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Piperazine modification in 2,4,6-triaminopyrimidine derivatives as histamine H_4 receptor ligands

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Dedicated to Prof. Dr. Theo Dingermann, Frankfurt, on the occasion of his 65th birthday

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The human histamine H_4 receptor (hH_4R) is a promising new target in the therapy of inflammatory and immunomodulatory diseases. The 2,4,6-triaminopyrimidine structure has been established as a potent hH_4R affinity scaffold. By using the inverse agonist ST-1012 as reference ligand, piperazine modifications were performed to get larger structural variations. Therefore, different spacers were introduced into the lead structure and the influence on affinity of this basic element was evaluated. While a short distance between aminopyrimidine and basic moiety is beneficial, a lipophilic group in the eastern part is necessary to maintain hH_4R affinity.

1. Introduction

The most recently discovered histamine receptor subtype, the hH_4R, belongs like the other histaminergic receptors to the family of class A (rhodopsin-like) G protein-coupled receptors (GPCR). It is mainly expressed peripherally on hematopoietic cells and plays a key role in chemotaxis, upregulation of inflammatory cells and hence, in inflammatory and immunomodulatory signaling (Zampeli et al. 2009; Walter et al. 2011). On molecular level hH_4R stimulation induces activation of $G\alpha_{i/o}$ protein and therefore inhibition of the adenylylcyclase. Additionally proteinkinase A and its downstream signals are modulated by receptor activation (Leurs et al. 2009; Liu et al. 2001; Morse et al. 2001; Nakamuira et al. 2000; Nguyen et al. 2001; Oda et al. 2000; Zhu et al. 2001). In the development of drug candidates that find their application in the therapy of chronic inflammatory diseases and immune disorders the hH_4R is a current target.

The first selective hH_4R antagonist was identified by Johnson&Johnson with the indole derivate JNJ-7777120 (Fig.) (Jablonski et al. 2003). Meanwhile the efficacy profile of this reference compound has been shown to be system, assay and species dependent (Rosethorne and Charlton 2010; Seifert et al. 2011). At the same time the poor pharmacokinetic properties like the short half-life (rat $t_{1/2} = 3$ h (Venable et al. 2005)) due to its weak metabolic stability, led to new compound classes for further investigation of H_4R dependent effects.

Based on a virtual screening we have previously deduced a blueprint structure for hH_4R ligands constructed on a 2,4,6-triaminopyrimidine core structure (Sander et al. 2009, 2010). It contains a central 2,4,6-triaminopyrimidine as conjugated donor/acceptor group, a basic group in the western and a lipophilic moiety in the eastern molecule part (Fig.). Thereby, slight structural changes of the aryl substituent led to a great variability in efficacy of the compounds mostly maintaining their affinities. Molecular Dynamics (MD) simulation studies indicate different ligand-receptor-binding modes of the inverse agonist ST-1012 and the structurally related partial agonist ST-1006 with a pseudo-ionic lock mechanism (Tanrikulu et al. 2009; Werner et al. 2012).

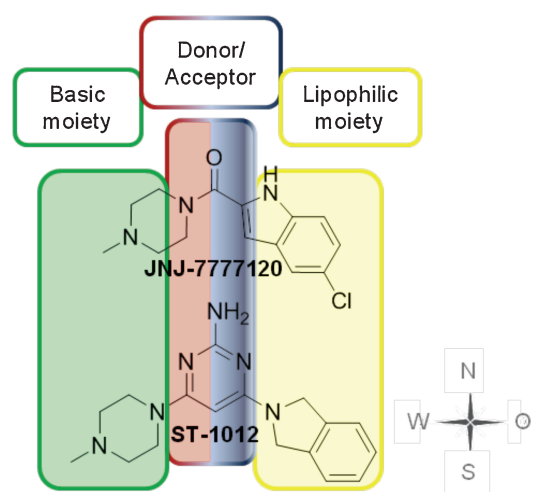


Fig.: Blueprint for hH_4R ligands.

Structural modifications of the basic moiety on hH_4R inverse agonists as pyrimidine derivatives led to dramatic changes in affinity and stability (Cramp et al. 2010). To investigate the influence of the piperazine moiety and some spacers thereof, the 2,4,6-triaminopyrimidine lead element was taken for further structural modifications and the hH_4R affinity was measured on membranes of a transient expression system with co-expression of G proteins (Schneider et al. 2009; Kottke et al. 2011). Here we describe two approaches for the modification of ST-1012 and related structures:

1. Elongation of the spacer between 4-methylpiperazino and pyrimidine by introduction of different unbranched alkyl linkers.
2. Exchange of the lipophilic residue by piperazine moieties as potential second basic binding element.

2. Investigations, results and discussion

Different alkyl spacer lengths for the connection of the piperazine element on hH_4R ligands can already be found in the class of benzimidazole derivatives (Savall et al. 2010). Consequently different linkers from two to five methylene groups were introduced between the aliphatic basic moiety and aromatic aminopyrimidine moiety while keeping the lipophilic element with the isoindoline substituent constant. All compounds were prepared stepwise in microwave assisted nucleophilic substitution reactions of 4,6-dichloropyrimidine-2-amine and an appropriate aliphatic amine. In general, secondary amines reacted faster and in higher yields than primary ones because of their increased nucleophilicity. As polar, protic solvent either 2-propanol or ethanol was used and diisopropyl ethyl amine (DIPEA) acted as basic auxiliar. Compounds **5 - 9** were prepared by exchange of the lipophilic moiety (isoindoline) by a second basic structure based on piperazine. Compounds **5** and **7** could be obtained in a single step reaction with an excess of the respective amine (piperazine or 1-methylpiperazine, Scheme).
A series of compounds **1 - 9** could be prepared in moderate to good yields using the well-established conditions for microwave assisted nucleophilic substitution reactions. The pharmacological results on hH_4R obtained are listed in the Table. Although all compounds showed moderate to good affinity at hH_4R, none of the compounds achieved the high binding affinity of the lead structure ST-1012 (hH_4R $K_i = 0.248$ μM, Sander et al. 2009).
Increasing spacer length also increased the K_i values of the compounds (**1** < **2** < **3**) with the exception of the longest alkyl chain tested (five methylene groups) for compound **4** which is in the same affinity range as the trimethylene spacer derivative **2**. Although the higher degree of freedom in combination with an increased lipophilicity may be the reason for this outlier, it has not been further investigated due to the drop in affinity of about two orders of magnitude in comparison to that of ST-1012. Despite the observed decrease in affinity of compound **1** with the ethylene linker in comparison to that of the reference compound ST-1012, it strikingly shows still moderate to good binding properties even though this large structural modification.
Exchange of the lipophilic isoindole moiety of ST-1012 by another 4-methylpiperazino element (**5**) led to a reduction in affinity of about one order of magnitude, but is in a similar range as that of the one ST-1012 homologue with the dimethylene spacer **1**. Change from the two tertiary amine functionalities to one (**6**) or two (**7**) secondary aliphatic amine/s with the piperazine introduction led to decrease in affinities of a factor of 3 and 30, respectively. This is in accordance with structural variations in hH_4R ligands in different series in which balanced basicity seems to be the main factor for this moiety and not hydrogen bond acceptor/donator function depending on the protonation status (Werner et al. 2010; Cramp et al. 2010).
The combination of the elongated spacer approach with the exchange of the lipophilic residue with the 4-methylpiperazino moiety has been performed with the design and synthesis of compounds **8** and **9**. As both compounds showed a dramatic loss in affinities, we have not followed this unsuccessful approach in further extent.
For verification of druglikeness and evaluation as a potential new lead the ligand efficiency (LE) was calculated for each compound based on their hH_4R affinity. LE is an important concept in drug discovery partly due to the realization that large ligands have a disadvantage in terms of the molecular properties necessary for bioavailability or related pharmacokinetic parameters and is calculated by the following equation:

$$LE = \frac{-0.592 \cdot \ln(IC_{50})}{\text{n}^\circ \text{ of heavy (non-hydrogen) atoms}}$$

LE with values of > 0.3 are generally validated as favorable (Abad-Zapatero 2007; Hopkins 2004; Meanwell 2011). Table shows that compounds **5**, **6** and the reference compound ST-1012 revealed promising structure concerning their LE values whereas for the remaining compounds a reduction in ligand efficiency could be calculated.
Due to controversial results regarding the pharmacological behavior of hH_4R ligands and especially the influence of the basic moiety we have performed structural modification on the basic piperazine-based element in the class of 2,4,6-triaminopyrimidine derivatives. Taking the reference compound ST-1012 as starting lead we have introduced some spacer variations as well as an exchange of the lipophilic isoindole residue by piperazine elements and a combination of both. All new compounds showed affinities at hH_4R in the micromolar concentration range which is somewhat lower than that of ST-1012. Surprisingly compounds **5** and **6** with one or two 4-methylpiperazino moieties and without the lipophilic isoindole substituent on the pyrimidine core element showed low micromolar affinity values and promising ligand efficiencies.

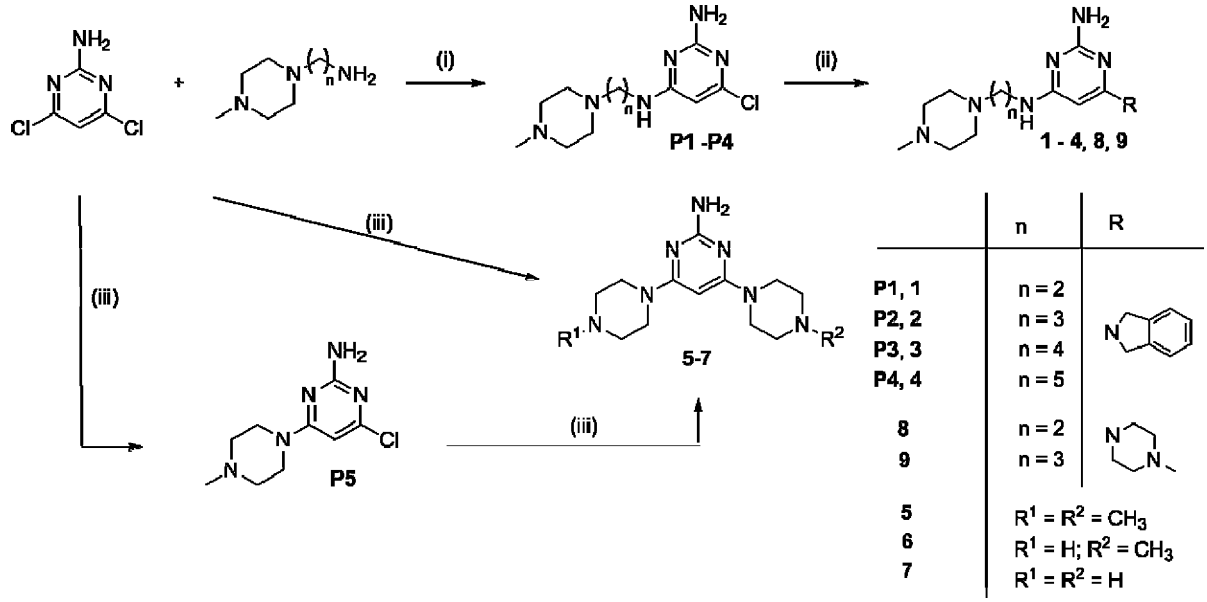
3. Experimental

3.1. Chemistry

Reagents and solvents were purchased from commercial suppliers. ^{1}H NMR spectra were recorded on a Bruker AMX 250 (250 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; number and assignment of protons (but, butyl, eth, ethyl; isoind, isoindoline; prop, propyl; pent, pentyl, pipaz, piperazine; pyr, pyrimidine). ^{13}C NMR spectra were recorded on a Bruker AMX 250 (250 MHz) spectrometer (Bruker, Germany). 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 (%). High Resolution Mass Spectrometry (HRMS) was performed on a Thermo Scientific MALDI LTQ XL Orbitrap (Waltham, USA) and were within ± 5 ppm of the theoretical values for final compounds. Elemental analyses (C, H, N) were measured on CHN-Rapid (Heraeus, Germany) and were within ± 0.4% of the theoretical values for final compounds. The microwave oven used was a Biotage Initiator 2.0 (Biotage, Sweden).
Substitution reactions were carried out under microwave irradiation. 4,6-Dichloropyrimidine-2-amine (1 eq), the designated amine (0.9 eq) and diisopropyl ethyl amine (DIPEA) (2 eq) were dissolved in 2-propanol. In sealed vials reactants were stirred at 130 °C for 30-60 min. Reaction termination was indicated by thin layer chromatography. After cooling the solvent was removed under reduced pressure and the crude product was purified using flash-chromatography (eluent dichloromethane (DCM): ammonia saturated methanol = 9:1). The second substitution step was accomplished in the same manner but by adding an excess (2 eq) of the amine and stirring the reactants at 75 W (160 °C) for 1-1.5 h. Products were purified by flash chromatography on silica gel (eluent DCM: ammonia saturated methanol = 9:1).

3.1.1. 6-Chloro-N^4-(2-(4-methylpiperazin-1-yl)ethyl)pyrimidine-2, 4-diamine (P1)

169 mg (43%), $C_{11}H_{19}ClN_6$, ^{1}H NMR (250 MHz, DMSO-d_6) δ = 7.08 (br s, 1H, NH), 6.45 (s, 2H, NH_2), 5.82 (s, 1H, pyr-5H), 3.40 (m, 2H, eth-1H_2), 2.45 (m, 8H, pipaz-2,3,5,6H_2), 2.37 (m, 5H, eth-2H_2), 2.19 (s, 3H, CH_3) ppm; ESI MS: 271.0 $[M+H^+]$.



Scheme: Synthesis of compounds: (i) DIPEA, 2-propanol, MW, 130 °C, 30-90 min.; (ii) Isoindoline or 1-methylpiperazine, DIPEA, 1-propanol, MW, 160 °C, 1 h; (iii) 1-Methylpiperazine or piperazine, DIPEA, ethanol, MW, 160 °C.

3.1.2. 6-(Isoindolin-2-yl)-N⁴-(2-(4-methylpiperazin-1-yl)ethyl) pyrimidine-2,4-diamine hydrate (1)

484 mg (72%), $C_{19}H_{27}N_7 \cdot 0.25H_2O$, ^{1}H NMR(250 MHz; $CDCl_3$-*d*) δ = 7.24 (m, 4H, isoind-4,5,6,7H), 5.01 (br s, 1H, NH), 4.84 (s, 1H, pyr-5H), 4.63 (s, 4H, isoind-1,3H_2), 4.59 (s, 2H, NH_2), 3.22 (m, 2H, eth-1H_2), 2.55 (m, 2H, eth-2H_2), 2.42 (m, 8H, pipaz-1,3,5,6H_2); 2.23 (s, 3H, CH_3) ppm; ^{13}C NMR (250 MHz; $CDCl_3$-*d*) δ = 164.30 (pyr-6C), 162.72 (pyr-4C), 162.47 (pyr-2C), 137.58 (isoind-3a, 7aC), 127.00 (isoind-5,6C), 122.50 (isoind-4,7C), 73.82 (pyr-5C), 56.88 (eth-2C), 55.03 (isoind-1,3C)53.18 (pipaz-2,3,5,6C), 46.57 (eth-1C), 38.10 (CH_3) ppm; ESI MS: 354.2 [M + H⁺]. Anal. Calc.: C, 63.75; H, 7.74; N27.39. Found: C, 64.02; H, 7.45; N, 27.63.

3.1.3. 6-Chloro-N⁴-(3-(4-methylpiperazin-1-yl)propyl)pyrimidine-2,4-diamine (P2)

268 mg (65%), $C_{12}H_{21}ClN_6$, ^{1}H NMR (250 MHz, DMSO-d_6) δ = 7.12 (br s, 1H, NH), 6.41 (s, 2H, NH_2), 5.77 (s, 1H, pyr-5H), 3.22 (m, 2H, prop-1H_2), 2.28 (m, 8H, pipaz-2,3,5,6H_2), 2.14 (m, 5H, CH_3, prop-3H_2), 1.61 (m, 2H, prop-CH_2) ppm; ESI MS: 285.0 [M + H⁺].

3.1.4. 6-(Isoindolin-2-yl)-N⁴-(3-(4-methylpiperazin-1-yl)propyl) pyrimidine-2,4-diamine trihydrogenoxalate hydrate (2)

277 mg (44%), $C_{20}H_{29}N_7 \cdot 3(COOH)_2 \cdot 0.25H_2O$, ^{1}H NMR(250 MHz; DMSO-d_6) δ = 7.71 (br s, 1H, NH), 7.49 (br s, 2H, NH_2), 7.39 (m, 4H, isoind-4,5,6,7H), 5.13 (s, 1H, pyr-5H), 4.80 (s, 4H, isoind-1,3H_2), 3.30 (m, 2H, prop-1H_2), 3.09 (m, 4H, pipaz-3,5H_2), 2.73 (m, 2H, prop-3H_2); 2.68 (s, 3H, CH_3); 2.52 (m, 4H, pipaz-2,6H_2); 1.75 (m, 2H, prop-2H_2) ppm; ^{13}C NMR (250 MHz; DMSO-d_6) δ = 164.09 (pyr-6C), 162.51 (pyr-4C), 161.29 (pyr-2C), 137.35 (isoind-3a, 7aC), 127.21 (isoind-5,6C), 122.85 (isoind-4,7C), 73.42 (pyr-5C), 55.92 (prop-3C), 55.04 (isoind-1,3C); 52.60 (pipaz-2,3,5,6C), 51.89 (prop-1C), 45.96 (CH_3); 26.56 (prop-2C) ppm; ESI MS: 368.5 [M + H⁺]. Anal. Calc.: C, 48.63; H, 5.57; N, 15.27. Found: C, 48.34; H, 5.60; N, 15.71.

3.1.5. 6-Chloro-N⁴-(4-(4-methylpiperazin-1-yl)butyl)pyrimidine-2,4-diamine (P3)

407 mg (57%), $C_{13}H_{23}ClN_6$, ^{1}H NMR (250 MHz, DMSO-d_6) δ = 7.10 (br s, 1H, NH), 6.36 (s, 2H, NH_2), 5.73 (s, 1H, pyr-5H), 3.17 (m, 2H, but-1H_2), 2.31-2.23 (m, 10H, pipaz-2,3,5,6H_2, but-4H_2), 2.14 (s, 3H, CH_3), 1.46 (m, 4H, but-2,3H_2) ppm; ESI MS: 299.0 [M + H⁺].

3.1.6. 6-(Isoindolin-2-yl)-N⁴-(4-(4-methylpiperazin-1-yl)butyl) pyrimidine-2,4-diamine trihydrogenoxalate (3)

277 mg (44%), $C_{21}H_{31}N_7 \cdot 3(COOH)_2$, ^{1}H NMR(250 MHz; DMSO-d_6) δ = 7.91 (br s, 1H, NH), 7.40 (br s, 2H, NH_2), 7.38-7.36 (m, 4H, isoind-4,5,6,7H), 5.132 (s, 1H, pyr-5H), 4.80 (s, 4H, isoind-1,3H_2), 3.26 (m, 2H, but-1H_2), 3.01 (m, 4H, pipaz-3,5H_2), 2.80 (m, 4H, pipaz-2,6H_2); 2.60 (br s, 5H, but-3H_2, CH_3); 1.59 (m, 4H, but-2,3H_2) ppm; ^{13}C NMR (250 MHz; DMSO-d_6) δ = 164.10 (pyr-6C), 164.04 (pyr-6C), 154.52 (pyr-4C), 146.89(pyr-2C), 136.03 (isoind-3a, 7aC), 127.49 (isoind-5,6C), 122.81 (isoind-4,7C), 71.36 (pyr-5C), 55.91 (isoind-1,3C), 52.70 (but-1C), 52.11 (pipaz-3,5C), 49.73 (pipaz-2,6C), 46.78 (CH_3), 42.90 (but-4C), 25.81 (but-3C), 22.48 (but-2C) ppm; ESI MS: 380.86 [M + H⁺]. Anal. Calc.: C, 49.77; H, 5.72; N, 15.05. Found: C, 50.09; H, 6.04; N, 14.74.

3.1.7. 6-Chloro-N⁴-(5-(4-methylpiperazin-1-yl)pentyl)pyrimidine-2,4-diamine (P4)

196 mg (38%), $C_{14}H_{25}ClN_6$, ^{1}H NMR (250 MHz, DMSO-d_6) δ = 7.08 (br s, 1H, NH), 6.35 (s, 2H, NH_2), 5.72 (s, 1H, pyr-5H), 3.34 (m, 2H, but-1H_2), 2.31-2.20 (m, 10H, pipaz-2,3,5,6H_2, pent-3H_2), 2.14 (s, 3H, CH_3), 1.45 (m, 4H, pent-2,4H_2), 1.28 (m, 2H, pent-3H_2) ppm; ESI MS: 313.3 [M + H⁺].

3.1.8. 6-(Isoindolin-2-yl)-N⁴-(5-(4-methylpiperazin-1-yl)pentyl)pyrimidine-2,4-diamine (4)

149 mg (65%), $C_{22}H_{33}N_7$, ^{1}H NMR(250 MHz; DMSO-d_6) δ = 7.20(m, 4H, isoind-4,5,6,7H), 4.78 (m, 3H, NH_2, pyr-5H), 4.15 (m, 4H, isoind-1,3H_2), 3.12 (m, 2H, pent-1H_2), 2.37 (m, 8H, pipaz-1,3,5,6H_2), 2.28-2.20 (m, 5H, pent-5H_2, CH_3), 1.64-1.42 (m, 4H, pent-2,4H_2), 1.28 (m, 2H, pent-3H_2) ppm; ^{13}C NMR (250 MHz; DMSO-d_6) δ = 163.54 (pyr-6C), 162.14 (pyr-4C), 162.09 (pyr-2C), 137.19(isoind-3a,7aC), 127.27 (isoind-5,6C), 122.66 (isoind-4,7C), 72.87 (pyr-5C), 58.47 (pent-5C), 55.06 (pipaz-3,5C), 53.19 (pipaz-2,6C), 52.56 (isoind-1,3C), 45.99 (CH_3), 41.70 (pent-1C), 29.24 (pent-2C), 26.56 (pent-4C), 24.98 (pent-3C) ppm; ESI MS: 396.9 [M + H⁺]. Anal. Calcd for HRMS: 396.28702 Found: 396.28747.

3.1.9. 4,6-Bis(4-methylpiperazin-1-yl)pyrimidine-2-amine (5)

320 mg (36%), $C_{14}H_{25}N_7$, ^{1}H NMR(250 MHz; DMSO-d_6) δ = 5.55 (s, 2H, NH_2), 5.27 (s, 1H, pyr-5H), 3.41 (m, 8H, pipaz-2,6CH_2, pipaz'-2,6CH_2), 2.28 (m, 8H, pipaz-3,5CH_2, pipaz'-3,5CH_2), 2.16 (s, 6H, CH_3, CH_3') ppm; ^{13}C NMR (250 MHz; DMSO-d_6) δ = 164.30 (pyr-4,6C), 162.13 (pyr-2C), 73.28 (pyr-5C), 54.44 (pipaz-3,5C, pipaz'-3,5C), 45.78 (CH_3, CH_3'), 43.62 (pipaz-2,6C, pipaz'-2,6C) ppm; ESI MS: 292.2 [M + H⁺]. Anal. Calcd for HRMS: 292.22442 Found: 292.22496.

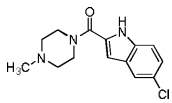
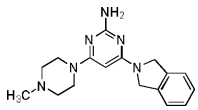
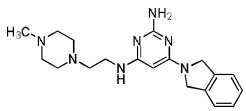
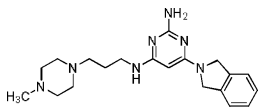
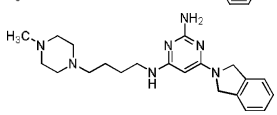
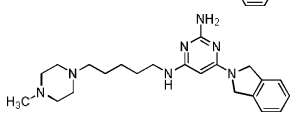
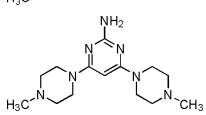
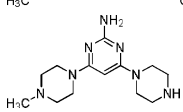
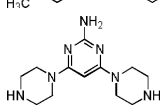
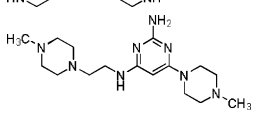
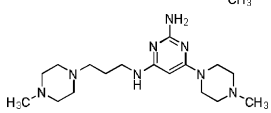
3.1.10. 4-Chloro-6-(4-methylpiperazin-1-yl)pyrimidin-2-amine (P5)

299 mg (43%), $C_9H_{14}ClN_5$, ^{1}H NMR (250 MHz, DMSO-d_6) δ = 6.45 (s, 2H, NH_2), 6.07 (s, 1H, pyr-5H), 3.51 (m, 4H, pipaz-2,6H_2), 2.30 (t, 4H, pipaz-3,5H_2), 2.17 (s, 3H, CH_3) ppm; ESI MS: 208.8 [M + H⁺].

3.1.11. 4-(4-Methylpiperazin-1-yl)-6-(piperazin-1-yl)pyrimidine-2-amine (6)

100 mg (38%), $C_{13}H_{23}N_7$, ^{1}H NMR(250 MHz; DMSO-d_6) δ = 5.68 (s, 2H, NH_2), 5.38 (s, 1H, pyr-5H), 3.54 (m, 8H, pipaz- 2,6CH_2, pipaz'-2,6CH_2), 2.88 (m, 4H, pipaz'-3,5CH_2), 2.38 (m, 4H, pipaz-3,5CH_2), 2.27 (s, 3H, CH_3)

Table 1: Histamine H_4 receptor binding data on novel 2,4,6-triaminopyrimidine derivatives

Compd.	Structure	MW [g·mol^{-1}]	hH_4R K_i [μM][i]	n	Ligand efficiency
JNJ7777120		277.75	0.014 ± 0.01	3	0.54
ST-1012		310.4	0.25 ± 0.06	2	0.37
1		353.5	2.33 ± 1.09	3	0.28
2		367.5	14.9 ± 5.7	3	0.23
3		381.5	30.7 ± 8.9	3	0.21
4		395.5	12.8 ± 1.0	3	0.22
5		291.4	2.2 ± 0.2	2	0.35
6		277.4	6.1 ± 0.3	2	0.34
7		263.3	66.9 ± 6.8	2	0.29
8		334.5	123.0 ± 36.8	2	0.21
9		348.5	118.3 ± 123.9	2	0.19

[i] [^{3}H]Histamine displacement assay with membrane preparation of Sf9 cells transiently expressing hH_4R, co-expressed with $G_{\alpha i2}$ and $G_{\beta 1\gamma 2}$ subunits; mean values with standard error of the mean (SEM) of at least two independent measurements, each in triplicates.

ppm; ^{13}C NMR (250 MHz; DMSO-d_6) δ = 164.32 (pyr-6C), 164.20 (pyr-4C), 162.14 (pyr-2C), 73.29 (pyr-5C), 54.43 (pipaz-3,5C), 45.78 (pipaz'-2,6C), 44.35 (pipaz'-3,5C), 43.62 (CH_3), 43.48 (pipaz-2,6C) ppm; ESI MS: 278.0 [M + H$^+$]; Anal. Calcd for HRMS: 278.20877 Found: 278.20866.

3.1.12. 4,6-Di(piperazin-1-yl)pyrimidine-2-amine (7)

361 mg (45%), $C_{12}H_{21}N_7$, ^{1}H NMR(250 MHz; DMSO-d_6) δ = 5.72 (s, 2H, NH_2), 5.39 (s, 1H, pyr-5H), 3.60 (m, 8H, pipaz-2,6CH_2, pipaz'-2,6CH_2), 2.92 (m, 8H, pipaz-3,5CH_2, pipaz'-3,5CH_2) ppm; ^{13}C NMR (250 MHz; DMSO-d_6) δ = 163.82 (pyr-4,6C), 162.20 (pyr-2C), 73.62 (pyr-5C), 42.66 (pipaz-2,6C, pipaz'-2,6C), 41.30 (pipaz-3,5C, pipaz'-3,5C) ppm; ESI MS: 264.0 [M + H$^+$]; Anal. Calcd for HRMS: 264.19312 Found: 264.19318.

3.1.13. 6-(4-Methylpiperazin-1-yl)-N^4-(2-(4-methylpiperazin-1-yl)ethyl)pyrimidine-2,4-diamine (8)

200 mg (54%), $C_{16}H_{30}N_8$, ^{1}H NMR(250 MHz; DMSO-d_6) δ = 5.86 (br s, 1H, NH), 5.45 (s, 2H, NH_2), 5.03 (s, 1H, pyr-5H), 3.34 (m, 4H, pipaz-2,6H_2), 3.20 (m, 2H, eth-1H_2), 2.38 (m, 6H, pipaz- 3,5H_2, eth-2H_2), 2.28 (m, 8H, pipaz'-2,3,5,6H_2), 2.17 (s, 3H, CH_3), 2.13 (s, 3H, CH_3') ppm; ^{13}C NMR (250 MHz; DMSO-d_6) δ = 164.33 (pyr-6C), 163.57 (pyr-4C), 162.39 (pyr-2C), 73.41 (pyr- 5C), 57.07 (eth-2C), 54.73 (pipaz'-3,5C), 54.40 (pipaz-3,5C), 52.68 (pipaz'-2,6C), 45.82 (CH_3'), 45.74 (CH_3), 43.61 (pipaz- 2,6C), 37.69 (eth-1C) ppm; ESI MS: 335.1 [M + H$^+$]; Anal. Calcd for HRMS: 335.26662 Found: 335.26642.

3.1.14. 6-(4-Methylpiperazin-1-yl)- N^4-(3-(4-methylpiperazin-1-yl) propyl)pyrimidine-2,4-diamine (9)

172 mg (47%), $C_{17}H_{32}N_8$, ^{1}H NMR(250 MHz; DMSO-d_6) δ = 6.12 (br s, 1H, NH), 5.41 (s, 2H, NH_2), 4.98 (s, 1H, pyr-5H), 3.13 (m, 2H, prop-1H_2), 2.68 (t, J = 5.0, 4H, pipaz-2,6H_2), 2.30-2.20 (m, 12H, pipaz-3,5H_2, pipaz'-2,3,5,6H_2), 2.16 (m, 2H, prop-3H_2), 2.12 (s, 3H, CH_3), 2.10 (s, 3H, CH_3'), 1.57 (m, 2H, prop-2H_2) ppm; ^{13}C NMR (250 MHz; DMSO-d_6) δ = 164.45 (pyr-6C), 163.51 (pyr-4C), 162.37 (pyr-2C), 73.35 (pyr-5C), 55.60 (prop-3C), 54.71 (pipaz'-3,5C), 54.38 (pipaz-3,5C), 52.66 (pipaz'-2,6C), 45.80 (CH_3'), 45.66 (CH_3), 43.61 (pipaz- 2,6C), 38.75 (prop-1C), 26.44 (prop-2C) ppm; ESI MS: 349.3 [M + H$^+$]; Anal. Calcd for HRMS: 349.28227 Found: 349.28235.

3.2. Pharmacology

Competition binding data were analyzed by the software GraphPad PrismTM (200, version 3.02, San Diego, CA, USA), using non-linear least square fit. Affinity values (K_i) were expressed as mean from at least two

experiments in triplicates. K_i values were calculated from IC_{50} values according to the Cheng-Prusoff equation (Cheng and Prusoff 1997).

3.2.1. Binding assay on hH_4R

Binding assay has been performed as previously described (Schneider et al. 2009; Kottke et al. 2011). In brief, competition binding experiments were carried out by incubating membranes, 40 μg/well (prepared from Sf9 cells transiently expressing hH_4R, co-expressed with G protein Gαi2 and $G\beta_1\gamma_2$ subunits) in a final volume of 0.2 mL containing binding buffer and [^{3}H]histamine (10 nM, 392 GBq/mmol; K_d([^{3}H]histamine) = 11.13 nM). Prior to the experiments, cell 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). Assays were run in triplicates with seven to eight appropriate concentrations between 0.001 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 JNJ-7777120. 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.

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