectively. The main focus of this project was the development of H2R agonists. This also includes the characterization of numerous compounds at the other histamine receptors (H1,3,4R), which led to the identification of selective H2R ligands.

2. RESULTS AND DISCUSSION

2.1. Chemistry. The structures of amines 6−9, which were used for the synthesis of compounds 5, 53−63, 127−141, and...
in the presence of sodium hydride resulted in the guanidinylating reagent two diacid (TFA, Scheme 1). It should be mentioned that the use of deprotection of the intermediates more than 2 equiv of HgCl₂ led to a decrease in yield, presumably due to conversion of one heterodimeric ligands, the diamines (Scheme 2). Although the alkylated thioureas followed by Boc deprotection of the intermediates (Scheme 2), commercially available, were used for the synthesis of the monomeric compounds (Scheme 2). Finally, the precursors were deprotected using TFA to give (Scheme 2).

The synthetic strategy for the dimeric compounds was also used for the synthesis of the monomeric compounds (Scheme 2). Although the alkylated thioureas were commercially available, had to be synthesized by nucleophilic addition of the corresponding amine with 10 to give 70–75, followed by alkaline hydrolysis yielding the desired compounds (cf. Scheme 2). S-methylation was accomplished as described for the dimeric ligands with adapted amount of substance (Scheme 2). Finally, the precursors were deprotected using TFA to give (Scheme 2).

The cyclic guanidine derivative N-[3-((1H-imidazol-4-yl)-propyl]-1,4,5,6-tetrahydropyrimidin-2-amine (145) was prepared in a three-step synthesis starting from tetrahydropyrimidine-2(1H)-thione (142) (Scheme 3). Di-Boc-protection of 142 in the presence of sodium hydride resulted in 143 (Scheme 3), as described. After guanidinylation with 6, intermediate 144 was treated with TFA to receive 145 (Scheme 3).

Stability of Dimeric Alkylguanidines. The chemical stability of the bisalkylguanidine-type dimeric HR ligands was exemplarily investigated for compounds 57 and 58, which were incubated in a binding buffer (BB, pH 7.4) at a concentration of 100 μM at room temperature for 12 months. Reversed-phase high-performance liquid chromatography (RP-HPLC) analysis revealed that 57 and 58 exhibited, in contrast to the previously reported acylguanidine-type HR ligands, excellent chemical stabilities (Figure 3).

2.3. Pharmacology. The synthesized dimeric (5, 53–63) and monomeric (127–141 and 145) ligands were investigated in radioligand competition binding assays (hH₁,₂,₃,₄R), in the [³⁵S]GTPγS binding assay (hH₂,₃,₄R, gp/hH₂R), in the guinea pig ileum assay (gpH₂R), and in the guinea pig right atrium assay (gpH₂R). For the radioligand and the GTPγS binding assay, membranes of S9 cells, expressing hH₁R + RGS₄, h/gp/rH₂R + G₄α₅, hH₂R + G₄α₅ + Gβδ₂, or hH₂R G₄α₅ + Gβδ₂ were used.

2.3.1. Receptor Subtype Selectivity. To investigate the affinities at hH₁R, hH₂R, hH₃R, and hH₄R of the imidazol-4-yl-type homodimeric ligands 56 and 57, containing a decamethylene and a dodecamethylene spacer, respectively, exhibited the highest affinities at every HR subtype with the following selectivity profile: pKᵢ H₁R < H₂R ≈ H₃R ≈ H₄R (Table 1). The same selectivity profile was evident for the homodimeric compounds 5 and 53–55. The 2-amino-4-methylthiazol-5-yl- and 2-aminothiazol-5-yl-type homodimeric ligands 58 and 59, respectively, as well as the 2-amino-4-methylthiazol-5-yl-2-aminothiazol-5-yl-type heterodimeric compound 63 proved to be selective H₂R ligands with at least 1.5 log units difference in pKᵢ (H₂R) over pKᵢ (H₁,₂,₃,₄R) (Table 1). This demonstrated that replacement of the imidazol-4-yl by an 2-aminothiazol-5-yl moiety is bioisosteric with respect to H₂R binding, but not in...
case of H3 and H4 receptor binding, and was in accordance with previous reports on 2-aminothiazol-5-yl-type H2R selective ligands.\textsuperscript{22} The binding data at the hH1R showed values, which were dependent from the spacer length, respectively the lipophilicity. The replacement of imidazol-4-yl by a imidazol-1-yl ring (60) leads to a collapse of the affinities at all histamine receptor subtypes. The monomeric reference substances \textsuperscript{127−141} and \textsuperscript{145} displayed increasing affinities at the H2R with respect to their chain length, but lower affinities compared to the respective dimeric compounds. Overall, the affinities at the hH3,4Rs were higher, except of the aminothiazole-containing structures \textsuperscript{139−140}. Molecules with a small side chain like \textsuperscript{129} are of special interest as highly affine H4R ligands. Sigmoidal radioligand displacement curves at all HR subtypes are shown for 58, which exhibited the highest H2R selectivity (Figure 4).

2.3.2. Functional Characterization at the hH2,3,4R ([\textsuperscript{35}S]GTP\textsubscript{γ}S Binding Assay).

The compounds 5, 57−59, 61−63, and 136 were chosen to be investigated in the

ACS Omega 2018, 3, 2865−2882

Doi: 10.1021/acsomega.8b00128
GTP\(^{\gamma}S\) binding assay to determine their agonistic or antagonistic activities at the \(hH_{2,3,4}R\) (Table 2). All compounds proved to be partial agonists at the \(hH_{2}R\) and silent antagonists at the \(hH_{3}R\) and \(hH_{4}R\), except the monomeric ligands 136 and 139, which exhibited inverse agonistic activity at the \(hH_{3}R\) and the \(hH_{4}R\), respectively. The homodimeric ligand 58, containing a \(C_{12}\)-spacer, showed the highest \(H_{2}R\) potency with a \(pEC_{50}\) of 7.27 and a maximal response of 52% relative to histamine. At the \(H_{3}R\), the antagonistic activities of imidazole-containing ligands (5, 57, 61, 62, and 136) were considerably higher than those of compounds with an amino(methyl)thiazole moiety (58, 59, 63, and 139) (e.g., \(pK_B\) values of 57 and 59: 7.33 vs 4.05; Table 2). Antagonistic activities at the \(hH_{1}R\) were throughout low (\(pK_B < 4\)).

### H\(_{2}\) Species Selectivity

To study the \(H_{2}\) species selectivity of the bisalkylguanidine-type dimeric ligands, the agonistic activities of compounds 5, 57, 58, and 63 were also investigated at the \(gpH_{2}R\) and \(rH_{2}R\) in the GTP\(^{\gamma}S\) binding assay. The studied compounds exhibited slightly higher agonistic potencies at the \(gpH_{2}R\) and \(rH_{2}R\) compared to the
hHR, with pEC_{50} values >7.2 and >6.8, respectively (Table 3). Compound 5 showed the highest ghHR potency (pEC_{50} = 7.60) with a high efficacy (E_{\max} = 0.95), and 57 displayed the highest rhHR potency (pEC_{50} = 7.61) with an efficacy of 0.80. In general, the efficacies were considerably higher at the ghHR and rhHR (E_{\max} > 0.7) compared to the hhHR (E_{\max} < 0.52), reaching full agonism in case of compound 63 (ghHR, E_{\max} = 1.02). In sum, these investigations revealed that the highest potencies and efficacies were observed at the ghHR followed by the rhHR and the hhHR.

2.3.4. Organ Bath Studies. In addition to the pharmacological characterization by radioligand competition binding (cf. Table 1) and by functional studies using the [35S]GTP\gammaS binding assay (cf. Tables 2 and 3), organ bath experiments at the guinea pig ileum (ghHR) and at the spontaneously beating guinea pig right atrium (rhHR) were carried out. All ligands (5, 53−63, 127−141, and 145) displayed antagonistic activities at the ghHR (Table 4). The imidazol-4-yl-type homodimeric ligands 57 and the monomeric compound 137, containing a dodecamethylene spacer and a decamethyl side chain, respectively, showed the highest pA_{2} values (6.91 and 6.74, respectively, cf. Table 4). A variation of the heteroaromatic moieties did almost not affect the pA_{2} value (ghHR), as shown for compounds containing a C_{8}-spacer (5 and 58−63). The experiments at the ghHR provided interesting information about the structure−activity relationship from monomeric and dimeric HR ligands. Regarding the imidazole-containing ligands (5, 53−57, and 127−138), longer alkyl spacers resulted in higher agonistic potencies (e.g., pEC_{50} of 53 (C_{3}-spacer) and 57 (C_{12}-spacer): 7.31 vs 8.11, pEC_{50} of 127 (methyl) and 138 (dodecyl): 5.10 vs 6.63). By contrast, an inverse correlation was observed for the dimeric compounds with respect to the efficacy: compound 53, containing a C_{3}-spacer, acted nearly as a full agonist (E_{\max} = 0.96), and 57, containing a C_{12}-chain, exhibited a maximum response of 0.63 compared to histamine (Table 4). With the aim of getting compounds of highest potency and efficacy, 58−63 were equipped with a C_{8}-spacer as a prototype. The replacement of imidazol-4-yl (5 and 138) by imidazol-1-yl (60 and 141) led to a drastic decrease in potency and efficacy (5 vs 60: pEC_{50}: 7.98 vs 5.31, E_{\max}: 0.91 vs 0.20; 138 vs 141: pEC_{50}: 6.63 vs not active, E_{\max}: 0.91 vs 0). The introduction of amino(methyl)thiazole moieties (58, 59, and 61−63) resulted in potent ghHR agonists (pEC_{50}: 7.69−8.56, E_{\max}: 0.78−1.02, Table 4). Overall, a switch from monomeric to dimeric ligands revealed compounds, which are approximately 100 times more potent than their monomeric analogues. The concentration−
**Table 1. Binding Data (pKᵢ Values) of Compounds Diphenhydramine (DPH), 1, 2, 5, 53–63, 127–141, and 145 Determined at Human HᵢRₐs (ᵦ = 1–4)**

| compound | hHᵢR⁺<sub>β</sub> | hHᵢR⁺<sub>c</sub> | hHᵢR⁺<sub>b</sub> | hHᵢR⁺<sub>c</sub> |
|-----------|------------------|------------------|------------------|------------------|
| DPH | 7.62 ± 0.01 | n.d.<sup>a</sup> | n.d.<sup>a</sup> | n.d.<sup>a</sup> |
| 1 | 5.62 ± 0.03<sup>b</sup> | 6.58 ± 0.04 | 7.59 ± 0.01 | 7.60 ± 0.01 |
| 2 | <4 | 5.39 ± 0.04 | 7.42 ± 0.04 | 8.13 ± 0.08 |
| 5 | 5.50 ± 0.01 | 7.05 ± 0.02 | 7.52 ± 0.01 | 7.06 ± 0.01 |
| 53 | <5 | 6.76 ± 0.03 | 6.95 ± 0.02 | 6.70 ± 0.01 |
| 54 | <5 | 6.39 ± 0.02 | 6.84 ± 0.01 | 6.18 ± 0.04 |
| 55 | <5.5 | 6.82 ± 0.04 | 7.28 ± 0.03 | 6.37 ± 0.02 |
| 56 | 5.90 ± 0.01 | 7.47 ± 0.12 | 7.72 ± 0.03 | 7.68 ± 0.04 |
| 57 | 6.45 ± 0.01 | 7.41 ± 0.03 | 7.79 ± 0.01 | 7.70 ± 0.01 |
| 58 | <5.5 | 7.33 ± 0.05 | 5.25 ± 0.05 | 5.00 ± 0.05 |
| 59 | <5 | 6.63 ± 0.03 | 4.96 ± 0.05 | 4.28 ± 0.02 |
| 60 | <5 | 5.35 ± 0.03 | 5.56 ± 0.02 | 4.47 ± 0.03 |
| 61 | <5 | 6.93 ± 0.04 | 7.49 ± 0.03 | 7.13 ± 0.04 |
| 62 | <5 | 7.27 ± 0.04 | 7.43 ± 0.03 | 6.97 ± 0.05 |
| 63 | <5.5 | 6.91 ± 0.04 | 5.40 ± 0.05 | 5.14 ± 0.04 |
| 127 | <4.5 | 5.56 ± 0.07 | 6.81 ± 0.03 | 7.58 ± 0.07 |
| 128 | <4.5 | 5.31 ± 0.05 | 7.03 ± 0.04 | 7.87 ± 0.01 |
| 129 | <4.5 | 5.52 ± 0.05 | 7.21 ± 0.02 | 8.04 ± 0.05 |
| 130 | <5 | 5.38 ± 0.07 | 7.04 ± 0.02 | 7.42 ± 0.01 |
| 131 | <5 | 6.11 ± 0.06 | 7.21 ± 0.04 | 8.04 ± 0.02 |
| 132 | <4.5 | 6.12 ± 0.05 | 7.18 ± 0.03 | 7.75 ± 0.03 |
| 133 | <4 | 5.60 ± 0.10 | 6.43 ± 0.03 | 6.66 ± 0.06 |
| 134 | <5 | 6.03 ± 0.06 | 7.04 ± 0.02 | 8.17 ± 0.04 |
| 135 | <5 | 6.10 ± 0.06 | 6.94 ± 0.04 | 7.60 ± 0.01 |
| 136 | <5.5 | 6.96 ± 0.07 | 6.97 ± 0.04 | 6.90 ± 0.01 |
| 137 | 5.70 ± 0.01 | 6.85 ± 0.09 | 7.50 ± 0.03 | 7.01 ± 0.03 |
| 138 | 5.53 ± 0.01 | 6.22 ± 0.01 | 7.53 ± 0.02 | 7.90 ± 0.03 |
| 139 | <5.5 | 6.33 ± 0.04 | 5.69 ± 0.01 | 5.25 ± 0.03 |
| 140 | <5.5 | 6.57 ± 0.03 | 4.85 ± 0.06 | 4.95 ± 0.05 |
| 141 | <5.5 | 5.90 ± 0.01 | 5.19 ± 0.01 | 4.89 ± 0.06 |
| 145 | <5 | 5.50 ± 0.02 | 6.73 ± 0.05 | 7.42 ± 0.01 |

<sup>a</sup>Data represent mean values ± standard error of the mean (SEM) from at least two independent experiments (N), each performed in triplicate.

<sup>b</sup>Dissociation constant (pA₂, 30 nM) determined by radioligand binding experiments in the presence of cimetidine (30 nM) at membranes of Sf9 cells expressing the respective receptor. Values are given for the respective radioligand, i.e., [³H]mepyramine (hHᵢR, Kᵢ = 4.5 nM, c = 5 nM), [³H]H₂R⁻ (hHᵢR, Kᵢ = 19.7 nM, c = 10 nM), [³H]hantagonist (hHᵢR, Kᵢ = 8.6 nM, c = 3 nM), [³H]H₁R⁻ (hHᵢR, Kᵢ = 16.0 nM, c = 5 nM), and [³H]H₄R⁻ (hHᵢR, Kᵢ = 31.3 nM, c = 20 nM).

The maximum positive effect of the investigated (partial) agonists in the guinea pig right atrium assay was mediated via the H₄R.
Aiming at an attempt to explain aminergic GPCR activation and ligand binding,27 by the fact that they were proven to act as key player in but varying a account for receptor selectivity (D186 5.42 at −K

Figure 4. Radioligand displacement curves from radioligand competition binding experiments performed with compound S8 and [3H]mepyramine ([H]R, K, 4.5 nM, c = 5 nM), [3H]tiotidine ([H]R, K, 19.7 nM, c = 10 nM), [3H]N(-methylhistamine ([H]R, K, 8.6 nM, c = 3 nM), or [3H]histamine ([H]R, K, 16.0 nM, c = 15 nM) at membranes of S9 cells expressing the respective hHR. Data represent mean values ± SEM from at least two independent experiments, each performed in triplicate.

D94,32, E182,5,46 for 129 at hHR (cf. Figure S32). The probability for an interaction with these residues is supported by the fact that they were proven to act as key player in aminergic GPCR activation and ligand binding.27 D94,32, E182,5,46 for 129 at hHR (cf. Figure S32). The probability for an interaction with these residues is supported by the fact that they were proven to act as key player in aminergic GPCR activation and ligand binding.27 Interestingly, both S and S8 showed high binding affinities at hHR (K < 100 nM), low affinities at the hH2R (K > 1 μM), but varying affinities at the hH2R and hH4R (5: K < 100 nM; S8: K > 1 μM). Although, by contrast, the monomeric imidazole-type analogue 129 bound unexpectedly poor to the hH2R, and, comparable to the dimeric compounds, also to the hH2R (K > 1 μM), it bound to the hH4R and hH4R with high affinity (K < 100 nM). In this context, an amino(methyl)-thiazole moiety present in S8, unlike an imidazole moiety present in S or 129, was shown to trigger hHR selectivity also in the case of other ligands.25,52,33 Aiming at an attempt to explain this behavior, different steric effects of amino acids enclosing the orthosteric binding pocket may come into play due to less voluminous residues at the hH4R (V99,32,33, V176,32,54, Q177,32,53) compared to hH4R (Y109,32,33, F184,32,54, Y185,32,53), hH2R (Y115,32,33, F192,32,54, F193,32,53), and hH3R (Y95,32,33, F168,32,54, F169,32,53). In addition, an absence (hH4R) or different locations (hH4R: E206,46; hH2R: E182,46) of fundamental acidic amino acids in TM5 compared to hH4R, may contribute to a lower hH4R selectivity of amino(methyl)thiazole-type compounds, but to preferential binding of imidazole-type ligands, such as 5 or 129, to the hH2R and hH2R. Although 5 is still capable of binding to hH2R, most probably due to its larger and more flexible chain, 129 merely binds to the hH2R and hH2R with high affinity in a well-defined binding mode. Noteworthy, the lowest free-energy pose (pose 1) of 129 bound to the hH4R showed H-bond contacts with E163,32,54 and T178,42 in addition to contacts with D94,32 and E182,46, compared to relatively few and weak H-bond interactions when bound to hH2R (Figures 7 and S32). This may contribute to a lower 129 binding free energy when bound to the hH4R, compared to hH2R (Figure S28).

3. SUMMARY AND CONCLUSIONS

Homo- (5 and S3–60) and heterodimeric (61–63) as well as monomeric (127–141 and 145) hetarylpropylguanidinetype HR ligands were obtained in excellent yield by a six-step synthesis. The replacement of the imidazolyl by an amino-thiazolyl moiety led, in accordance with previous reports on acylguanidine- and carbamoylguanidine-type HR ligands,9,10,22 to highly selective and potent H-R agonists. The variation of the spacer length revealed best results for compounds containing a Cγ-c, Cσ-c, or Cα-c spacer (5, S6–59, and 61–63). The heterodimeric compounds showed potencies in a one-digit nanomolar range (up to 250 times the potency of histamine) as full agonists in the gpHR-R assay. In comparison to the monomeric ligands, the dualistic structures showed up notably higher hH4R selectivity, higher affinities in the hH4R binding assay, and higher potencies at the functional H4R assays (e.g., guinea pig right atrium). The dimeric ligands displayed a slightly higher sensitivity for gpHR-R and H2R compared to the

Table 2. Agonistic (pEC50) and Antagonistic (pKb) Activities of 1, 2, 5, 57–59, 61–63, 136, and 139 at the hH3,3,4,5 Determined in the [35S]GTPγS Binding Assay

| compound | pEC50 (μM) | pKb | N   | pEC50 (μM) | pKb | N   |
|---------|------------|-----|-----|------------|-----|-----|
| 1       | 6.01 ± 0.07 | 100 | 7   | 8.52 ± 0.10 | 100 | 6   |
| 2       | 5.59 ± 0.01 | 6.66 ± 0.02 | 3   | 8.12 ± 0.10 | 6.69 ± 0.04 | 3   |
| 5       | 6.78 ± 0.01 | 0.50 ± 0.03 | 3   | (6.87 ± 0.05) | 0   | 3   |
| 57      | 7.27 ± 0.05 | 0.52 ± 0.03 | 6   | (7.33 ± 0.07) | 0   | 3   |
| 68      | 6.61 ± 0.03 | 0.33 ± 0.03 | 5   | (4.53 ± 0.05) | 0   | 3   |
| 59      | 6.53 ± 0.08 | 0.40 ± 0.05 | 3   | (4.05 ± 0.10) | 0   | 3   |
| 61      | 6.23 ± 0.09 | 0.54 ± 0.05 | 3   | (7.18 ± 0.02) | 0   | 3   |
| 62      | 6.51 ± 0.03 | 0.45 ± 0.04 | 6   | (7.09 ± 0.01) | 0   | 3   |
| 63      | 6.60 ± 0.02 | 0.47 ± 0.03 | 3   | (4.67 ± 0.05) | 0   | 3   |
| 136     | 6.88 ± 0.08 | 0.48 ± 0.02 | 3   | (7.54 ± 0.06) | −0.53 ± 0.03 | 3   |
| 139     | 5.16 ± 0.03 | −0.43 ± 0.01 | 3   | (3.57 ± 0.04) | 0   | 3   |

Data represent mean values ± SEM from at least three independent experiments (N), each performed in triplicate. Data were analyzed by nonlinear regression and were best-fitted to sigmoidal concentration–response curves (CRCs).19 [35S]GTPγS binding assay at membranes of S9 cells expressing the hH4R + Gα16, [35S]GTPγS binding assay at membranes of S9 cells expressing the hH4R + Gα6, Gβγ−γ, pEC50 = −log EC50, Emax: maximal response relative to histamine (Emax = 1.00). For determination of antagonism, reaction mixtures contained histamine (1) (100 nM), and ligands were at concentrations from 10 nM to 1 mM; pKb = −log Kb. Determined in a steady-state [32P]GTPase assay on S9 cells expressing the related receptors.
5 guanidine groups with the acidic residues D98, 32 and D186, 42 relative to the histamine reference curve by equation pEC50 = 6.16 + Gs.

Studies at the role in di...induced by histamine (ΔpEC50 = 141, and 145 Determined by Organ Bath Studies at the gh1R (Ileum) and the gh2R (Atrium)44.

Table 3. Agonistic Activities of 1, 5, 57, 58, and 63 at the h1R/h2R Determined in the [35S]GTPγS Binding Assay44

| Compound | pEC50 | Emax | N | pEC50 | Emax | N |
|----------|-------|------|---|-------|------|---|
| 1        | 6.01 ± 0.07 | 1.00 | 7 | 5.82 ± 0.02 | 1.00 | 3 | 5.97 ± 0.02 | 1.00 | 3 |
| 5        | 6.78 ± 0.01 | 0.50 ± 0.03 | 6 | 7.60 ± 0.02 | 0.95 ± 0.03 | 5 | 7.18 ± 0.07 | 0.80 ± 0.01 | 3 |
| 57       | 7.27 ± 0.05 | 0.52 ± 0.03 | 6 | 7.53 ± 0.03 | 0.89 ± 0.07 | 3 | 7.61 ± 0.10 | 0.80 ± 0.03 | 3 |
| 58       | 6.61 ± 0.03 | 0.33 ± 0.03 | 6 | 7.28 ± 0.05 | 0.97 ± 0.06 | 3 | 6.83 ± 0.09 | 0.70 ± 0.04 | 3 |
| 63       | 6.60 ± 0.02 | 0.47 ± 0.03 | 6 | 7.33 ± 0.03 | 1.02 ± 0.04 | 3 | 6.85 ± 0.07 | 0.87 ± 0.02 | 3 |

Data represent mean values ± SEM from at least three independent experiments (N), each performed in triplicate. Data were analyzed by nonlinear regression and were best-fitted to sigmoidal CRCS. 4pEC50 = 6.16 + Gs. 5pEC50 was calculated from the mean-corrected shift ΔpEC50. 6pEC50 was calculated from the mean-corrected shift ΔpEC50 of the agonist curve relative to the histamine reference curve by equation pEC50 = 6.16 + ΔpEC50. 7Emax: maximal response relative to histamine (Emax = 1.00). 

Table 4. Agonistic (pEC50) and Antagonistic (pA2) Activities of 1, 2, 5, 53–63, 127–141, and 145 Determined by Organ Bath Studies at the gh1R (Ileum) and the gh2R (Atrium)44

| Compound | gh1R | gh2R |
|----------|------|------|
| 1        | (6.68 ± 0.03) | 255 |
| 2        | n.a.  | 24   |
| 5        | 5.77 ± 0.04 | 9    |
| 53       | 6.20 ± 0.03 | 9    |
| 54       | 5.89 ± 0.03 | 9    |
| 55       | 5.69 ± 0.04 | 9    |
| 56       | 6.64 ± 0.05 | 8    |
| 57       | 6.91 ± 0.04 | 9    |
| 58       | 5.88 ± 0.03 | 9    |
| 59       | 6.08 ± 0.03 | 9    |
| 60       | 5.37 ± 0.05 | 9    |
| 61       | 5.85 ± 0.04 | 6    |
| 62       | 6.01 ± 0.05 | 6    |
| 63       | 6.06 ± 0.04 | 6    |
| 127      | 4.23 ± 0.02 | 4    |
| 128      | 5.08 ± 0.03 | 10   |
| 129      | 4.83 ± 0.05 | 8    |
| 130      | 5.27 ± 0.04 | 10   |
| 131      | 5.39 ± 0.04 | 11   |
| 132      | 4.99 ± 0.03 | 14   |
| 133      | n.d.  | 0    |
| 134      | 5.35 ± 0.04 | 12   |
| 135      | 5.14 ± 0.03 | 19   |
| 136      | 6.10 ± 0.06 | 8    |
| 137      | 6.74 ± 0.05 | 12   |
| 138      | 6.71 ± 0.05 | 12   |
| 139      | 6.36 ± 0.06 | 6    |
| 140      | 6.43 ± 0.07 | 6    |
| 141      | 6.43 ± 0.06 | 6    |
| 145      | 5.24 ± 0.04 | 9    |

As key contributions to H2R binding. As the bisalkylguanidines turned out to be very stable chemical entities, the synthesized H2R selective aminothiazole derivatives represent promising lead structures for the development of pharmacological tools for the H2R, such as S8, 59, and 63. Moreover, the presented data could be of interest for the development of CNS penetrating H2R agonists—in consideration of changing the basicity of the strongly basic alkylguanidine structure, using.
bioisosteric approaches, and the lipophilicity—and H₂R ligands useful for the treatment of acute myeloid leukemia.

Figure 5. CRCs of histamine (reference) and 63 in the absence (A) and presence (B) of 30 μM cimetidine at the gpH₂R (atrium). Displayed curves were calculated by endpoint determination (N = 1).

Figure 6. Most probable binding mode of 5 (A) and 58 (B) at the hH₂R, showing the respective most predominant clustered structure with the lowest binding free energy of the docking poses investigated by 30 ns MD simulations. Fundamental amino acids involved in ligand binding are shown as light-blue sticks, 5 is colored in green and 58 in magenta. H-bond contacts are illustrated as dashed yellow lines. The allosteric binding site is located most likely between ECL2 and ECL3.
4. EXPERIMENTAL SECTION

4.1. General Conditions. Commercially available chemicals (9, 10, 11–16, 64–69, and 82–87) and solvents were purchased from Acros Organics (Geel, Belgium), Alfa Aesar GmbH & Co. KG (Karlsruhe, Germany), Iris Biotech GmbH (Marktredwitz, Germany), Merck KGaA (Darmstadt, Germany), Sigma-Aldrich Chemie GmbH (München, Germany), or TCI Europe (Zwijndrecht, Belgium) and were used as received. Deuterated solvents for nuclear magnetic resonance (1H NMR and 13C NMR) spectra were purchased from Deutero GmbH (Kastellaun, Germany). Compounds 6–8 were prepared as previously described.22,32,36,37 The synthesis steps described in Section 2.1 were carried out according to reported procedures.8,17–19 All reactions involving dry solvents were accomplished in dry flasks under nitrogen or argon atmosphere. Millipore water was used for the preparation of buffers, HPLC eluents, and stock solutions. Column chromatography was carried out using Merck silica gel Geduran 60 (0.063–0.200 mm) or Merck silica gel 60 (0.040–0.063 mm) (flash column chromatography). The reactions were monitored by thin-layer chromatography (TLC) on Merck silica gel 60 F254 aluminum sheets, and spots were visualized under UV light at 254 nm, by iodine vapor, ninhydrin, or fast blue B staining.

Nuclear magnetic resonance (1H NMR and 13C NMR) spectra were recorded on a Bruker (Karlsruhe, Germany) Avance 300 (1H: 300 MHz, 13C: 75 MHz) or Avance 400 (1H: 400 MHz, 13C: 101 MHz) spectrometer using perdeuterated solvents. The chemical shift δ is given in parts per million (ppm). Multiplicities were specified with the following abbreviations: s (singlet), d (doublet), t (triplet), q (quartet), quint (quintet), m (multiplet), and br (broad signal) as well as combinations thereof. 13C NMR peaks were determined by distortionless enhancement by polarization transfer (DEPT) 135 and DEPT 90: “+” primary and tertiary carbon atom (positive DEPT 135 signal), “−” secondary carbon atom (negative DEPT 135 signal), and “quat” quaternary carbon atom. NMR spectra were processed with MestReNova 11.0

Figure 7. Most probable binding modes of 129 at both the hH3R (A) and hH4R (B), showing the respective most predominant clustered structure with the lowest binding free energy of the docking poses investigated by 30 ns MD simulations. Fundamental amino acids involved in ligand binding are shown as light-blue sticks, 129 is colored in cyan when bound to the hH3R (A) and in orange when bound to the hH4R (B). H-bond contacts are illustrated as dashed yellow lines. The allosteric binding site is located most likely between ECL2 and ECL3 in both cases.
(Mestrelab Research, Compostela, Spain). High-resolution mass spectrometry (HRMS) was performed on an Agilent 6540 UHD Accurate-Mass quadrupole time-of-flight liquid chromatography/mass spectrometry (MS) system (Agilent Technologies, Santa Clara, CA) using an electrospray ionization (ESI) source. Elemental analyses (EA) were performed on a Heraeus Elementar Vario EL III and are within ±0.4% unless otherwise noted. Melting points (mp) were measured on a Büchi (Essen, Germany) B-545 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 and a K-2001 detector. A Eurospher-100 C18 (250 × 32 mm², 5 µm) (Knauer, Berlin, Germany) or a Kinetex XB-C18 (250 × 21.2 mm², 5 µm) (Phenomenex Ltd., Aschaffenburg, Germany) served as stationary phase. As mobile phase, 0.1% TFA in millipore water and acetonitrile (MeCN) were used. The temperature was 25 °C, the flow rate was 15 mL/min, and UV detection was performed at 220 nm. Analytical HPLC was performed on system from Merck-Hitachi (Darmstadt/Duernberg, Germany) composed of an L-6200-A pump, an AS 2000A autosampler, an L-4000A UV detector, and a D-6000 interface. Stationary phase was a Merck-Hitachi (Darmstadt/Duernberg, Germany) or a Kinetex XB-C18 (250 × 46 mm², 5 µm) (Phenomenex, Aschaffenburg, Germany). As mobile phase, mixtures of MeCN and aqueous TFA were used (linear gradient: MeCN/TFA (0.1%) (v/v) 0 min: 5:95, 25 min: 50:50, 26—35 min: 95:5 (method A); 0 min: 10:90, 25 min: 50:50, 26—35 min: 95:5 (method B); flow rate = 0.80 mL/min, t₀ = 3.32 min). Capacity factors were calculated according to k = (tᵢ − t₀)/t₀. Detection was performed at 220 nm. All compounds were analyzed using method A, except for 139−141 (method B). Furthermore, filtration of the stock solutions with poly(tetrafluoroethylene) filters (25 mm, 0.2 µm) (Phenomenex Ltd., Aschaffenburg, Germany) was accomplished before testing. Compound purities determined by HPLC were calculated as the peak area of the analyzed compound in % relative to the total peak area (UV detection at 220 nm). The HPLC purities (see Supporting Information) of the final compounds were ≥95% except for compound 58 (94.3%). The tested compounds were screened for pan-assembly interference compounds (PAINS) and aggregation by publicly available filters (http://zinc15.docking.org/patterns/home, http://advisor.docking.org).56 None of the screened structures have been previously reported as PAINS or an aggregator. Since Devine et al. described 2-aminothiazoles as a promiscuous frequent hitting scaffold at different enzymes,40 full dose−response curves for all experiments and compounds, not only for the 2-aminothiazoles, were measured. None of the curves showed abnormalities, e.g., high Hill slopes, which could be a hint for PAINS.59

4.2. Chemical Synthesis and Analytical Data.

4.2.1. General Procedure for the Synthesis of the Dibenzo[ghi]thiaoareas 17−22. To an ice-cold solution of the pertinent diamine (11−16, 1 equiv) in dichloromethane (DCM), benzyol isothiocyanate (2 equiv) in DCM was added dropwise. The reaction was allowed to stir at room temperature (rt) overnight, and the organic solvent was concentrated under vacuum. The residue was suspended in 80 mL of methanol (MeOH) for 1 h and filtered to give the pure title compound.

4.2.1.1. N,N′-(Octane-1,8-diyli bis(azanediyl))bis[carbonothiolylidibenzoamide] (20). The title compound was prepared from octane-1,8-diamine (14, 1.08 g, 7.50 mmol) and 10 (2.02 mL, 15.00 mmol) in DCM (30 mL) according to the general procedure (Rᵢ = 0.40 in ethyl acetate (EtOA)/Hex 1:3). The product was obtained as a yellow solid (3.30 g, 93%), mp 146.8 °C. 1H NMR (300 MHz, CDCl₃) δ (ppm) 10.74 (brs, 2H), 9.00 (brs, 2H), 7.88−7.78 (m, 4H), 7.68−7.57 (m, 2H), 7.56−7.45 (m, 4H), 3.71 (q, J = 7.2 Hz, 4H), 1.72 (quint, J = 6.7 Hz, 4H), 1.54−1.29 (m, 8H). 13C NMR (75 MHz, CDCl₃) δ (ppm) 179.7, 166.9, 133.6, 131.8, 129.2, 127.4, 45.9, 29.1, 28.2, 26.8. HRMS (ESI-MS): m/z [M + H⁺] calculated for C₂₄H₃₁N₄O₂S₂: 471.1883, found 471.1884, C₂₃H₂₉N₄O₂S₂ (470.65).

4.2.2. General Procedure for the Synthesis of the Bisthioureas 23−28. The corresponding dibenzo[ghi]thiaoarea (17−22, 1 equiv) was stirred in a solution of K₂CO₃ (4.1 equiv) in MeOH/H₂O (7/3 v/v) for 3−5 h at rt. The proportion of MeOH was evaporated, and the resulting suspension was stirred for 1 h. The pure product was filtered with a Büchner funnel.

Because of the thione−thiol tautomeration, a splitting of the NH−CH₂−(CH₂)₆−CH₂−NH signal could be observed in the following NMR spectra. Two broad singlets could be noted right next to each other. In each case, the integration value was exactly 4. For all of the other symmetric CH₂ peaks, this peak splitting was not shown.

4.2.2.1. 1,1′-(Octane-1,8-diyli)bis(thiourea) (26). The title compound was prepared from 20 (3.30 g, 7.01 mmol) and K₂CO₃ (3.88 g, 28.75 mmol) in MeOH/H₂O (7/3 v/v, 100 mL) according to the general procedure (Rᵢ = 0.43 in DCM/MeOH/NH₃ (29−34)). The reaction mixture was stirred for 1 h under refluxing and the solvent was evaporated under vacuum. The resulting product (di-HI salt) was washed with three times with 20 mL of diethylether (Et₂O) and dried under vacuum.

4.2.3. General Procedure for the Synthesis of the Bis-S-methylthiaoareas (29−34). The appropriate bithiaoarea (23−28, 1 equiv) was dissolved in 50 mL of acetonitrile (MeCN) and treated with methyl iodide (2.1 equiv). The reaction mixture was stirred for 1 h under refluxing and the solvent was evaporated under vacuum. The resulting product (di-HI salt) was washed three times with 20 mL of diethyl ether (Et₂O) and dried under vacuum.

4.2.3.1. 1,1′-(Octane-1,8-diyli)bis(5-methylthiaoarea) (32). The compound 26 (1.80 g, 6.86 mmol) was dissolved in MeCN (50 mL) and treated with methyl iodide (0.90 mL, 14.40 mmol) according to the general procedure (Rᵢ = 0.16 in DCM/MeOH/NH₃ (29−34)). The resulting product was obtained as a yellow oil (32−2H, 3.70 g, 99%). 1H NMR (300 MHz, CD₃OD, hydrogen iodide) δ (ppm) 3.38 (t, J = 7.2 Hz, 4H), 2.65 (s, 6H), 1.66 (quint, J = 7.2 Hz, 4H), 1.44−1.36 (m, 8H). 13C NMR (75 MHz, CD₃OD, hydrogen iodide) δ (ppm) 170.0, 45.5, 30.2, 29.0, 27.7, 14.4. HRMS (ESI-MS): m/z [M + H⁺] calculated for C₁₂H₁₈N₂S₂: 291.1672, found 291.1674; C₁₁H₁₆N₂S₂·2Hİ (546.32).

4.2.4. General Procedure for the Synthesis of the Bis-N′-boc-S-methylthiaoareas (35−40). To a solution of the pertinent isothiourea (29−34) and 2 equiv of triethylamine (NE₃) in 50 mL of DCM, a solution of Boc₂O (2 equiv) in 20 mL of DCM was added dropwise at rt. The reaction mixture was stirred overnight (rt) and washed with H₂O and a saturated
solution of NaCl. The organic layer was dried over Na₂SO₄ and the crude product was purified by column chromatography (EtOAc/petroleum ether (PE) 1/4–1/2 v/v).

4.2.4.1. 1,1’-(Octane-1,8-diyl)bis(N-tert-butoxycarbonyl-S-methylthioioureia) (38). The reaction was carried out with 32 (3.70 g, 6.77 mmol), NEt₃ (1.88 mL, 13.55 mmol), and Boc₂O (2.96 g, 13.55 mmol) according to the general procedure (Rₑ = 0.62 in EtOAc/Hex 1:2), yielding a colorless oil (3.30 g, 99%). ¹H NMR (300 MHz, CDCl₃) δ (ppm) 9.56 (brs, 2H), 3.28 (q, J = 7.0 Hz, 4H), 2.45 (s, 6H), 1.65–1.56 (m, 4H), 1.49 (s, 18H), 1.38–1.30 (m, 8H). ¹³C NMR (75 MHz, CDCl₃) δ (ppm) 173.5, 162.3, 79.2, 43.8, 29.3, 29.0, 28.3, 26.7, 13.6. HRMS (ESI-MS): m/z [M + H⁺] calculated for C₃₂H₆₃N₂O₆S₂: 491.2720, found 491.2721; C₂₂H₄₅N₂O₆S₂ (490.72).

4.2.5. General Procedure for the Guanidinylation Reaction of 41–49. To a suspension of the corresponding amine 6, 7, 8, or 9 (2 equiv), the pertinent bis-N-boc-S-methylthioureia 35–40 (1 equiv), and HgCl₂ (2 equiv) in DCM, NEt₃ (6 equiv) was added. The mixture was stirred overnight at rt. A possible excess of HgCl₂ was quenched with 7 N NH₃ in DCM/MeOH/NH₃ 98:2:0.1). The product was obtained as a yellow foamlike solid (520 mg, 54%). ¹H NMR (300 MHz, CDCl₃) δ (ppm) 9.52 (brs, 2H), 7.38–7.19 (m, 20H), 7.15–7.01 (m, 12H), 6.53 (d, J = 1.4 Hz, 2H), 3.46–3.02 (m, 8H), 2.55 (t, J = 6.4 Hz, 4H), 1.84 (quint, J = 6.8 Hz, 4H), 1.44 (m + s, 4 + 18H), 1.22–1.12 (m, 8H). ¹³C NMR (75 MHz, CDCl₃) δ (ppm) 164.4, 160.5, 142.3, 140.6, 138.0, 129.7, 128.1, 128.1, 118.3, 77.5, 75.2, 41.4, 40.7, 29.2, 28.8, 28.7, 26.9, 26.9. HRMS (ESI-MS): m/z [M + H⁻⁺] calculated for C₄₄H₇₇N₁₀O₈S₂ (923.5208), found 923.5204, C₅₂H₆₉N₁₀O₈S₂ (923.25).

4.2.7. General Procedure for the Synthesis of the Bivalent Ligands (5 and 53–63). TFA (4.0 mL) was added to a solution of the protected precursors 41–52 in DCM (16.0 mL, 20% TFA in DCM), and the mixture was refluxed overnight until the protecting groups were removed (TLC control). Subsequently, the solvent was evaporated in vacuo and the residue was washed three times with Et₃O (each 20 mL). The crude product was purified by preparative RP-HPLC (MeCN/0.1% TFA (aq): 5%–40/60%). All compounds were dried by lyophyllization and obtained as tetra-trifluorooacetates.

4.2.7.1. 1,1’-(Octane-1,8-diyl)bis[3-3’-((1H-imidazo[4,5-d]-pyrrol-yl)guanidine) (53). Prepared from 44 (280 mg, 0.25 mmol) in DCM (16.0 mL) and TFA (4.0 mL) according to the general procedure, S was yielded as a yellow oil (150 mg, 67%): RP-HPLC: 98% (tₑ = 13.97, k = 3.21). ¹H NMR (300 MHz, CD₃OD, tetra-trifluoroacetate) δ (ppm) 8.83 (d, J = 1.2 Hz, 2H), 7.39 (s, 2H), 3.28 (t, J = 7.5 Hz, 4H), 3.20 (t, J = 7.0 Hz, 4H), 2.83 (t, J = 7.8 Hz, 4H), 1.98 (quint, J = 7.4 Hz, 4H), 1.59 (quint, J = 6.9 Hz, 4H), 1.42–1.35 (m, 8H). ¹³C NMR (75 MHz, CD₃OD, tetra-trifluoroacetate) δ (ppm) 163.1 (q, J = 35.2 Hz, 157.6, 154.3, 134.9, 134.5, 117.1, 42.7, 41.7, 30.3, 30.0, 28.9, 27.7, 22.6. HRMS (ESI-MS): m/z [M + H⁺] calculated for C₅₂H₄₉N₁₀O₄Cl⁴⁺: 445.3510, found 445.3506; C₅₂H₄₉N₁₀O₄F⁴⁺ (400.72).

4.2.7.2. 1,1’-(Dodecane-1,12-diyl)bis[3-3’-((1H-imidazo[4,5-d]-pyrrol-yl)guanidine) (57). Prepared from 46 (240 mg, 0.20 mmol) in DCM (16.0 mL) and TFA (4.0 mL) according to the general procedure, 57 was yielded as a yellow oil (120 mg, 62%): RP-HPLC: 99% (tₑ = 17.84, k = 4.37). ¹H NMR (300 MHz, CD₃OD, tetra-trifluoroacetate) δ (ppm) 8.77 (d, J = 1.3 Hz, 2H), 7.32 (d, J = 0.9 Hz, 2H), 3.27 (t, J = 7.0 Hz, 4H), 3.17 (t, J = 7.1 Hz, 4H), 2.80 (t, J = 7.7 Hz, 4H), 1.95 (quint, J = 7.3 Hz, 4H), 1.56 (quint, J = 6.8 Hz, 4H), 1.34–1.26 (m, 16H). ¹³C NMR (75 MHz, CD₃OD, tetra-trifluoroacetate) δ (ppm) 163.1 (q, J = 34.8 Hz, 157.6, 157.6, 134.8, 134.6, 129.0 (q, J = 44.3 Hz), 117.0, 42.7, 41.6, 30.7, 30.6, 30.4, 30.0, 28.9, 27.8, 22.6. HRMS (ESI-MS): m/z [M + H⁺] calculated for C₅₄H₄₉N₁₀O₄Cl⁴⁺: 501.4136, found 501.4131; C₂₄H₄₉N₁₀O₄F⁴⁺ (956.83).

4.2.7.3. 1,1’-(Octane-1,8-diyl)bis[3-3’-((2-amino-4-methyl-thiazol-5-yl)propyl)guanidine] (58). Prepared from 47 (520 mg, 0.55 mmol) in DCM (16.0 mL) and TFA (4.0 mL) according to the general procedure, 58 was yielded as a yellow oil (280 mg, 51%): RP-HPLC: 94% (tₑ = 15.53, k = 3.68). ¹H NMR (300 MHz, CD₃OD, tetra-trifluoroacetate) δ (ppm) 3.26–3.10 (m, 8H), 2.71 (t, J = 7.1 Hz, 4H), 2.20 (s, 6H), 1.86 (quint, J = 7.3 Hz, 4H), 1.61–1.52 (m, 4H), 1.37–1.30 (m, 8H). ¹³C NMR (75 MHz, CD₃OD, tetra-trifluoroacetate) δ (ppm) 170.4, 162.8 (q, J = 35.6 Hz), 157.6, 157.6, 132.4, 118.5, 42.6, 41.5, 30.6, 30.2, 29.9, 27.7, 23.5, 11.5. HRMS (ESI-MS):
m/z [M + H+] calculated for C₃₀H₄₇N₁₀S₂+: 537.3265, found 537.3261; C₃₀H₄₇N₁₀S₂-4TFA (992.90).

4.2.7.4. 1-[3-(2-Amino-4-methylthiazol-5-yl)propyl]-3-[3-(2-aminothiazol-5-yl)propyl]guanidino]octylguanidine (63). Prepared from 53 (without purification) in DCM (16.0 mL) and TFA (4.0 mL) according to the general procedure, 63 was yielded as a yellow oil (28.3 mg, 7.1%). 1H NMR (300 MHz, CDCl₃, tetra-trifluoroacetoacetate) δ (ppm) 7.02 (s, 1H), 3.29–3.05 (m, 8H), 2.85–2.55 (m, 4H), 2.19 (s, 3H), 2.00–1.73 (m, 4H), 1.69–1.50 (m, 4H), 1.46–1.30 (m, 8H). 13C NMR (75 MHz, CDCl₃, tetra-trifluoroacetoacetate) δ (ppm) 171.7, 170.3, 157.5, 132.4, 126.7, 123.7, 118.9, 43.1, 42.0, 41.9, 31.0, 30.8, 30.4, 30.2, 27.9, 25.3, 24.0, 12.4. HRMS (ESI-MS): m/z [M + H+] calculated for C₂₃H₄₃N₁₀S₂: 523.3108, found 523.3106; C₂₃H₄₃N₁₀S₂-4TFA (978.87).

4.2.8. General Procedure for the Synthesis of the Benzoylthioureas 70–75. To an ice-cold solution of the pertinent amine (64–69, 1 equiv) in MeCN, benzoyl isothiocyanate (1 equiv) was added dropwise. The reaction was stirred at room temperature (rt) for 2 h, and the organic solvent was concentrated under vacuum. The residue was dissolved in DCM (50 mL) and washed three times with H₂O and saturated solution of NaCl. The organic layer was dried over Na₂SO₄, and the crude product was purified by column chromatography (EtOAc/PE 1/1–1/6 v/v).

4.2.8.1. N-(Isobutylcarbamothioyl)benzamide (70). The title compound was prepared from isobutylammonium (64, 1.00 mL, 10.00 mmol) and 10 (1.34 mL, 10.00 mmol) in MeCN (30 mL) according to the general procedure (Rₐ = 0.41 in EtOAc/Hex 1:7). The product was obtained as a beige-colored solid (2.10 g, 89%), mp 79.6–80.1 °C. 1H NMR (300 MHz, CDCl₃) δ (ppm) 7.38 (m, 5H), 7.04–6.93 (m, 3H), 6.83 (s, 1H), 6.70–6.58 (m, 2H), 3.55 (td, J = 6.9, 5.4 Hz, 2H), 2.06 (m, 1H), 1.03 (d, J = 6.7 Hz, 6H). 13C NMR (75 MHz, CDCl₃) δ (ppm) 179.85, 166.90, 133.59, 131.81, 129.19, 127.41, 53.38, 27.61, 20.29. HRMS (ESI-MS): m/z [M + H+] calculated for C₁₂H₁₆N₂O₂S: 236.33.

4.2.9. General Procedure for the Synthesis of the Thioureas 76–81. The corresponding benzoylthiourea (70–75, 1 equiv) was stirred in a solution of K₂CO₃ (2.1 equiv) in MeOH/H₂O (7/3 v/v) for 3–5 h at rt. The proportion of MeOH was evaporated, and the resulting suspension was extracted with DCM and stirred for 1 h. The organic layer was dried over Na₂SO₄, and the crude product was purified by column chromatography (DCM/MeOH/7/3 NH₃ in MeOH 95/3/2 v/v).

Because of the thione–thiol tautomeration, a splitting of the NH—CH₃—R—signal could be observed in the following NMR spectra. Two broad singlets could be noted right next to each other. In each case, the integration value was exactly 2. For all of the other symmetric CH₃ peaks, this peak splitting was not shown.

4.2.9.1. N-Isobutylthiourea (76). The title compound was prepared from 70 (2.00 g, 8.46 mmol) and K₂CO₃ (2.46 g, 17.77 mmol) in MeOH/H₂O (7/3 v/v, 50 mL) according to the general procedure (Rₐ = 0.38 in DCM/MeOH/NH₃, 95:5:0.1), yielding a colorless oil (0.78 g, 70%). 1H NMR (300 MHz, CDCl₃) δ (ppm) 6.96 (brs, 1H), 6.32 (brs, 2H), 3.34 + 2.93 (2 brs, 0.9H + 1.1H (thione–thiol tautomeration)), 1.88 (m, 1H), 0.94 (d, J = 6.6 Hz, 6H). 13C NMR (75 MHz, CDCl₃) δ (ppm) 180.76, 51.82, 27.85, 20.23. HRMS (ESI-MS): m/z [M + H+] calculated for C₁₃H₁₅N₃S+: 213.0794, found 213.0796; C₁₃H₁₅N₃S-3TFA (583.05).
(75 MHz, CDCl₃) δ (ppm) 164.49, 160.52, 142.38, 140.67, 138.07, 129.73, 128.34, 128.06, 118.25, 75.20, 53.49, 43.03, 40.67, 29.63, 28.69, 28.57, 22.52, 11.53. HRMS (ESI-MS): m/z [M + H⁺] calculated for C₁₄H₂₄N₂O₄S: 355.1339, found 355.1338; C₁₄H₁₈N₂O₄S (511.74).

4.2.13. General Procedure for the Synthesis of the Monomeric Ligands (127–141 and 145). TFA (4.0 mL) was added to a solution of the protected precursors 112–126 or 144 in DCM (16.0 mL, 20% TFA in DCM), and the mixture was refluxed overnight until the protecting groups were removed (TLC control). Subsequently, the solvent was evaporated in vacuo and the residue was washed three times with Et₂O (each 20 mL). The crude product was purified by preparative RP-HPLC (MeCN/0.1% TFA (aq): 5/95).

All compounds were dried by lyophilization and obtained as di-fluoroacetates.

4.2.13.1. 1-[3-(1H-Imidazol-4-yl)propyl]-3-propylguanidine (129) 44 Prepared from 114 (450 mg, 0.82 mmol) in DCM (16.0 mL) and TFA (4.0 mL) according to the general procedure, 129 was yielded as a beige-colored solid (310 mg, 88% yield). 1H NMR (300 MHz, CDCl₃, δ (ppm) 7.36 (t, J = 7.0 Hz, 2H), 3.15 (t, J = 7.1 Hz, 2H), 2.80 (t, J = 7.2 Hz, 2H), 1.96 (quint, J = 7.3 Hz, 2H), 1.61 (m, 2H), 0.97 (t, J = 7.4 Hz, 3H). 13C NMR (75 MHz, CDCl₃, δ (ppm)) 157.58, 134.92, 134.57, 116.97, 44.27, 41.58, 28.82, 23.25, 22.56, 11.41. HRMS (ESI-MS): m/z [M + H⁺] calculated for C₁₀H₁₅N₅O₂S²⁻: 287.1317, found 287.1321; C₁₀H₁₆N₅O₂S (286.1282).
KG, Schwabach, Germany). Afterward, bound radioligand was separated from free radioligand by filtration through GF/C filters (Whatman, Maidstone, U.K.) using a 96-well Brandel harvester (Brandel Inc., Unterföhring, Germany). After two washing steps with binding buffer, the filter pieces were punched and transferred into 1450-401 96-well sample plates (PerkinElmer). Unspecific binding was determined in the presence of 10 μM GTPyS. Each well was supplemented with 200 μL of scintillation cocktail (Rotiszint Eco plus, Roth, Karlsruhe, Germany), and the plates were sealed with Plateseel 1450-461 (PerkinElmer, Rodgau, Germany) and incubated in the dark for 12 h. Radioactivity (cpm) was measured with a MicroBeta 1450 scintillation counter (PerkinElmer). Data were analyzed by nonlinear regression and were best-fitted to sigmoidal concentration–response curves using Prism 5.0c software (GraphPad, San Diego, CA).

4.4. Computational Methods. 4.4.1. Model Preparation. To examine possible binding modes of the bivalent ligands 5 and 58 at hH1R and of the monovalent ligand 129 at both hH1R and hH3R, homology models of these receptors were prepared as follows: For hH1R, the described hH1R model was used,84 which was based on the inactive state crystal structure of the hH1R55 (PDB ID: 3RZE), and the models comprising hH1R and hH3R were adapted from this model. Model preparation was essentially performed as described in Wilting et al.85 using the modeling suite SYBYL-X 2.0 (Tripos Inc., St. Louis, MO).

4.4.2. Molecular Docking. The most interesting bivalent compounds 5 and 58 on the one hand and the monovalent ligand 129 on the other hand were geometry-optimized by means of Gaussian 09690 at the HF/6-31(d,p) level, attributing the ligands a formal charge of +2 and +1, respectively. Upon file conversion by means of Open Babel67 and assignment of physiological ionization states by means of ChemAxon (http://www.chemaxon.com) Marvin 16.3.28.0, 2016 Calculator Plugins, flexible docking was performed with the software package Autodock Vina.58 The following hH1R amino acids were kept as flexible: K18, S75, Q79, Y94, T95, D98, Y99, C102, R161, N168, H169, T170, T171, S172, K173, K175, V176, V178, N179, E180, G183, D186, G187, T190, W247, Y250, F251, F254, R257, R260, N262, E267, E270, L274, W275, G277, Y278. For hH3R, the amino acids T34, M41, Y91, Y94, V95, W110, L111, D114, Y115, C118, E185, H187, F192, F193, Y194, W196, L199, A202, S203, E206, W371, Y374, T375, M378, R381, H387, D391, D394, E395, D398, W399, L401, W402 were kept as flexible, and for hH4R the residues R15, M22, Y72, H75, T76, W90, L91, D94, Y95, C98, M150, K158, E160, S162, E163, F168, F169, S170, W172, L175, T178, S179, E182, W316, Y319, S320, T323, L326, S330, S331, T333, S337, Y340, R341, F344, W345, Q347, W348. The search box was set to a size of 28 Å × 32 Å × 32 Å, centered at the binding pocket. Up to 20 binding poses were exported for each ligand. Taking into account the results of the scoring function as well as experimental data, the respective best poses were selected for downstream molecular dynamics simulations.

4.4.3. Molecular Dynamics Simulations. To identify the respective most probable binding pose, 30 ns molecular dynamics simulations were performed in a water box at 310 K, and subsequently, free energy (MM-GBSA89) calculations were performed using the Amber 1490 molecular dynamics package. To determine ligand force-field parameters, RESP61 charges were determined by means of Gaussian 0990 at the HF/6-31(d,p) level, and general amber force field92 atom types as well as RESP61 charges were assigned using antechamber.50 parmchk290 and tleap90 were used for input file generation. For ligand guanidine atoms and protein residues, the Amber ff99SB93 force-field parameters were used. The simulation steps were carried out in an octahedral box comprising an 8.0 Å TIP3P84 water layer and neutralizing chloride ions.65 The mbondi2 parameter set was utilized.6667 The respective systems were minimized using the steepest descent (2500 steps) and conjugated gradient (7500 steps) methods without restraints.
and were subsequently heated from 0 to 100 K over 50 ps in the NVT ensemble as well as from 100 to 310 K over 450 ps in the NPT ensemble. During the first ns of the 5 ns equilibration period, initial harmonic restraints of 5 kcal/mol Å² were applied to all ligand and receptor atoms, and, beginning from 1 ns, harmonic restraints on receptor main chain atoms were reduced from 5.0 to 0.5 kcal/mol Å² in a stepwise manner and maintained during the 30 ns simulation. Bonds involving hydrogen atoms were constrained using SHAKE to enable a frame step size of 2 fs. Nonbonded interactions were cut off at 8.0 Å, and long-range electrostatics were computed using the particle-mesh Ewald method. The Langevin thermostat with a collision frequency of 1.0 ps⁻¹ and randomly assigned initial velocities was used to maintain a target temperature of 310 K. The Berendsen barostat with isotropic position scaling and a pressure relaxation time of 1.0 ps was employed to keep the pressure constant at 1 bar. Data were collected every 10 ps. H-bond and cluster analysis were performed by means of CPPTRAJ for the entire 30 ns trajectories. Moreover, the average linkage algorithm was applied for cluster analysis, setting a cluster size of 5, and the programming language R was used for the preparation of H-bond plots. Binding free-energy (MM-GBSA) calculations of the 30 ns trajectories were performed using MMPBSA.py and a frame size step of 10 ps, and the corresponding plots were obtained using the Prism 5.01 software (GraphPad, San Diego, CA). All other figures were created with PyMOL Molecular Graphics system, version 1.8.2.1 (Schrödinger LLC, Portland, OR).

**ASSOCIATED CONTENT**

1 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b00128.

Synthesis and analytical data of compounds 17−19, 21−25, 27−31, 33−37, 39−43, 45−46, 48−51, 53−56, 59−62, 71−75, 77−81, 88−89, 91−101, 103−113, 115−128, 130−141, and 144−145; RP-HPLC images and elemental analyses (purity control) of the target compounds; 1H and 13C NMR spectra of 5, 57, 58, 63, and 129; guinea pig right atrium experiments in the presence of cimetidine; and computational data and figures (free energy of binding, cluster analysis) (PDF) SMILES (XLSX)

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Notes

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

The authors thank Christine Braun, Kerstin Röhr, and Maria Beer-Kron for expert technical assistance. Furthermore, special thanks go to Tim Clark and Jonas Kaindl for providing infrastructure and scientific expertise.

**DEDICATION**

This paper is dedicated to Prof. Dr. Armin Buschauer, in memoriam (died on July 18, 2017).

**ABBREVIATIONS**

BB, binding buffer; Boc₂O, di-tert-butyl carbonate; br, broad signal; CRC, concentration−response curve; DPH, diphenylhexylamine; ECL, extracellular loop; E₄₅, efficacy; ESI, electrospray ionization; ESI-MS, electrospray ionization mass spectrometry; EtOAc, ethyl acetate; GBSA, generalized born surface area; GαS, α₂-subunit of the G, protein; gH₂R, guinea pig histamine H₂ receptor; gH₄R, guinea pig histamine H₄ receptor; Gα₂, α₂-subunit (short splice variant) of the G, protein; Gβ₁γ₂, G-protein β₁- and γ₂-subunit; GTPγS, guanosine 5′-O-(β-thio)triphosphate; H₁R, histamine H₁ receptor; Hex, hexane; hH₁R, human histamine H₁ receptor; hH₄R, human histamine H₄ receptor; hH₂R, human histamine H₂ receptor; hH₃R, human histamine H₃ receptor; hH₄R, human histamine H₄ receptor; Kᵦ, dissociation constant of a antagonist-receptor complex; m, multiplet; MeCN, acetonitrile; MeOH, methanol; MM-GBSA, molecular mechanics generalized born surface area; PE, petroleum ether; pEC₅₀, negative decadic logarithm of EC₅₀; pKᵦ, negative decadic logarithm of Kᵦ; pKᵦ, negative decadic logarithm of Kᵦ; RGS4, regulator of G-protein signaling proteins; R₂H₂R, rat histamine H₂ receptor; RP-HPLC, reversed-phase HPLC; SEM, standard error of mean; Trt, trit"
(49) Schnell, D.; Burleigh, K.; Trick, J.; Seifert, R. No Evidence for Functional Selectivity of Proxafan at the Human Histamine H 3 Receptor Coupled to Defined G i/G o Protein Heterotrimers. J. Pharmacol. Exp. Ther. 2010, 332, 996–1005.

(50) GraphPad Prism, version 5.01; GraphPad Software Inc.: San Diego, CA, 2007, www.graphpad.com.

(51) Schneider, E. H.; Seifert, R. Histamine H 4 receptor-RGS Fusion Proteins Expressed in Sf9 Insect Cells: A Sensitive and Reliable Approach for the Functional Characterization of Histamine H 4 Receptor Ligands. Biochem. Pharmacol. 2009, 78, 607–616.

(52) Schneider, E. H.; Schnell, D.; Papa, D.; Seifert, R. High Constitutive Activity and a G-Protein-Independent High-Affinity State of the Human Histamine H 3 Receptor. Biochemistry 2009, 48, 1424–1438.

(53) Lennartz, H.-G.; Hepp, M.; Schunack, W. Synthese Und Wirkung 5-Alkylsubstituierter Histamine Und N°-Methylhistamine. Eur. J. Med. Chem. 1978, 13, 229–234.

(54) Willing, D.; Löffel, K.; Nordemann, U.; Strasser, A.; Bernhardt, G.; Dove, S.; Seifert, R.; Buschauer, A. Molecular Determinants for the High Constitutive Activity of the Human Histamine H 1 Receptor: Functional Studies on Orthologues and Mutants. Br. J. Pharmacol. 2015, 172, 785–798.

(55) Shimamura, T.; Shiroishi, M.; Weyand, S.; Tsuji moto, H.; Winter, G.; Katritch, V.; Abagyan, R.; Cherezov, V.; Liu, W.; Han, G. W.; et al. Structure of the Human Histamine H 3 Receptor Complex with Doxepin. Nature 2011, 475, 65–70.

(56) Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Scalmani, G.; Barone, V.; Mennucci, B.; Petersson, G. A. Gaussian09, revision D.01; Gaussian Inc.: Wallingford, CT, 2013.

(57) O’Boyle, N. M.; Banck, M.; James, C. A.; Morley, C.; Vandermeersch, T.; Hutchison, G. R. Open Babel: An Open Chemical Toolbox. J. Cheminf. 2011, 3, 33.

(58) Trotz, O.; Ölsön, A. J. AutoDock Vina: Improving the Speed and Accuracy of Docking with a New Scoring Function, Efficient Optimization, and Multithreading. J. Comput. Chem. 2010, 31, 455–461.

(59) Miller, B. R., III; McGee, T. D., Jr.; Swails, J. M.; Homeyer, N.; Gohlke, H.; Roitberg, A. E. MMPSA: Py: An Efficient Program for End-State Free Energy Calculations. J. Chem. Theory Comput. 2012, 8, 3314–3321.

(60) Case, D. A.; Babin, V.; Berryman, J.; Betz, R. M.; Cai, Q.; Cerutti, D. S.; Cheatham III, T. E.; Darden, T. A.; Duke, R. E.; Gohlke, H., et al. Amber 14, University of California, San Francisco, 2014, ambermd.org.

(61) Bayly, C. I.; Cieplak, P.; Cornell, W.; Kollman, P. A. A Well-Behaved Electrostatic Potential Based Method Using Charge Restraints for Deriving Atomic Charges: The RESP Model. J. Phys. Chem. 1993, 97, 10269–10280.

(62) Wang, J.; Wolf, R. M.; Caldwell, J. W.; Kollman, P. A.; Case, D. A. Development and Testing of a General Amber Force Field. J. Comput. Chem. 2004, 25, 1157–1174.

(63) Hornak, V.; Abel, R.; Okur, A.; Strockbine, B.; Roitberg, A.; Simmerling, C. Comparison of Multiple Amber Force Fields and Development of Improved Protein Backbone Parameters. Proteins: Struct., Funct., Bioinf. 2006, 65, 712–725.

(64) Jorgensen, W. L.; Chandrasekhar, J.; Madura, J. D.; Impey, R. W.; Klein, M. L. Comparison of Simple Potential Functions for Simulating Liquid Water. J. Chem. Phys. 1983, 79, 926–935.

(65) Möller, D.; Kling, R. C.; Skultety, M.; Leuner, K.; Hübner, H.; Gmeiner, P. Functionally Selective Dopamine D 3 Receptor Partial Agonists. J. Med. Chem. 2014, 57, 4861–4875.

(66) Onufriev, A.; Bashford, D.; Case, D. A. Modification of the Generalized Born Model Suitable for Macromolecules. J. Chem. Phys. B 2000, 104, 3712–3720.

(67) Onufriev, A.; Bashford, D.; Case, D. A. Exploring Protein Native States and Large-scale Conformational Changes with a Modified Generalized Born Model. Proteins: Struct., Funct., Bioinf. 2004, 55, 383–394.

(68) Ryckaert, J.-P.; Ciccotti, G.; Berendsen, H. J. C. Numerical Integration of the Cartesian Equations of Motion of a System with Constraints: Molecular Dynamics of N-Alkanes. J. Comput. Phys. 1977, 30, 237–341.

(69) Pogoli, A.; She, X.; Willing, D.; Hüblner, H.; Bernhardt, G.; Gmeiner, P.; Keller, M. Radiolabeled Dibenzoazepinone-Type Antagonists Give Evidence of Dualistic Binding at the M 2 Muscarinic Acetylcholine Receptor. J. Med. Chem. 2017, 60, 3314–3334.

(70) Darden, T.; York, D.; Pedersen, L. Particle Mesh Ewald: An N-Log (N) Method for Ewald Sums in Large Systems. J. Chem. Phys. 1993, 98, 10089–10092.

(71) Uberuaga, B. P.; Anghel, M.; Voter, A. F. Synchronization of Trajectories in Canonical Molecular-Dynamics Simulations: Observation, Explanation, and Exploitation. J. Chem. Phys. 2004, 120, 6363–6374.

(72) Sindhikara, D. J.; Kim, S.; Voter, A. F.; Roitberg, A. E. Bad Seeds Sprout Perilous Dynamics: Stochastic Thermostat Induced Trajectory Synchronization in Biomolecules. J. Chem. Theory Comput. 2009, 5, 1624–1631.

(73) Berendsen, H. J. C.; Postma, J. P. M.; van Gunsteren, W. F.; DiNola, A.; Haak, J. R. Molecular Dynamics with Coupling to an External Bath. J. Chem. Phys. 1984, 81, 3684–3690.

(74) Sokal, R.; Michener, C. A Statistical Method for Evaluating Systematic Relationships. Univ. Kans. Sci. Bull. 1958, 38, 1409–1438.

(75) R Development Core Team R: A Language and Environment for Statistical Computing; Vienna, Austria: the R Foundation for Statistical Computing, 2014. ISBN 3-900051-07-0. Available online at http://www.R-project.org/.

(76) Sootaert, K. plot3D: Plotting Multi-Dimensional Data, R package version, 2013, https://cran.r-project.org/package=plot3D.

(77) Johnson, P. Graphics Output Device, 2015, https://cran.R-project.org/package=devEMF.

(78) Lemon, J. Plotrix: A Package in the Red Light District of R, R-News, 2006; Vol. 6, pp 8–12.