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2,4-Diaminopyrimidines as histamine H_4 receptor ligands—Scaffold optimization and pharmacological characterization ☆

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ABSTRACT

The human histamine H_4 receptor (hH$_4$R) is a promising new target in the therapy of inflammatory diseases and disorders of the immune system. For the development of new H_4R antagonists a broad ligand-based virtual screening was performed resulting in two hits. The dissection of their common annelated aromatic core into its heteromonocyclic components showed that 2,4-diaminopyrimidine is a potent hH$_4$R affinity scaffold, which was comprehensively investigated. Structure–activity relationship studies revealed that slight structural changes evoke extensive differences in functional activities and potencies: while *o*- and *p*-substituted benzyl amines mainly showed partial agonism, *m*-substituted and rigidified ones exhibited inverse agonist efficacy.

1. Introduction

The biogenic amine histamine (**1**), 2-(1*H*-imidazol-4-yl)ethanamine, mainly acts via four distinct histamine receptor subtypes (H_1R–H_4R). Antagonists for H_1R and H_2R are used in the therapy of allergic diseases and gastrointestinal ulcer diseases, respectively, and have reached blockbuster status.[1,2] The first H_3R antagonists are progressing into clinical phase III to be investigated for the treatment of sleep-wake and cognition disorders, epilepsy, neuropathic pain or obesity.[3] As the H_4R is the last reported receptor subtype researchers are making efforts to find potent ligands with inverse agonist/antagonist efficacy, and only preclinical studies of a few structures have been reported.

Like all other histamine receptors, the H_4R belongs to class A (rhodopsin-like) of G protein-coupled receptors (GPCRs). It interacts with heterotrimeric $G_{i/o}$ proteins.[4] Receptor activation leads to G protein α-subunit-mediated inhibition of some adenylyl cyclase isoforms. βγ-Subunits released from the heterotrimer cause phospholipase Cβ activation and an increase of the intracellular calcium ion concentration.[5] The H_4R shows an extraordinary high constitutive activity.[6] According to the two-state model of receptor activation[7] the equilibrium between the active G protein-coupling conformation (R^*) and the uncoupled inactive state (R) is shifted to the active state, which promotes G protein signalling in the absence of a ligand. In the model, agonists are thought to stabilize the active state, whereas inverse agonists change the receptor conformation to stabilize the inactive state. Partial agonists stabilize the active state less efficiently than full agonists and neutral antagonists have no preference for the active or inactive state, yet block the effects of both agonists and inverse agonists. In the case of hH$_4$R, full and inverse agonists have been reported to stabilize the active state.[8]

The H_4R is predominantly expressed peripherally on hematopoietic mononuclear cells, especially on eosinophils, basophils, T-lymphocytes, dendritic cells, and mast cells. Hence, it shows highest concentration levels in tissues involved in immune responses such as bone marrow, lung, and spleen.[6] The H_4R is involved in immune or inflammatory responses because histamine signalling induces cell shape change and chemotaxis of mast cells as well as eosinophils, mast cell migration, and upregulation of adhesion molecules on monocytes, and all these effects can be

☆ Main parts of this investigation have recently been presented at international conferences: Sander, K., et al. Lead Structure Identification by Virtual Screening and Scaffold Modification for New Histamine H_4 Receptor Ligands. 20th International Symposium on Medicinal Chemistry, Vienna/Austria (August 31–September 4, 2008). *Drugs Future* **2008**, *33*, 267–268 (P490)—Sander, K., et al. From Hit to Lead on Histamine H_4 Receptor Ligands—Diaminopyrimidines as Potential Anti-Inflammatory Agents. Frontiers in Medicinal Chemistry 2009, Heidelberg/Germany (March 15–18, 2009), Abstract book, p 66 (INF02).

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blocked by H_4R antagonists.[9–13] From these physiological reactions, one can deduce several potential clinical uses for H_4R inverse agonists/antagonists in the broad field of anti-inflammatory therapy,[14] like allergy and asthma,[15] pruritus[16] associated with allergy[17] or autoimmune skin conditions, inflammatory bowel disease,[18] rheumatoid arthritis, and pain.[19]

Since the hH$_4$R is structurally most closely related to hH$_3$R (37% protein sequence identity, 58% in transmembrane domains)[20] the identification of selectively acting hH$_4$R ligands is a major challenge in current histamine research. Because some imidazole-containing hH$_3$R ligands turned out to be hH$_4$R ligands as well[21] a reclassification of frequently used reference structures has taken place.[22] Some of those ligands such as thioperamide (**2**), which exhibits similar affinities at both hH$_3$R (pK_i = 7.2)[23] and hH$_4$R (pK_i = 7.3),[20] are now used as standard compounds in hH$_4$R binding assays. In addition, new radioligands with improved selectivity and specific activity compared to that of [^{3}H]histamine are under investigation.[24] So far, only a few potent and selectively acting H_4R ligands are published.[25] JNJ 7777120 (**3**; pK_i = 8.4) is the most profiled among a series of indolylpiperazines and has become the de facto standard reference H_4R antagonist in literature studies. The compound was the first reported H_4R antagonist with anti-inflammatory properties.[26] Elongation of the spacer between the basic moiety and the aromatic scaffold led to 2-arylbenzimidazoles like **4**. On the one hand, H_4R binding in the low nanomolar concentration range has been reported for these compounds but, on the other hand, they show high structural similarity to H_3R antagonists.[27] The atypical neuroleptic clozapine has become a lead in H_4R research (pK_i = 6.8)[28] despite exhibiting high affinities to multiple biogenic amine receptors.[29] It is thought that it mainly mediates its antipsychotic effects via dopamine D_2-like receptors as well as serotonin 5-HT$_2$ receptor subtypes though clozapine exhibits substantial affinity for other biogenic amine receptors such as histamine H_1 and adrenergic α_1 receptors.[30] Optimized oxazepines possess improved H_4R affinities and agonist efficacy while showing H_1R antagonism.[28] A scaffold hopping approach resulted in quinazolin-4-amine VUF10497, which was reported as a dual acting H_1R/H_4R ligand (pK_i = 7.7/8.2).[31] The combination of tricyclic structural elements and a methylpiperazine group can also be found in the patent literature[32] (e.g., compound **5**), where its H_4R affinity has been claimed. A group of conformationally constrained aminopyrimidines was reported as potent antagonists on human, rat and mouse H_4R, highlighted by A-943931, a potent (pK_i = 8.3) and selective H_4R antagonist.[33] Another constrained series led to the tetracyclic H_4R antagonist A-987306, which also showed efficacy in pain models.[34] Non-annelated aminopyrimidines like **6** have been discovered simultaneously and described previously by a few groups and have been published in broad patent applications. In a way these structures are built up quite similar. They all consist of an aminopyrimidine substituted with an aliphatic basic chain or heterocycle. However, almost all structures differ with regard to the specific substitution patterns and ring systems of the pyrimidine core. Chronologically, other 2,4-diaminopyrimidines were first detailed by chemists from Palau Pharma[35] and UCB[36] though without giving specific description of pharmacological data. Pfizer scientists also published related pyrimidines as hH$_4$R ligands, substituted with aliphatic heterocycles,[37] while the Johnson & Johnson group prepared carbon-analog 2-amino-4-alkyl-pyrimidines.[38]

The objective of our study was the development of novel hH$_4$R ligands with inverse agonist/antagonist efficacy as well as selectivity over other aminergic receptors and off-targets, especially the hH$_3$R. We initiated our project with a virtual screen search of a SPECS library using compound **3** as reference structure. The search identified a five-membered heterocycle [*d*]annelated to pyrimidine. This fused pyrimidine scaffold was theoretically dissected into its fragments, which were synthetically prepared and tested separately to figure out the structural requirements for hH$_4$R binding. The resulting 2,4-diaminopyrimidine core structure was fur-

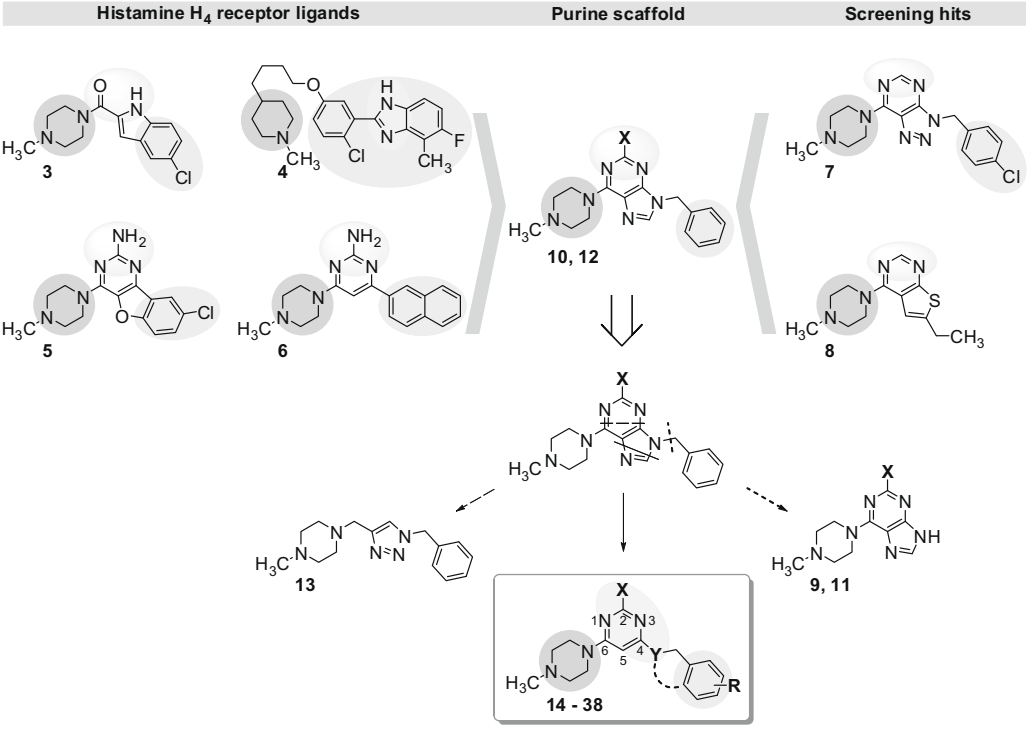


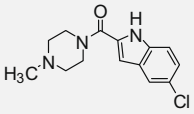
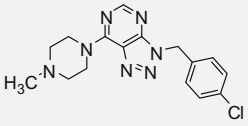
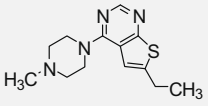
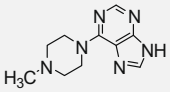
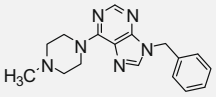
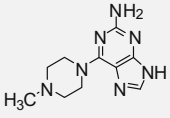
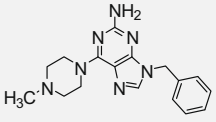
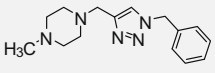
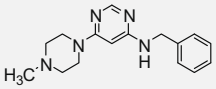
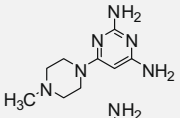
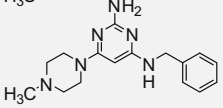
Figure 1. Scaffold modification of virtual screening hits based on known compounds. X = H or NH_2; Y = NH, N, O or S; R = aromatic substituents. Elements of the hH$_4$R blueprint are highlighted as follows: the basic moiety (in most cases methylpiperazine) in dark grey, the hydrogen donor/acceptor system in light grey, and the aromatic moiety in medium grey.

ther optimized by variation of the key substituents. Affinities, efficacies and potencies of the resulting compounds were investigated in in vitro assays.

2. Scaffold determination

A variety of pyrimidines have been reported as active hH$_4$R ligands. With the aim to find novel compounds, a ligand-based virtual screening[39] was performed and resulted in two hits with affinities in the low micromolar concentration range, namely a 3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidine and a thieno[2,3-*d*]pyrimidine (**7** and **8**, respectively). Both hits consist of a cyclopenta[*d*]pyrimidine scaffold substituted with 4-methylpiperazine. 4-Methylpiperazine is frequently used as basic moiety in hH$_4$R ligands[31,40] and in most cases it increases affinity. Observing the hits and some representative reference ligands in Figure 1 it becomes clear that the majority of known hH$_4$R ligands have some similarities sharing the mentioned basic piperazine, a hydrogen donor/acceptor system and an aromatic moiety allowing for π-interactions. For reasons of comparison, in our study the 4-methylpiperazine moiety was kept constant, while the heterocyclic core was modified (Fig. 1) to study the structure–affinity/efficacy relationships at this position.

Table 1
hH$_4$R Affinities of reference **3**, screening hits **7** and **8**, and structural fragments thereof

Compound	Structure	Formula	Affinity on hH$_4$R[a] K_i (μM)
3 (JNJ 7777120)		$C_{14}H_{16}ClN_3O$	0.014 ± 0.007
7		$C_{16}H_{18}ClN_7$	3.5
8		$C_{13}H_{18}N_4S$	6.6
9		$C_{10}H_{14}N_6$	18.4
10		$C_{17}H_{20}N_6 \cdot C_4H_4O_4$	14.3
11		$C_{10}H_{15}N_7$	15.6
12		$C_{17}H_{21}N_7 \cdot 2(COOH)_2$	8.1
13		$C_{15}H_{21}N_5 \cdot 2C_4H_4O_4$	35.0
14		$C_{16}H_{21}N_5 \cdot 2C_4H_4O_4$	0.417 ± 0.165
15		$C_9H_{16}N_6 \cdot 2C_4H_4O_4$	0.290 ± 0.050
16		$C_{16}H_{22}N_6 \cdot 2C_4H_4O_4$	0.098 ± 0.013

[a] [^{3}H]Histamine displacement assay with membrane preparation of Sf9 cells expressing hH$_4$R, co-expressed with Gαi2 and Gβ$_1$γ$_2$ subunits;[8] mean values of at least two independent measurements, each in triplicates; SEM given for K_i values below 1 μM.

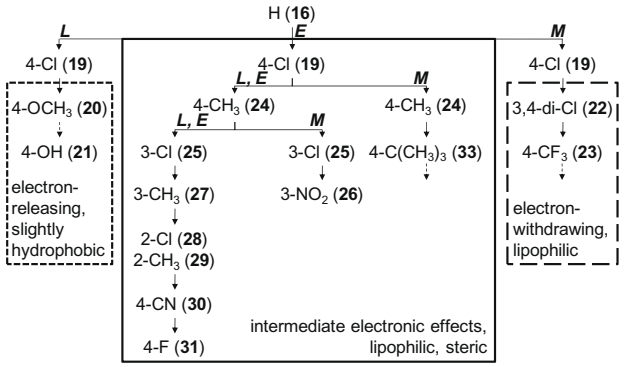


Figure 2. Topliss Tree on scaffold optimization. L = lower affinity; E = equal affinity; M = higher affinity. In parenthesis corresponding compound numbers are given.

Due to their convenient synthetic accessibility the first synthesized ligands were purine **10** and its corresponding fragment desbenzyl **9**, which showed lower affinities than hits **7** and **8** (Table 1). Introduction of the 2-amino group known from other *h*H_4R ligands in patent literature[32] (cf. Fig. 2) led to a slight improvement in *h*H_4R binding of compounds **11** and **12**.

The central aromatic moiety of hit **7** was analyzed by dissection of the components into a triazole (**13**) and a pyrimidine (**14**) scaffold. These fragments evoked pronounced differences in receptor interaction: the triazole did not show any affinity at all, whereas the pyrimidine retained affinity in the submicromolar concentration range. As can be seen from comparing the affinities of compounds **10** and **14** only removal of purine N-7 and C-8 caused a fortyfold increase in receptor binding. This is consistent with the pyrimidine as the source of affinity increase.

Incorporation of a 2-amino group into compound **14** provided compound **16** and led to an improvement of receptor binding. A similar effect could be seen in the increase of affinities of compounds **9** and **10** versus **11** and **12**. Compound **15** showed affinity in the submicromolar concentration range and partial agonist efficacy in two functional assays (vide infra), and hence its scaffold was kept as a basic pharmacophore. Since benzyl substitution further improved binding strength, structure **16** was defined as lead structure (Table 2). Compounds **15** and **16** have meanwhile been previously reported as *h*H_4R ligands in a FLIPR assay (pEC_{50} of 6.4 and 7.0, respectively), consistent with our findings.[40]

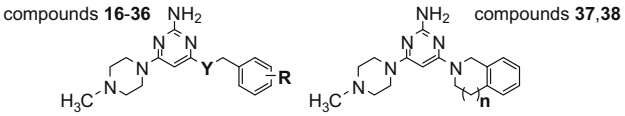
To investigate the influence of substituents on the benzylic portion of the molecule and to optimize the partial agonist **16** with respect to *h*H_4R affinity and efficacy the latter compound was modified according to classical Topliss scheme.[41] Effects of electron-withdrawing, electron-donating and/or steric substituents were studied and led to nanomolar ligands with differing functionality (cf. Tables 2–4).

3. Chemistry

Fragments **9** and **11** were prepared by substitution of a chloropurine derivative with *N*-methylpiperazine under basic conditions. Further addition of benzyl bromide and caesium carbonate led to compounds **10** and **12** (Scheme 2). Triazole **7** was prepared in a one-pot-reaction via click chemistry[42] (Scheme 1). The Huisgen 1,3-dipolar cycloaddition is an efficient approach to couple azides and terminal acetylenes to achieve 1,2,3-triazoles.[43] Copper(I) ion catalysis selectively leads to 1,4-substituted triazoles.[44] Azide **13a** was prepared from benzyl bromide in the microwave oven using water as the solvent.[45]

Pyrimidin-4-amines **14**–**16** and **19**–**38** were synthesized by microwave-assisted aromatic nucleophilic substitution reactions[46] of 2-amino-4,6-dichloropyrimidine and an appropriate amine (Scheme 2). As adjuvant base *N*,*N*-diisopropylethylamine was added, and reactions were conducted in the polar protic solvent

Table 2
hH_4R Affinities of compounds **16**–**38**



Compound	Y	R	*n*	Formula	Affinity on *h*H_4R[a] K_i (μM)
16	NH	H		$C_{16}H_{22}N_6 \cdot 2C_4H_4O_4$	0.098 ± 0.013
17	O	H		$C_{16}H_{21}N_5O$	2.5
18	S	H		$C_{16}H_{21}N_5S \cdot 2C_4H_4O_4 \cdot 0.5\ H_2O$	13.7
19	NH	4-Cl		$C_{16}H_{21}ClN_6 \cdot 2C_4H_4O_4$	0.071 ± 0.010
20	NH	4-OCH_3		$C_{17}H_{24}N_6O \cdot 2C_4H_4O_4$	0.542 ± 0.260
21	NH	4-OH		$C_{16}H_{22}N_6O \cdot 2C_4H_4O_4$	0.237 ± 0.047
22	NH	3,4-Di-Cl		$C_{16}H_{20}Cl_2N_6$	0.150 ± 0.023
23	NH	4-CF_3		$C_{17}H_{21}F_3N_6 \cdot 2C_4H_4O_4$	1.2
24	NH	4-CH_3		$C_{17}H_{24}N_6 \cdot 2C_4H_4O_4$	0.205 ± 0.033
25	NH	3-Cl		$C_{16}H_{21}ClN_6$	0.290 ± 0.044
26	NH	3-NO_2		$C_{16}H_{21}N_7O_2 \cdot 2C_4H_4O_4$	0.538 ± 0.152
27	NH	3-CH_3		$C_{17}H_{24}N_6 \cdot 2C_4H_4O_4$	0.282 ± 0.112
28	NH	2-Cl		$C_{16}H_{21}ClN_6 \cdot 2C_4H_4O_4$	0.018 ± 0.007
29	NH	2-CH_3		$C_{17}H_{24}N_6 \cdot 2C_4H_4O_4$	0.025 ± 0.008
30	NH	4-CN		$C_{18}H_{22}N_7 \cdot 2C_4H_4O_4$	0.341 ± 0.099
31	NH	4-F		$C_{16}H_{21}FN_6 \cdot 2C_4H_4O_4$	0.046 ± 0.020
32	NH	2,6-Di-Cl		$C_{16}H_{20}Cl_2N_6 \cdot 2C_4H_4O_4$	0.012 ± 0.003
33	NH	4-*tert*-Butyl		$C_{20}H_{30}N_6 \cdot 2C_4H_4O_4$	3.4
34	NH	4-*iso*-Propyl		$C_{19}H_{28}N_6 \cdot 2C_4H_4O_4$	2.2
35	NH	4-Phenyl		$C_{22}H_{26}N_6$	0.716 ± 0.203
36	NH	4-COOH		$C_{17}H_{22}N_6O_2$	376.6
37			1	$C_{18}H_{24}N_6 \cdot 2C_4H_4O_4$	0.146 ± 0.073
38			0	$C_{17}H_{22}N_6 \cdot 2C_4H_4O_4$	0.248 ± 0.071

[a] [^{3}H]Histamine displacement assay with membrane preparation of Sf9 cells expressing *h*H_4R, co-expressed with Gαi2 and $G\beta_1\gamma_2$ subunits;[8] mean values ± SEM of at least three independent measurements, each in triplicates; SEM given for K_i values below 1 μM.

Table 3
Efficacy values for partial agonists determined in two functional assays

Compound	Code[a]	Efficacy and potency on hH_4R				Functionality
		GTPγS[b]		GTPase[c]		
		E_{max}[d] (%)	p EC_{50}[e]	E_{max}[d] (%)	p EC_{50}[e]	
1 (Histamine)		100.0	7.86 ± 0.20	100.0	7.93 ± 0.06	Agonist
28	ST-997	32.5	8.22 ± 0.18	30.5	7.71 ± 0.25	Partial agonist
29	ST-998	23.7	8.07 ± 0.16	19.2	7.93 ± 0.15	Partial agonist
31	ST-999	28.5	7.68 ± 0.16	26.6	7.04 ± 0.27	Partial agonist
32	ST-1006	28.4	8.95 ± 0.29	17.5	8.85 ± 0.39	Partial agonist
2 (Thioperamide)		−100.0	7.15 ± ± 0.18	−100.0	7.05 ± 0.11	Inverse agonist

[a] Laboratory code for later identification of these compounds.
[b] Functional binding assay with [^{35}S]GTPγS on membrane preparation of Sf9 cells expressing hH_4R, co-expressed with Gαi2 and $G\beta_1\gamma_2$ subunits.[8]
[c] Functional binding assay with GTPase on membrane preparation of Sf9 cells expressing hH_4R, co-expressed with Gαi2 and $G\beta_1\gamma_2$ subunits.[8]
[d] Values relating to maximal response of **1** as reference full agonist (100%).
[e] Mean values ± SEM of at least three independent experiments, each at least in duplicates and six point measurements.

Table 4
Functional characterization of partial and inverse agonists

Compound	Substitution of benzyl amine	GTPγS[a]		Functionality
		Efficacy E_{max}[b] (%)	Potency pEC_{50}[c]/pK_b[d]	
1 (Histamine)		100.0	7.86 ± 0.20	Full agonist
21	*p*-OH	37.7	6.99 ± 0.14	Partial agonist
16		34.5	7.67 ± 0.29	Partial agonist
30	*p*-CN	24.3	6.22 ± 0.05	Partial agonist
19	*p*-Cl	24.0	7.50 ± 0.27	Partial agonist
24	*p*-CH_3	—	6.98 ± 0.07	Antagonist
20	*p*-OCH_3	−32.3	6.79 ± 0.07	Inverse agonist
22	*m*,*p*-Di-Cl	−52.9	7.25 ± 0.22	Inverse agonist
3 (JNJ 7777120)		−58.7	7.80 ± 0.21	Inverse agonist
25	*m*-Cl	−66.4	6.70 ± 0.25	Inverse agonist
27	*m*-CH_3	−79.0	6.57 ± 0.24	Inverse agonist
26	*m*-NO_2	−85.3	6.32 ± 0.19	Inverse agonist
37	Tetrahydroisoquinolin-2-yl	−95.6	7.36 ± 0.07	Inverse agonist
2 (Thioperamide)		−100.0	7.15 ± 0.18	Inverse agonist
38	Isoindoline-2-yl	−110.7	7.43 ± 0.16	Inverse agonist

[a] Functional binding assay with [^{35}S]GTPγS on membrane preparation of Sf9 cells expressing hH_4R, co-expressed with Gαi2 and $G\beta_1\gamma_2$ subunits.[8]
[b] Values relating to maximal response of **1** as reference full agonist (100%), or to maximal inhibition of constitutive activity by **2** as reference inverse agonist (−100%).
[c] pEC_{50} for full, partial, and inverse agonists determined in agonist mode; mean values ± SEM of at least three independent experiments, each at least in triplicates and seven point measurements.
[d] pK_b for neutral agonist **24** determined in antagonist mode (after prestimulation by **1**); mean value ± SEM of three independent experiments, each in triplicates and seven point measurements.

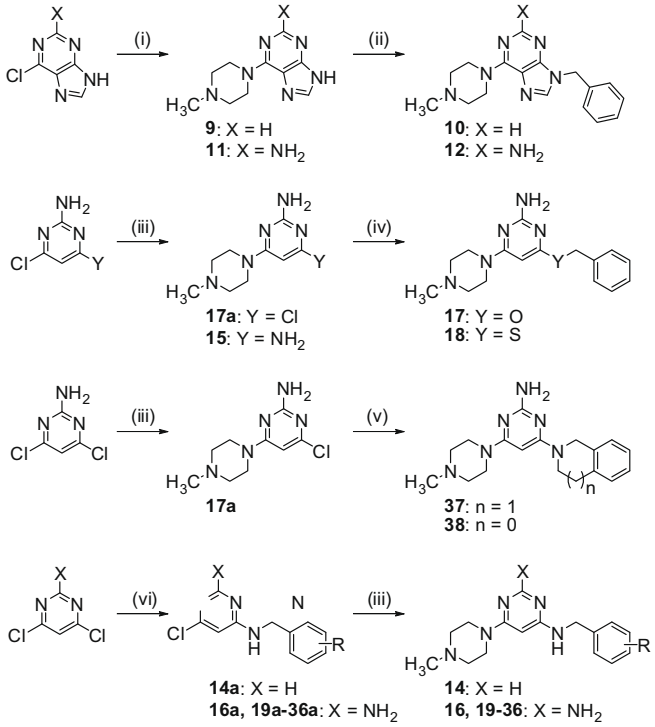
iso-propanol. These conditions make it suitable for microwave heating because the irradiation is well absorbed. Under common conditions, which is heating the reagents in solvents like ethanol, transformation to the product hardly took place, even after three days of reflux. Hence, microwave irradiation was necessary to obtain high yields in a reasonable time (30–60 min). Typically, due to the higher basicity of secondary amines, higher overall yields were obtained when, in a sequential synthesis, first the benzyl amine and second 1-methylpiperazine was coupled. Ether **17** and thioether **18** resulted from the reaction of 4-chloro-6-(4-methylpiperazin-1-yl)pyrimidin-2-amine (**17a**) with benzyl alcohol,[47] and benzyl mercaptane,[48] respectively. Sodium hydride was needed to deprotonate the alcohol, whereas for thiol activation sodium hydroxide was sufficient. Rigidified structures **37** and **38** resulted from the reaction of **17a** with tetrahydroisochinoline and isoindoline, respectively.

All final compounds were re-crystallized from ethanol, a few as free bases, but most as salts of maleic acid due to ease of handling. According to NMR spectra, diprotonation takes place on N^4-piperidine and pyrimidine-4-amine, whereas the latter might be preferred due to its mesomeric stabilization.

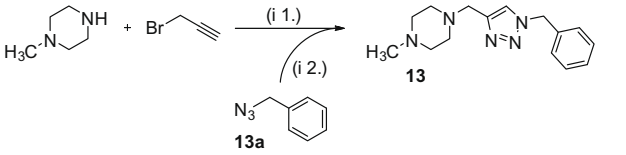
4. Pharmacology

To determine hH_4R affinities all compounds (**7**–**38**) as well as reference structures were examined in a [^{3}H]histamine competition binding assay (cf. Tables 1 and 2). For this purpose a membrane preparation from Sf9 cells expressing hH_4R, co-expressed with G protein Gαi2 and $G\beta_1\gamma_2$ subunits, was made.[8] Similar experiments were performed to measure affinities of the most potent compounds **28**, **29**, **31**, and **32** on the remaining three histamine receptor subtypes, in each case using an appropriate radioligand, namely [^{3}H]pyrilamine, [^{3}H]tiotidine, and [^{3}H]N^α-methylhistamine for hH_1R,[49] hH_2R,[50] and hH_3R[51] binding, respectively (cf. Table 5).

Two functional assays[8] were used to determine the efficacy of compounds **28**, **29**, **31**, and **32** on hH_4R (cf. Table 3). Both were carried out with the Sf9 cell membrane preparation containing hH_4R, co-expressed with G protein Gαi2 and $G\beta_1\gamma_2$ subunits. The first assay is based on the exchange of GDP to [^{35}S]GTPγS after a ligand has bound to the receptor. As the complex of the Gαi2 subunit and [^{35}S]GTPγS hardly dissociates, agonist-induced [^{35}S]GTPγS binding can be measured. The second assay determines the steady-state GDP/GTP exchange by measuring hydrolysis of [γ-^{32}P]GTP catalysed by the enzyme GTPase. Its activity is determined by counting the signals of the released $^{32}P_i$. Reference structures in both assays are the endogenous hH_4R ligand **1** as agonist and the imidazole-containing H_3R/H_4R ligand **2** as inverse agonist. In these membrane systems, relative to the baseline (equating with the basal constitutive activity) compound **1** evokes a stimulation of ~60% (63% and 60% in [^{35}S]GTPγS and GTPase assay, respectively). Inverse agonist efficacy of **2** also shows comparable values (−38% and −40%, respectively).



Scheme 2. Synthesis of compounds **9**–**12** and **14**–**38** via substitution reactions. R = aromatic substituents. Reagents and conditions: (i) 1-methylpiperazine, TEA, DMF, 12 h, rt; (ii) benzyl bromide, Cs_2CO_3, DMF, 12 h, 110 °C; (iii) 1-methylpiperazine, DIPEA, 2-propanol, μW, 30–60 min, 160 °C; (iv) Y = O: benzyl alcohol, NaH, THF, 24 h, 0 °C→rt; Y = S: benzyl mercaptane, NaOH, H_2O, ethanol, 12 h, 80 °C; (v) tetrahydroisoquinoline ($n = 1$) or isoindoline ($n = 0$), DIPEA, 2-propanol, μW, 60 min, 160 °C; (vi) benzyl amine, DIPEA, 2-propanol, μW, 30–60 min, 130 °C.



Scheme 1. Synthesis of compound **13** via click chemistry. Reagents and conditions: (i) (1) TEA, H_2O, 1 h, rt; (2) CuI, 12 h, rt.

Table 5
Histamine receptor subtype affinities and hH$_3$R/hH$_4$R selectivity ratio of selected compounds

Compound	hH$_1$R[a] pK_i	hH$_2$R[b] pK_i	hH$_3$R[c] pK_i	hH$_4$R[d] pK_i	hH$_3$R/hH$_4$R K_i/K_i
28	<6.0	<6.0	6.46	7.77	19.2
29	<6.0	<6.0	6.35	7.63	17.8
31	<6.0	<6.0	6.49	7.37	7.1
32	<6.0	<6.0	6.29	7.94	42.5

[a] [^{3}H]Pyrilamine displacement on Sf9 cell membranes co-expressing hH$_1$R and RGS4;[49] mean values of three independent experiments, each in triplicates and one point measurements.

[b] [^{3}H]Tiotidine displacement on Sf9 cell membranes with hH$_2$R-Gsαs fusion protein;[50] mean values of three independent experiments, each in triplicates and one point measurements.

[c] [^{3}H]N^α-Methylhistamine displacement on Sf9 cell membranes co-expressing hH$_3$R, Gαi2, Gβ1γ2 and RGS4;[51] mean values of three independent experiments, each at least in duplicates and five point measurements.

[d] [^{3}H]Histamine displacement on Sf9 cell membranes co-expressing hH$_4$R, Gαi2 and Gβ$_1$γ$_2$;[8] mean values of three independent experiments, each in triplicates and seven point measurements.

Compounds **16**, **19**–**22**, **24**–**27**, **30**, **37**, and **38** were additionally analyzed in the [^{35}S]GTPγS binding assay (cf. Table 4). Partial agonists and inverse agonists were characterized in the agonist mode, compound **24** additionally in the antagonist mode (prestimulation with **1**).

5. Results and discussion

Purines **10** and **12** exhibited hH$_4$R affinity in the same range as hit **7** did. Fragmentation of the annelated heterocycle of **7** led to triazole **13** and pyrimidine **14**. Triazoles can be used as bioisosters of amides since nitrogen atoms two and three replace the carbonyl's weak acceptor function.[43] Hence, this heterocycle in compound **13** was thought to take the place of the amide moiety in compound **3**. However, the donor function of the indole nitrogen in compound **3** is missing in compound **13** explaining the loss of affinity. In contrast, the pyrimidine in compound **14** includes both, hydrogen bond donor and acceptor. Hence it is able to replace the carbonyl-indole moiety of **3**. Because 2-amino substitution of the pyrimidine led to a fivefold increase of affinity (**14**→**16**) this beneficial moiety was kept constant. Structure–activity relationships of compound **16** are shown in Table 2. The exocyclic 4-amino group is incorporated in the aromatic system of the pyrimidine and might act as a hydrogen donor important for receptor interactions. Changing this heteroatom (Y) to O (**17**) or S (**18**) led to a 25- and 140-fold loss of affinity, respectively. These results reinforce previous descriptions of 2,4-diaminopyrimidines as a potent hH$_4$R affinity scaffold.[35,36,40] Replacement of the 4-amino moiety by a methylene group considerably improved receptor binding to the low nanomolar concentration range.[38] This indicates that differences in the electrostatic charge distribution also influence the affinity of these compounds.

Initially, optimization was carried out for hH$_4$R affinity. The 4-chloro derivative **19** is nearly equipotent to non-substituted compound **16**. When referring to Topliss' scheme (Fig. 2) the equality of hydrogen and chlorine at this site already gave a hint, that steric rests with lipophilic and intermediate electronic effects improve hH$_4$R binding. This classical approach was used to systematically examine the ability of various substituents to enhance affinities.[41] Indeed, 2-chloro (**28**), 2-methyl (**29**), and 4-fluoro (**31**) derivatives showed affinities in the low nanomolar concentration range. Compared to these substituents, electron-releasing but hydrophilic groups like 4-methoxy (**20**) or 4-hydroxy (**21**) and electron-withdrawing rests like 3,4-di-Cl (**22**) or 4-trifluoromethyl (**23**) decrease binding potency (pK_i <7). Nevertheless, all exhibit stronger affinities than hits **7** and **8**. Additional acidic moieties as in compound **36** seem to be detrimental to hH$_4$R binding (pK_i ~3.5).

To evaluate the effect of the 2-Cl substitution and to investigate the steric interactions and the lipophilic properties of compound **28** a 2,6-di-Cl-substituted compound (**32**) was prepared. It shows a similar binding behaviour to that of compound **28**. Most likely, only one chlorine is required to interact with a lipophilic area within the receptor's binding pocket. No additional fixation is achieved by inserting additional steric or lipophilic properties. This assumption was supported with a series of ligands with steric substituents: compounds with bulky rests like 4-*tert*-butyl (**33**) and 4-*iso*-propyl (**34**) possess affinities in the micromolar concentration range, whereas biphenyl **35** evokes submicromolar receptor binding. The planar phenyl moiety seemed to allow stronger receptor interactions than *iso*-alkyl rests. Rigidification may be a useful concept to increase binding potency and/or selectivity of ligands.[52] This is not found with tetrahydroisoquinoline **37** and isoindoline **38**. They show a slightly reduced affinity when compared to that of parent structure **16**, which might be caused by conformational change of the molecule.

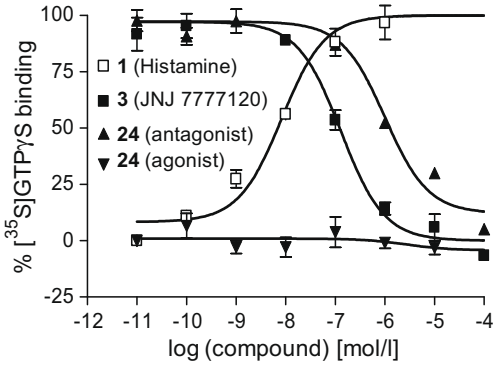


Figure 3. Determination of efficacy and potency of compound **24** (black triangles) in the agonist and antagonist mode of [^{35}S]GTPγS binding assay. Functional binding assay with [^{35}S]GTPγS on membrane preparation of Sf9 cells expressing hH_4R, co-expressed with Gαi2 and $G\beta_1\gamma_2$ subunits;[8] values relating to maximal response of **1** as reference full agonist (100%); mean values ± SEM of three independent experiments, each in triplicates and seven point measurements.

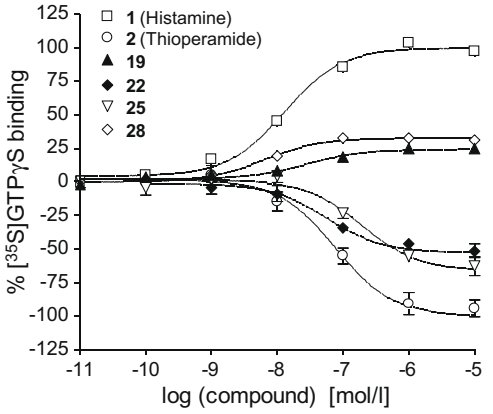


Figure 4. Structure–efficacy relationships of compounds **19**, **22**, **25**, **28**, and reference structures. Functional binding assay with [^{35}S]GTPγS on membrane preparation of Sf9 cells expressing hH_4R, co-expressed with Gαi2 and $G\beta_1\gamma_2$ subunits;[8] values relating to maximal response of **1** as reference full agonist (100%), or to maximal inhibition of constitutive activity by **2** (−100%); mean values ± SEM of at least three independent experiments, each in triplicates and seven point measurements.

Selected compounds with high affinities, that is pK_i values >7.3, were further characterized with respect to their potency and efficacy on hH_4R (Table 3) and their selectivity relative to other histamine receptor subtypes (Table 5). A functional [^{35}S]GTPγS binding assay provided evidence that these pyrimidine-2,4-diamines can show partial agonist efficacy: *o*-substituted compounds **28**, **29**, and **32** cause partial receptor activation. Among all tested structures, 2-chloro derivative **28** exhibits the highest agonist efficacy of ~30%, relative to maximal response of **1** as reference full agonist (100%). With addition of one more chlorine (**32**) agonist efficacy somewhat decreases, while potency increases. Compared to functional characteristics of **28**, 2-methyl derivative **29** shows reduced efficacy and similar potency, whereas 4-fluoro substituted compound **31** evokes a similar efficacy but decreased potency. To confirm these results the compounds were reassessed by a different functional assay, which is based on GTPase activity. Profiles were consistent with the ones obtained from [^{35}S]GTPγS assay.

Compounds with pK_i values <7.3 were functionally characterized in the [^{35}S]GTPγS binding assay. 4-Hydroxy, 4-cyano and 4-chloro derivatives **21**, **30** and **19** exhibit receptor activation in the same range as partial agonist **31** does, whereas 4-methyl derivative **24** does not show receptor activation nor reduction of its constitutive activity indicating that it possesses neutral antagonist properties in the respective in vitro system. Hence, this compound was reinvestigated in the antagonist mode of the [^{35}S]GTPγS binding assay and showed the inhibition of receptor activation induced by the endogenous ligand **1** (Fig. 3).

Among the *p*-substituted compounds only the 4-methoxy derivative **20** exhibits weak inverse agonist efficacy. Possibly steric substituents in *p*-position evoke inverse agonism but reduced affinity. In contrast to *o*- and *p*-substituted partial agonists, *m*-substituted compounds **25**–**27** more clearly are characterized as inverse agonists showing a reduction of constitutive activity between 65% and 85%, compound **2** being the reference. When comparing the *meta*-chlorinated inverse agonist **25** with its corresponding *para*-substituted analogue and partial agonist **19** and the dichlorinated compound **22** it is found that the 3,4-di-Cl-substitution still allows for inverse agonism. In this series of aminopyrimidines the *meta*-site seems to have a major influence on the overall efficacy of the compound.

The conformationally restrained structure **37** possesses a similar and **38** even a superior potency compared to that of **2** (Table 4). Compared to reference structure **3**, which is characterized as inverse agonist in both functional assays (cf. Table 4 and Ref. 8), the *meta*-substituted and rigidified compounds exhibit stronger inverse agonist efficacy.

Functional performance of reference structures and the differentially chloro-substituted derivatives (results of [^{35}S]GTPγS assay) are shown graphically in Figure 4 displaying a range of partial to inverse agonist efficacy, depending on the substitution site. Although the number of compounds and structural variation within the described scaffold is too low to draw a definite conclusion, our results provide first hints to structure–efficacy relationships. Further investigations will be needed to elucidate the structure–efficacy relationships for agonism/partial agonism/antagonism/inverse agonism in this series of differentially substituted diaminopyrimidines. Especially **27** is an interesting compound for follow-up development as it exhibits an inverse agonistic constitutive activity of about -80%, relative to reference **2**. Cyclic structures **37** and **38** possess increased inverse agonist efficacy similar to that of **2** but more limited possibilities for structural modifications. However, taking into consideration that the reference structure is not a full inverse agonist[8] it might be possible to enhance inverse agonist efficacy towards the hH_4R beyond **2**.

To investigate the selectivity of the most potent hH_4R ligands, affinities on the three other histamine receptor subtypes, hH_1R–hH_3R, were determined for selected compounds (Table 5). These structures possess good selectivity over hH_1R and hH_2R because pK_i values are below 6. For compound **32** at least a 80-fold selectivity for hH_4R was seen. With regard to hH_3R the ligands do show significant affinities but like other pyrimidine-containing compounds[34,40] they preferentially bind to hH_4R. Compound **19** is the least selective of compounds tested, the other three ligands favour the hH_4R: affinities of **28** and **29** differ to one order of magnitude, whereas the best selectivity (more than 40-fold) is found in compound **32**. Although ligands are at least monoprotonated under physiological conditions and calculated *S*log *P* values are below 1.5 it can be assumed that these compounds are able to penetrate the blood/brain-barrier. Structurally related aminopyrimidines of similar physicochemical properties are reported to achieve brain/plasma ratios ranging up to 20.[33,40] Therefore, some of the compounds reported here might also be able to penetrate the central nervous system, though this was not tested. All ligands discussed in this context are partial agonists and unlikely to be developed as drug candidates. However, the structures are active on both hH_3R and hH_4R and potentially occurring hH_3R effects have to be considered in further investigations. In the periphery dual acting ligands might be useful in the treatment of pain or cancer. For example, the endogenous ligand **1** influences proliferation of cancer cells via

hH$_3$R and hH$_4$R.[53] To probe such applications the diaminopyrimidines' efficacy towards both receptors will be further investigated in future studies.

6. Conclusions

New hH$_4$R lead structures were successfully identified by structural modifications and structure–affinity relationships of the hits obtained from a virtual screening. By employing classical medicinal chemical methods optimization was carried out to investigate hH$_4$R affinity with the outcome that hH$_4$R binding in the nanomolar concentration range was achieved with the best compounds **28**, **29**, and **32**. Functional profiling for receptor activation was determined for the compounds with pK_i values >6.2. The described series of diaminopyrimidines covers a broad range of functional properties. Substitution in *o*- and *p*-position obviously led to partial agonist efficacy, whereas *m*-substituted and conformationally restrained compounds exhibited inverse agonism. The latter conform to the goal of designing hH$_4$R ligands with inverse agonist functionality. Such property is aimed at enhancing efficacy as anti-inflammatory or immune modulating agents, compared to partial agonists and neutral antagonists.[54] Hence, compound **27** represents a new lead structure for further investigations on expanded structure–efficacy and structure–affinity relationships in this direction.

7. Experimental section

7.1. General chemical procedures

Melting points were determined on a Büchi 510 melting point apparatus (Büchi, Switzerland) and are uncorrected. ^{1}H NMR spectra were recorded on a Bruker AMX 300 (300 MHz) spectrometer (Bruker, Germany). ^{1}H NMR data are reported in the following order: chemical shift (δ) in ppm downfield from tetramethylsilane as internal reference; multiplicity (br, broad; s, singlet; d, doublet; dd, double doublet; t, triplet; m, multiplet); approximate coupling constants (J) in hertz (Hz); number and assignment of protons (mal, maleate; ox, oxalate; prz, piperazine; pur, purine; pyrim, pyrimidine; ph, phenyl). ^{13}C NMR spectra were recorded on a Bruker AC 200 (50 MHz) spectrometer (Bruker, Germany). Depending on scanning time of ^{13}C NMR spectra, signal intensity of pyrim-4*C* and pyrim-6C sometimes was too low to be detected. ESI MS was performed on a Fisons Instruments VG Platform II (Manchester, Great Britain) in positive polarity. Data are listed as mass number ([M+H]$^+$) and relative intensity (%). Elemental analyses (C, H, N) were measured on CHN-Rapid (Heraeus, Germany) and were within ±0.4% of the theoretical values for all final compounds. Preparative column chromatography was performed on silica gel 63–200 μM (Merck, Germany). Hydrogenations were carried out on an autoclave model IV, 500 mL (Roth, Germany). The microwave oven used is a Biotage Initiator 2.0 (Biotage, Sweden). Educts (6-chloro-9*H*-purine, 4,6-dichloropyrimidine, and the corresponding 2-amines, 1-methylpiperazine, propargyl bromide, benzyl bromide, benzyl alcohol, benzyl mercaptane, and benzyl amines) and all other reactants (DIPEA, TEA, Cs_2CO_3, NaH) were commercially obtained from Sigma–Aldrich, ABCR, and Acros Organics and were used without further purification unless otherwise stated.

Preparation of parent compounds and most affine hH$_4$R ligands as well as detailed pharmacological assay procedures are described in the following experimental part, whereas complete analytical data of final compounds can be found in the Supplementary data.

7.1.1. Preparation of purines 9 and 11

6-Chloro-9*H*-purines (4-5 mmol), 1-methylpiperazine (2 equiv) and TEA (4 equiv) were stirred in DMF (50 ml) at rt under argon atmosphere for 12 h. The solvent was evaporated, the residue purified by column chromatography (DCM/ammoniacal MeOH = 9/1) and the product re-crystallized from acetonitrile.

7.1.2. Example compound 6-(4-methylpiperazin-1-yl)-9*H*-purine (9)

Yield: 60%. ^{1}H NMR (DMSO-d_6): δ 13.01 (s, 1H, pur-N*H*), 8.19 (s, 1H, pur-2*H*), 8.10 (s, 1H, pur-8*H*), 4.19 (br s, 4H, prz-2,6H_2), 2.39 (t, J = 4.9, 4H, prz-3,5H_2), 2.19 (s, 3H, prz-CH_3). ^{13}C NMR (DMSO-d_6): δ 153.14 (pur-4*C*), 151.73 (pur-2*C*), 151.44 (pur-6*C*), 138.06 (pur-8*C*), 118.74 (pur-5*C*), 54.58 (prz-3,5*C*), 45.75 (prz-*C*H$_3$), 44.43 (prz-2,6*C*). ESI MS: 218.9 (100). Mp: 270 °C. Anal. ($C_{10}H_{14}N_6$): C, H, N.

7.1.3. Preparation of benzyl purines 10 and 12

Purines **9** or **11** (1.8–3 mmol), benzyl bromide (1.2 equiv), Cs_2CO_3 (2 equiv) and DMF (5 ml) were stirred overnight at 110 °C. After cooling and evaporating the solvent the mixture was purified by column chromatography (DCM/ammoniacal MeOH = 9.5/0.5). The oily products were crystallized as salts.

7.1.4. Example compound 9-benzyl-6-(4-methylpiperazin-1-yl)-9*H*-purine hydrogenmaleate (10)

Yield: 10%. ^{1}H NMR (DMSO-d_6): δ 12.16 (s, 1H, pur-N*H*), 7.65 (s, 1H, pur-8*H*), 5.71 (s, 2H, NH_2), 4.10 (br s, 4H, prz-2,6H_2), 2.35 (t, J = 4.9, 4H, prz-3,5H_2), 2.19 (s, 3H, prz-CH_3). ^{13}C NMR (DMSO-d_6): δ 159.50 (pur-2*C*), 153.80 (pur-4*C*), 153.50 (pur-6*C*), 134.67 (pur-8*C*), 113.16 (pur-5*C*), 54.69 (prz-3,5*C*), 45.85 (prz-*C*H$_3$), 44.14 (prz-2,6*C*). ESI MS: 233.9 (100). Mp: 255–258 °C. Anal. ($C_{10}H_{15}N_7$): C, H, N.

7.2. General procedure for preparation of 14a, 16a, 17a, 19a–36a, 14–16, and 19–38

Monosubstitution of dichloropyrimidines was carried out in a microwave oven using 4,6-dichloropyrimidines (3–12 mmol), the designated amine (0.9 equiv of either 1-methylpiperazine or a substituted benzyl amine), DIPEA (2 equiv), and 2-propanol (5–15 ml). In sealed vials reactants were stirred at 160 °C in the case of 1-methylpiperazine and at 130 °C in the case of benzyl amines. Reaction termination was indicated by TLC; usually 30–60 min were required, depending on scale and substitution pattern of benzyl amines. After cooling the solvent was evaporated and the crude product was purified by column chromatography (DCM/ammoniacal MeOH = 9.5/0.5). The second substitution step was accomplished in the same manner but by adding an excess (2 equiv) of the amine (in most cases 1-methylpiperazine). Final compounds, either as base or as hydrogenmaleate, were re-crystallized from ethanol.

7.2.1. Example compound *N*-benzyl-6-(4-methylpiperazin-1-yl)-pyrimidin-4-amine dihydrogenmaleate (14)

In a sealed vial 4,6-dichloropyrimidine (0.50 g, 3.4 mmol), benzyl amine (0.32 g, 3.1 mmol), DIPEA (0.87 g, 6.8 mmol) were suspended in 2-propanol (4 ml) and heated in the microwave oven at 130 °C for 30 min. After cooling the solvent was evaporated and the residue purified by column chromatography (DCM/ammoniacal MeOH = 9.5/0.5) to obtain *N*-benzyl-6-chloropyrimidin-4-amine **(14a)** as light yellow crystals (yield: 75%). **14a** (0.55 g, 2.5 mmol), together with 1-methylpiperazine (0.50 g, 5 mmol), DIPEA (0.65 g, 5 mmol) and 2-propanol (4 ml), was retreated in the microwave oven at 160 °C for 30 min. Purification by column chromatography led to a beige solid, which was crystallized as hydrogenmaleate salt and re-crystallized in ethanol (overall yield: 30%). ^{1}H NMR (DMSO-d_6): δ 8.07 (s, 1H, pyrim-2*H*), 7.48 (t, J = 6.1, 1H, N*H*), 7.33–7.22 (m, 5H, ph-2,3,4,5,6*H*), 6.13 (s, 4H, mal-(C*H*)$_2$), 5.77 (s, 1H, pyrim-5*H*), 4.46 (d, J = 5.0, 2H, CH_2), 3.71 (br s, 4H,

prz-2,6H_2), 3.22 (s, 4H, prz-3,5H_2), 2.81 (s, 3H, prz-CH_3). ^{13}C NMR (DMSO-d_6): δ 166.87 (mal-*C*OOH), 162.83 (pyrim-6*C*), 161.28 (pyrim-4*C*), 156.91 (pyrim-2*C*), 139.75 (ph-1*C*), 133.17 (mal-*C*H), 128.22 (ph-2,6*C*), 127.13 (ph-3,5*C*), 126.68 (ph-4*C*), 82.11 (pyrim-5*C*), 51.94 (prz-3,5*C*), 43.65 (NH–*C*H_2), 42.22 (prz-*C*H_3), 40.92 (prz-2,6*C*). ESI MS: 284.1 (100). Mp: 168 °C. Anal. ($C_{16}H_{21}N_5$. 2 $C_4H_4O_4$): C, H, N.

7.2.2. Example compound N^4-benzyl-6-(4-methylpiperazin-1-yl)pyrimidine-2,4-diamine dihydrogenmaleate (16)

2-Amino-4,6-dichloropyrimidine (1.00 g, 6.0 mmol), benzyl amine (0.56 g, 5.4 mmol), DIPEA (1.54 g, 12.0 mmol), and 2-propanol (10 ml) were heated under microwave irradiation at 130 °C for 60 min. Purification was accomplished by column chromatography (DCM/ammoniacal methanol = 9.5/0.5). The resulting yellowish powder (yield: 87%), *N^4-benzyl-6-chloropyrimidin-2,4-diamine* **(16a**, 1.10 g, 4.7 mmol**)**, was suspended in 2-propanol (10 ml), and 1-methylpiperazine (0.94 g, 9.4 mmol) and DIPEA (1.22 g, 9.4 mmol) were added. The mixture was stirred in the microwave oven at 160 °C for 30 min. After purification by column chromatography the resulting oil was crystallized by adding maleic acid to obtain a white solid (overall yield: 34%). ^{1}H NMR (DMSO-d_6): δ 7.75 (s, 1H, N*H*), 7.38–7.25 (m, 5H, ph-2,3,4,5,6*H*), 7.05 (br s, 2H, NH_2), 6.06 (s, 4H, mal-(C*H*)$_2$), 5.42 (s, 1H, pyrim-5*H*), 4.45 (d, J = 5.9, 2H, CH_2), 3.74 (br s, 4H, prz-2,6H_2), 3.17 (s, 4H, prz-3,5H_2), 2.76 (s, 3H, prz-CH_3). ^{13}C NMR (DMSO-d_6): δ 167.10 (mal-*C*OOH), 161.56 (pyrim-2*C*), 157.40 (pyrim-4*C*), 155.95 (pyrim-6*C*), 138.20 (ph-1*C*), 134.94 (mal-*C*H), 128.44 (ph-3,5*C*), 127.27 (ph-2,6*C*), 127.19 (ph-4*C*), 72.15 (pyrim-5*C*), 52.04 (prz-3,5*C*), 44.25 (NH–*C*H_2), 42.49 (prz-*C*H_3), 40.35 (prz-2,6*C*). ESI MS: 299.0 (100). Mp: 169 °C. Anal. ($C_{16}H_{22}N_6 \cdot 2C_4H_4O_4$): C, H, N.

7.2.3. Example compound 4-(benzyloxy)-6-(4-methylpiperazin-1-yl)pyrimidin-2-amine (17)

4,6-Dichloropyrimidin-2-amine (2.00 g, 12.2 mmol), 1-methylpiperazin (1.10 g, 10.9 mmol), DIPEA (3.20 g, 24.4 mmol), and 2-propanol (15 ml) were heated under microwave irradiation at 160 °C for 60 min. After cooling the product crystallized and was filtered out. *4-Chloro-6-(4-methylpiperazin-1-yl)pyrimidin-2-amine* **(17a)**, a white solid (yield: 68%), was used without further purification. Under argon atmosphere, NaH (60%, 0.23 g, 5.7 mmol) was suspended in THF (15 ml) and benzyl alcohol (0.48 g, 4.4 mmol) was added dropwise. The suspension was stirred at rt for 15 min, subsequently cooled to 0 °C and treated with a solution of **17a** (0.5 g, 2.2 mmol) in THF (5 ml). The solution was allowed to come to rt and was stirred for 24 h. The reaction was quenched with a saturated solution of NH_4Cl. THF was reduced under vacuum and the crude product extracted into ethyl acetate (2 × 20 ml). The organic layers were combined, washed with 2 N-NaOH and brine, dried over Na_2SO_4 and concentrated to dryness. Further purification was accomplished by column chromatography (DCM/ammoniacal methanol = 9.5/0.5) and the product re-crystallized from ethanol (white solid; yield: 20%). ^{1}H NMR (DMSO-d_6): δ 7.40–7.27 (m, 5H, ph-2,3,4,5,6*H*), 6.07 (s, 2H, NH_2), 5.38 (s, 1H, pyrim-5*H*), 5.23 (s, 2H, CH_2), 3.43 (t, J = 4.5, 4H, prz-2,6H_2), 2.28 (t, J = 4.7, 4H, prz-3,5H_2), 2.17 (s, 3H, prz-CH_3). ^{13}C NMR (DMSO-d_6): δ 170.55 (pyrim-4*C*), 164.70 (pyrim-6*C*), 162.34 (pyrim-2*C*), 137.45 (ph-1*C*), 128.24 (ph-3,5*C*), 127.78 (ph-2.6*C*), 127.58 (ph-4*C*), 75.64 (pyrim-5*C*), 66.00 (O–*C*H_2), 54.28 (prz-3,5*C*), 45.74 (prz-*C*H_3), 43.54 (prz-2,6*C*). ESI MS: 299.9 (100). Mp: 161 °C. Anal. ($C_{16}H_{21}N_5O$): C, H, N.

7.2.4. Example compound 4-(benzylthio)-6-(4-methylpiperazin-1-yl)pyrimidin-2-amine dihydrogenmaleate (18)

Compound **17a** (0.86 g, 3.8 mmol) and NaOH (0.18 g, 4.4 mmol) were dissolved in water (10 ml) and ethanol (15 ml). Benzyl mercaptane (0.71 g, 5.7 mmol) was added and the solution was refluxed for 12 h. After cooling to rt, ethanol was evaporated and water added leading to the crystallization of the product, which was washed with small amounts of diethyl ether. It was crystallized as dihydrogenmaleate and re-crystallized twice from ethanol to give a white solid (yield: 3%). ^{1}H NMR (DMSO-d_6): δ 7.41–7.21 (m, 5H, ph-2,3,4,5,6*H*), 6.36 (s, 2H, NH_2), 6.14 (s, 4H, mal-(C*H*)$_2$), 6.04 (s, 1H, pyrim-5*H*), 4.33 (s, 2H, CH_2), 3.72 (br s, 4H, prz-2,6H_2), 3.19 (s, 4H, prz-3,5H_2), 2.80 (s, 3H, prz-CH_3). ^{13}C NMR (DMSO-d_6): δ 167.35 (pyrim-4*C*), 166.91 (mal-*C*OOH), 161.76 (pyrim-6*C*), 161.60 (pyrim-2*C*), 138.43 (ph-1*C*), 133.12 (mal-*C*H), 128.95 (ph-3,5*C*), 128.33 (ph-2,6*C*), 126.91 (ph-4*C*), 88.92 (pyrim-5*C*), 51.92 (prz-3,5*C*), 42.19 (prz-*C*H_3), 40.73 (prz-2,6*C*), 32.06 (S–*C*H_2). ESI MS: 316.0 (100). Mp: 179 °C. Anal. ($C_{16}H_{21}N_5S \cdot 2C_4H_4O_4 \cdot 0.5H_2O$): C, H, N.

7.2.5. Preparation of 1-((1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl)-4-methylpiperazine dihydrogenmaleate (13)

Benzyl bromide (0.34 g, 2 mmol) and NaN_3 (0.33 g, 5 mmol) were mixed in water (4 ml) and heated in the microwave oven at 120 °C for 30 min. The product was extracted in diethyl ether and the solvent evaporated to give (*azidomethyl)benzene* **(13a)** as a colourless oil (yield: 100%). Propargyl bromide (0.29 g, 2.4 mmol), 1-methylpiperazine (0.24 g, 2.4 mmol), and TEA (0.40 g, 4 mmol) were stirred in water (4 ml) at rt for 1 h. CuI (0.046 g, 0.2 mmol) and **13a** (0.26 g, 2 mmol) were added and the mixture was stirred for further 12 h. After addition of water and extraction with methylene chloride the organic layer was dried (Na_2SO_4) and concentrated to dryness. The product was purified by column chromatography (DCM:MeOH/NH_3 = 9:1) and crystallized as dihydrogenmaleate (yield: 17%). ^{1}H NMR (DMSO-d_6): δ 7.85 (s, 1H, trz-5*H*), 7.17–7.05 (m, 5H, ph-2,3,4,5,6*H*), 5.92 (mal-(C*H*)$_2$), 5.35 (s, 2H, ph-CH_2), 3.46 (s, 2H, prz-CH_2), 2.91 (br s, 4H, prz-2,6H_2), 2.48 (s, 4H, prz-3,5H_2), 2.26 (s, 3H, prz-CH_3). ^{13}C NMR (DMSO-d_6): δ 166.92 (mal-*C*OOH), 142.26 (trz-4*C*), 136.04 (ph-1*C*), 133.11 (mal-*C*H), 128.75 (ph-3,5*C*), 128.12 (ph-4*C*), 127.87 (ph-2,6*C*), 124.35 (trz-5*C*), 52.72 (ph-CH_2), 52.55 (prz-3,5*C*), 51.11 (prz-CH_2), 48.89 (prz-2,6*C*), 42.25 (prz-CH_3). ESI MS: 272.1 (100). Mp: 186–189 °C. Anal. ($C_{15}H_{21}N_5$. 2 $C_4H_4O_4$): C, H, N.

7.3. Virtual screening

The Specs database[55] containing 199,272 compounds was virtually screened. After in silico washing of the virtual molecules, that is, protonating basic and deprotonating acidic compounds, the database was prefiltered with MOE[56] to select cationic compounds. The remaining 9867 molecules were straightforwardly resorted by their 2-dimensional topological pharmacophore similarity towards structural features of **3** by the software package CATS.[39] A 150-dimensional correlation vector-based molecular descriptor was compiled for the reference molecule and all residual molecules in the database. Next, each screening substance was ranked according to its pairwise Euclidean distance to the reference. Twenty seven compounds from the top percentiles of the ranked remnant database were selected by experienced medicinal chemists under consideration of the synthetic accessibility. Another selection criterion was the frequency of occurrence of certain scaffolds. These hand-picked molecules were tested in a competition binding assay. Nine compounds yielded an affinity <30 μM, which implied a hit rate of 33%, and two of these compounds, 3-(4-chlorobenzyl)-7-(4-methylpiperazin-1-yl)-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidine (**7**) and 6-ethyl-4-(4-methylpiperazin-1-yl)thieno[2,3-*d*]pyrimidine (**8**) showed an affinity in the low micromolar concentration range.

7.4. Binding assay on *h*H_1R, *h*H_2R, and *h*H_3R

Prior to the experiments, membranes were sedimented by a 10 min centrifugation at 4 °C and 16,000*g* and resuspended in

binding buffer (12.5 mM $MgCl_2$, 1 mM EDTA and 75 mM Tris/HCl, pH 7.4). For determination of K_d and B_{max} values, Sf9 membranes (30–70 μg of protein per tube, depending on the specific expression level) were suspended in 250 μl of binding buffer containing the radioligand and 0.2% (mass/vol) of bovine serum albumin.

For competition binding studies on hH$_1$R, Sf9 cell membranes co-expressing the hH$_1$R with RGS4 were incubated with [^{3}H]pyrilamine (5 nM, 954.6 GBq/mmol). Binding studies on hH$_2$R were performed using the hH$_2$R-Gsαs fusion protein and the radioligand [^{3}H]tiotidine (10 nM, 2.8564 TBq/mmol). Binding studies on hH$_3$R were performed by incubating membranes co-expressing hH$_3$R, Gαi2, Gβ1γ2 and RGS4 with [^{3}H]N^{α}-methylhistamine (1 nM, 2.6048 TBq/mmol). Non-specific binding was determined in the presence of 10 μM of diphenhydramine (hH$_1$R), famotidine (hH$_2$R) or thioperamide (hH$_3$R).

Incubations were performed for 60 min at 25 °C and shaking at 250 rpm. Bound radioligand was separated from free radioligand by filtration through GF/C filters (for binding on hH$_2$R and hH$_3$R pretreated with 0.3% (mass/vol) polyethyleneimine) and washed three times with 2 ml of ice-cold binding buffer (4 °C). Filter-bound radioactivity was determined by liquid scintillation counting.

7.5. Binding assay on hH$_4$R

Prior to the experiments, cell membranes were sedimented by a 10 min centrifugation at 4 °C and 16,000g. and resuspended in binding buffer (12.5 mM $MgCl_2$, 1 mM EDTA and 75 mM Tris/HCl, pH 7.4). Competition binding experiments were carried out by incubating membranes, 35 μg/well (prepared from Sf9 cells expressing hH$_4$R, co-expressed with G protein Gαi2 and Gβ$_1$γ$_2$ subunits) in a final volume of 0.2 ml containing binding buffer and [^{3}H]histamine (10 nM, 566.1 GBq/mmol). Assays were run in triplicates with seven appropriate concentrations between 0.1 nM and 100 μM of the test compound. Incubations were performed for 60 min at 25 °C and shaking at 250 rpm. Non-specific binding was determined in the presence of 100 μM unlabelled **1**. Bound radioligand was separated from free radioligand by filtration through GF/B filters pretreated with 0.3% (mass/vol) polyethyleneimine and washed three times with 0.5 ml of ice-cold binding buffer (4 °C). The amount of radioactivity collected on the filter was determined by liquid scintillation counting. Determination of affinity values (K_i) were calculated by Graph Pad Prism 3.02.

7.6. hH$_4$R [^{35}S]GTPγS binding assay

Prior to the experiments, cell membranes were sedimented by a 10 min centrifugation at 4 °C and 16,000g and resuspended in binding buffer (1 μM GDP, 12.5 mM $MgCl_2$, 100 mM NaCl and 75 mM Tris/HCl, pH 7.4). [^{35}S]GTPγS binding studies in the agonist mode were performed with binding buffer in the presence of 0.6 nM [^{35}S]GTPγS in a final volume of 0.2 ml. In the antagonist mode hH$_4$R was stimulated with 100 nM **1** resulting in a final volume of 0.25 ml. Membranes, 10 μg/well (prepared from Sf9 cells expressing hH$_4$R, co-expressed with G-protein Gαi2 and Gβ$_1$γ$_2$ subunits) and ligands were preincubated without [^{35}S]GTPγS for 30 min to obtain steady-state receptor occupation. Assays were run in triplicates with seven appropriate concentrations between 0.01 nM and 100 μM of test compound. After addition of [^{35}S]GTPγS, membranes were further incubated for 120 min at 25 °C and shaking at 250 rpm. Basal [^{35}S]GTPγS binding was measured in the absence of ligands. The reaction was terminated by rapid filtration through GF/B filters soaked in binding buffer followed by three washings with 0.5 ml of ice-cold binding buffer (4 °C). The amount of radioactivity collected on the filter was determined by liquid scintillation counting. The compounds were tested in three independent experiments ($n \geqslant 3$, mean ± SEM). Determination of potency of receptor activation (EC_{50}) was performed by GraphPad Prism 3.02.

7.7. Steady-state GTPase assay

Sf9 membranes were thawed, sedimented and resuspended in 10 mM Tris/HCl, pH 7.4. Each assay tube contained 80 μl of 50 mM Tris/HCl (pH 7.4) with 5.0 mM $MgCl_2$, 100 μM EDTA, 100 μM ATP, 100 nM GTP, 100 μM adenylyl imidodiphosphate, 1.2 mM creatine phosphate, 1 μg of creatine kinase, 0.2% (w/v) BSA, 100 mM NaCl and H$_4$R ligands at various concentrations. Additionally, each tube contained 10–20 μg of Sf9 membranes co-expressing the hH$_4$R with Gαi2 and Gβ1γ2. The tubes were incubated for 2 min at 25 °C before the addition of 20 μl of [γ-^{32}P]GTP to yield 0.1 μCi per tube (final volume 100 μl). The [γ-^{32}P]GTP work solutions were prepared in 20 mM Tris/HCl, pH 7.4.

Reactions were conducted for 20 min at 25 °C and terminated by the addition of 900 μl of slurry consisting of 5% (w/v) activated charcoal in 50 mM NaH_2PO_4 (pH 2.0). Charcoal adsorbs nucleotides but not $^{32}P_i$. Charcoal-quenched reaction mixtures were centrifuged for 15 min at room temperature at 15,000 × g. Supernatant fluid of the reaction mixtures was removed (600 μl) and $^{32}P_i$ was determined in water by detecting the Čerenkov radiation. Enzyme activities were corrected for spontaneous degradation of [γ-^{32}P]GTP, which was determined by adding a very high concentration of unlabelled GTP (1 mM) to tubes containing all of the above described components. Competing with [γ-^{32}P]GTP, the unlabeled GTP prevents hydrolysis of [γ-^{32}P]GTP by the enzymatic activities present in Sf9 membranes. The [γ-^{32}P]GTP, which was prepared by labelling of GDP with ^{32}P, was highly stable, showing a spontaneous degradation of <1% in 20 mM Tris/HCl, pH 7.4. The experimental conditions chosen ensured that not more than 10% of the total amount of [γ-^{32}P]GTP added was converted to ^{32}P.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.08.059.

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