Chapter 6

Mapping histamine H₄ receptor—ligand binding modes

Sabine Schultes, Saskia Nijmeijer, Harald Engelhardt, Albert J. Kooistra, Henry F. Vischer, Iwan J.P. de Esch, Eric E. J. Haaksma, Rob Leurs, Chris de Graaf

Abstract

The increasing number of G protein-coupled receptor (GPCR) crystal structures offers new opportunities for histamine receptor homology modeling. However, computational prediction of ligand binding modes in GPCRs such as the histamine H₄ receptor (H₄R), a receptor that plays an important role in inflammation, remains a challenging task. In the current work we have combined complementary in silico receptor modeling approaches with in vitro ligand structure—activity relationship (SAR) and protein site-directed mutagenesis studies to elucidate the binding modes of different ligand classes in H₄R. By systematically considering different H₄R modelling templates, ligand binding poses, and ligand protonation states in combination with docking and MD simulations we are able to explain ligand-specific mutation effects and subtle differences in ligand SAR. Our studies confirm that a combined theoretical and experimental approach represents a powerful strategy to map ligand–protein interactions.
Introduction

Rational drug design requires detailed knowledge and understanding of the molecular interactions between ligand and protein. Both ligand- and protein-based in silico modeling approaches have been successfully applied to rationalize structure-activity relationships (SAR) and receptor site-directed mutagenesis studies, for the histamine H4 receptor (H4R)\(^1\)\(^-\)\(^7\), a G protein-coupled receptor (GPCR) that plays an important role in inflammation.\(^8\)\(^-\)\(^20\) The increasing number of GPCR X-ray structures,\(^21\)\(^,\)\(^22\) including the recently solved histamine H1 receptor (H1R) crystal structure\(^23\) (Figure 1A), offers new opportunities for histamine receptor homology modeling and the structure-based design of new histamine receptor ligands.\(^24\)\(^-\)\(^27\) However, computational H4R-ligand binding mode prediction still remains a challenging task. The symmetric distribution of the two acetic residues (aspartate D94\(^3.32\) and glutamate E182\(^5.46\))\(^2\)\(^,\)\(^3\)\(^,\)\(^7\) in combination with different hydrophobic subpockets (I and II\(^28\), Figure 1B-C) that are complementary to (two) basic and several hydrophobic groups in H4R ligands (Figure 2)\(^29\)\(^-\)\(^31\) allows different plausible H4R-ligand binding modes. Site-directed mutagenesis studies have identified D94\(^3.32\) (a conserved binding residue in bioaminergic GPCRs\(^32\)) as an essential residue in H4R to bind both the non-imidazole antagonist JNJ 7777120 1b\(^3\) and the small imidazole-containing agonist histamine 3.\(^3\)\(^,\)\(^7\) In addition, site-directed mutagenesis (SDM) studies showed that the anionic carboxylate group of E182\(^5.46\) plays an important role in binding of H4R ligands that contain two basic groups, including 3, VUF 8430 4, and to a smaller degree clobenpropit 5 and clobenpropit analogue VUF5228 6, while 1b and clozapine 7 only require a H-bond acceptor at position 182\(^5.46\) (e.g. present in the E182\(^5.46\)Q mutant).\(^2\)\(^,\)\(^3\) In the H1R co-crystal structure\(^23\) the inverse agonist doxepin forms an ionic H-bond interaction with the conserved D107\(^3.32\), but does not form a H-bond with N198\(^5.46\). The aromatic ring systems of doxepin bind to two connected subpockets in the hydrophobic cavity between transmembrane (TM) helices 3-6 (pocket II\(^28\)), in the current study defined as subpocket IIa and subpocket IIb (Figure 1A). Receptor mutagenesis, ligand SAR, and receptor-ligand interaction modeling studies have indicated that different ligands bind to these different subpockets.\(^2\)\(^,\)\(^4\)\(^,\)\(^30\)\(^,\)\(^33\) Ligands 6 and 7 occupy both subpockets IIa and IIb simultaneously (Figure 1B-C).\(^2\)\(^,\)\(^4\) Triazole ligand 8 is proposed to bind only in subpocket IIa,\(^33\) while combined modeling and mutagenesis studies support two alternative binding modes of ligand 5 in which it accommodates its aromatic moiety in subpocket IIb or alternatively in subpocket I.\(^2\)
Mapping histamine H₄ receptor–ligand binding modes

Figure 1. Binding modes of doxepin in H₁R co-crystal structure²³ (A), and VUF 5228² (B) and clozapine⁴ (C) in (ADRB2 crystal structure²⁴ based) H₄R homology models. Compounds and pocket residues are depicted as ball-and-sticks, whereas for clarity Y95³,33 is shown as lines. H-bonds between the ligand and pocket residues are represented as black dotted lines. The backbone TM helices 5, 6, and 7 (right to left) are presented as yellow helices. Helix 3 is presented by yellow ribbons. Subpockets I, IIa and IIb are labeled in red.

Figure 2. Structures of H₄R ligands 1-9. Affinities (pKᵢ) for H₄R of 1 and 2 are from the current study, 3, 4 and 7 from ref.⁴, 5 and 6 from ref.², 8 from ref.³⁵ and 9 from ref.³⁵

In this study we have focused on the elucidation of the binding interactions of two different selective H₄R ligand classes: indolecarboxamides⁴,⁶,²⁷,³⁶-³⁹ (1a-b) and 2-aminopyrimidines⁴⁰-⁴⁶ (2a-b) (Figure 2). Recent ligand-binding mode predictions of dual H₃R/H₄R clobenpropit ligands 5 and 6 demonstrated that the integration of complementary in vitro and in silico modeling approaches is an efficient way to map protein-ligand interactions.² In the current study we used in silico guided mutation studies to compare the binding modes of substituted and unsubstituted indolecarboxamides and 2-aminopyrimidines. To obtain more detailed insights into ligand-specific molecular recognition features, molecular dynamics (MD) simulations were employed, a technique that has been successfully applied to describe subtle differences in ligand binding (e.g. ligand regio-, stereo-, and protein selectivity⁴⁷,⁴⁸). Furthermore, different ligand protonation states (i.e. 2-aminopyrimidines 2a-b with one versus two positively ionized
basic groups) and different H₄R modeling templates (*i.e.* the bioaminergic GPCR β₂-adrenergic receptor (ADRB2)³⁴ versus the more closely related H₁R²³) were considered to investigate the influence of modeling parameters. The combination of protein mutagenesis data with different binding mode hypotheses, molecular modeling and MD-simulation revealed similarities as well as differences in the molecular H₄R binding determinants for both ligand classes. These insights are useful for rational optimization of indolecarboxamides and 2-aminopyrimidines, and the design of new H₄R ligands.

**Results**

SAR (*Table 1, Figure 3*) and mutagenesis (*Table 2, Figure 4*) studies in combination with docking and MD-simulations (*Figure 5-7, Tables 3-4*) were used to elucidate the binding modes of indolecarboxamides 1a-b and 2-aminopyrimidines 2a-b (*Figure 2*) in the H₄R binding site and to give insights into the molecular determinants of H₄R-ligand interactions. For this procedure different starting poses of the ligands were generated (*Figure 5*) in different H₄R models (*Figure S1*). For the 2-aminopyrimidine ligands different protonation states were considered (*Table 3 and 4*). The H₄R-ligand binding mode models that could best explain the (ligand specific) mutation data were used to identify similarities and differences in the binding characteristics of the different ligand classes.

**Structure-activity relationships of substituted indolecarboxamides and 2-aminopyrimidines**

To determine the binding mode of indolecarboxamides and 2-aminopyrimidines we synthesized -H, -Cl, -NH₂, -NO₂, and -OCH₃ substituted indolecarboxamide (1a-e) and 2-aminopyrimidine (2a-e) analogues and investigated the effect of different substituents at for both ligand classes (*Table 1*). The synthesis of the indolecarboxamide ligands is described in literature³⁶,³⁷,³⁹ and *Figure 3* illustrates the synthesis route for the 2-pyrimidine-2-amine derivatives. Starting from 4,6-dichloro-pyrimidine-2-amine, methylpiperidine was introduced via a nucleophilic aromatic substitution reaction. In a second step the accordant phenyl moieties were installed using a Suzuki reaction.
Mapping histamine H₄ receptor–ligand binding modes

Figure 3. Reagents and conditions: (i) dichloromethane, N-ethyl-diisopropylamine, 20 °C, 16 h; (ii) dichloro[1,1′-bis(diphenylphosphino)ferrocalne] palladium (II) dichloromethane adduct, Cs₂CO₃, tetrahydrofurane, 1-methyl-2-pyrrolidinone, water, 100 °C, 16 h.

Radioligand displacement studies of [³H]histamine binding to the human H₄R of the indolecarboxamide (1a-e) and 2-aminopyrimidine (2a-e) analogues indicated that the SAR of the two ligand series is comparable for small substituents but deviates for the larger -OCH₃ substituent (Table 1). Substitution of the indolecarboxamide with the methoxy group (1e) resulted in a 63 fold decrease compared to the unsubstituted ligand (1a), while comparison of the methoxy- (2e) and unsubstituted (2a) 2-aminopyrimidines gave only a 3 fold affinity change. The Cl-substituted ligands showed the highest H₄R binding affinity for both the indolecarboxamide and 2-aminopyrimidine ligand classes.

Table 1. pKᵢ values of indolecarboxamides (1) and 2-aminopyrimidines (2) with different R substitutions.ª

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<th>b (Cl)</th>
<th>c (NH₂)</th>
<th>d (NO₂)</th>
<th>e (OCH₃)</th>
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ª Data shown are mean of at least two independent experiments performed in triplicates (SEM given between brackets).

Ligand binding affinities for H₄R, L175⁵.₃⁹V and E182⁵.⁴⁶Q

The binding affinities (pKᵢ) of the unsubstituted and Cl-substituted indolecarboxamide and 2-aminopyrimidine ligands were analysed for H₄R, L175⁵.₃⁹V and E182⁵.⁴⁶Q (Table 2, Figure 4,) using heterologous [³H]histamine displacement binding experiments. Previous studies have shown that E182⁵.⁴⁶Q affects the binding of H₄R ligands that contain two basic groups (3-6)²,³,⁷, but does not affect binding of ligands that contain only one basic moiety (1b, 7)³. The L175⁵.₃⁹V mutant affects the affinity for larger ligands 1b and 7, but
does not affect the binding of \( \text{3} \).\(^4\) The fact that these two mutants show ligand-dependent effects and that they have previously been used to investigate the binding mode of \( \text{1b} \) makes them attractive tools for investigating similarities and differences in binding modes of \( \text{1a-b} \) and \( \text{2a-b} \). Both ligand classes lost affinity for L175\(^{5.39}\)V. The Cl-substituted ligands, \( \text{1b} \) and \( \text{2b} \), were more affected (\( \Delta pK_i(\text{1b}): -1.3 \), \( \Delta pK_i(\text{2b}): -1.5 \)) than the unsubstituted ligands, \( \text{1a} \) and \( \text{2a} \), (\( \Delta pK_i(\text{1a}): -0.6 \), \( \Delta pK_i(\text{2a}): -0.6 \)). In contrast, E182\(^{5.46}\)Q had a different effect on the two ligand classes. While the affinities of the 2-aminopyrimidines for E182\(^{5.46}\)Q significantly decreased (\( \Delta pK_i(\text{2a}): -1.1 \), \( \Delta pK_i(\text{2b}): -2 \)) the mutations did not have a significant effect on the affinities of the indolecarboxamides (\( \Delta pK_i(\text{1a}): -0.4 \), \( \Delta pK_i(\text{1b}): -0.2 \)). Again the Cl-substituted 2-aminopyrimidine ligand \( \text{2b} \) was more affected than the unsubstituted ligand \( \text{2a} \).

![Figure 4](image-url)  
**Figure 4.** Radioligand displacement curves of ligands \( \text{1a} \) (A), \( \text{1b} \) (B), \( \text{2a} \) (C), \( \text{2b} \) (D) on \( \text{H}_4\text{R-WT} \) (●), \( \text{H}_4\text{R-L}^{5.39}\text{V} \) (●), \( \text{H}_4\text{R-E}^{5.46}\text{Q} \) (●). E\(^{5.46}\)Q binding studies were performed with radioligand \( [\text{H}]\text{-JNJ 777120} \), whereas \( [\text{H}]\text{-histamine} \) was used for \( \text{H}_4\text{R} \) and L\(^{5.39}\)V. Data shown are representative specific binding curves of at least two experiments performed in triplicate. Error bars indicate SEM values. To enable a better visualization of the pK\(_i\) shifts, each curve is corrected for the respective radioligand concentration and K\(_d\) values.
Table 2. pKᵯ values of ligands 1a-b and 2a-b for the H₄R, L175⁵.₃⁹V and E182⁵.₄⁶Q.

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Data shown are mean of at least two independent experiments performed in triplicate (SEM given between brackets).

Molecular docking studies

We used molecular docking simulations⁴⁹,⁵⁰ to generate different plausible poses for the two indolecarboxamides (1a and 1b) and 2-aminopyrimidines (2a and 2b) in a H₄R homology model that is based on the recently resolved H₁R crystal structure.²³ (Figure 5). For comparison we also investigated docking poses in a H₄R model that is based on the ADRB2 crystal structure³⁴ (Figure S1) as this model was as good as the H₁R-based H₄R model in retrospective virtual screening studies.⁵¹ While the TM helices of ADRB2 and H₁R crystal structures share that the same overall fold²³,³⁴, the conformations of particular the second extracellular loops (EL2) are different (Figure S1).

Figure 5. Initial binding poses of indolecarboxamide ligand 1a (A, B, C) and 2-aminopyrimidine ligand 2a (D, E, F) in H₁R crystal structure²³ based H₄R models used as starting structures for MD simulations. Rendering and labels are the same as in Figure 1.
As a result, the H₄R models based on ADRB2 and H₁R crystal structures have very similar TM domains and show overall the same receptor-ligand interactions, except in the EL2 loop region (Figure S1). Only docking poses where the ligands interact with both, aspartate D9⁴ ³.₃₂ and glutamate E1₈² ⁵.₄₆ (essential residues for binding of H₄R ligands ³.⁷) were considered. Both residues were modeled in the deprotonated (negatively charged) state. Three binding poses were generated for both ligand classes in the two different H₄R models as starting structures for MD simulations. The binding pose of indolecarboxamides in Figure 5A (pose 1) is similar to the binding mode proposed by Lim et al.⁴, in which the piperazine and indole nitrogen atoms of the ligand donate H-bonds to D9⁴ ³.₃₂ and E1₈² ⁵.₄₆, respectively, while the aromatic moiety of the ligand is accommodated in the upper hydrophobic subpocket IIb. The binding pose in Figure 5B (pose 2) is similar to the pose proposed by Kiss et al.³⁸ in which the piperazine and indole nitrogen atoms of the ligand donate H-bonds to E1₈² ⁵.₄₆ and D9⁴ ³.₃₂, respectively, while the aromatic moiety of the ligand is directed towards TM7 (subpocket I²⁸). The binding pose in Figure 5C (pose 3) is similar to the binding mode reported by Schneider et al.⁶, in which the piperazine and indole nitrogen atoms of the ligand donate H-bonds to D9⁴ ³.₃₂ and E1₈² ⁵.₄₆, respectively (similar in pose 1), while the aromatic ring of the ligand binds in subpocket IIa. To obtain this latter pose the χ₁ torsional angle of C9₈ ³.₃₆ was rotated from its t conformation to the g⁺ conformation.

Three docking poses were selected for the 2-aminopyrimidines that correspond to the respective poses of the indolecarboxamides (Figure 5D-F). The amino group of the pyrimidine interacts in a similar manner as the NH of the indole group in these poses. A binding pose for 2-aminopyrimidines that is comparable to pose 1 was proposed by Werner et al.⁴⁶ with the exception that the 2-aminopyrimidine moiety in that H₄R model is differently oriented in the H₄R binding pocket and H-bonds to E1₈² ⁵.₄₆ are formed via water molecules. The 2-aminopyrimidines can form an additional hydrogen bond with either E1₈² ⁵.₄₆, in pose 1 or 3, or D9⁴ ³.₃₂ in pose 2 when the pyrimidine ring is protonated. pKₐ measurements for ligand 2a (see Experimental section for procedure) revealed a pKₐ value of 7.5 for the piperazine nitrogen atom and a pKₐ value of 6.0 for the pyrimidine, indicating that in solution approximately 2.3% of ligand 2a will be double-protonated at physiological pH 7.4.
Mapping histamine H\textsubscript{4} receptor–ligand binding modes

Molecular dynamics simulations

Binding mode elucidation

All three binding poses were subjected to 1 ns MD simulations. During the course of each MD-simulation 500 snapshots were collected. H-bond interactions between the ligands 1a-b and 2a-b and the carboxylate groups of D94\textsuperscript{3.32} and E182\textsuperscript{5.46}, as well as hydrophobic contacts between the ligands and L175\textsuperscript{5.39} were monitored (Table 3, Figure 6) to validate the different binding mode models with mutagenesis data. Our data showed that the E182\textsuperscript{5.46}Q mutant affects binding of 2a-b, but not of 1a-b, while the L175\textsuperscript{5.39}V mutation affects the affinity of all ligands (Table 2 and Figure 4). In addition, previous mutation studies\textsuperscript{3,7,32,52} indicated that the conserved D94\textsuperscript{3.32} residue plays an important role in histamine receptor binding, while an H-bond donor in the side-chain of residue 182\textsuperscript{5.46} in H\textsubscript{4}R (e.g. present in the E182\textsuperscript{5.46}Q mutant) is required to bind 1b.\textsuperscript{3} Table 3 shows the frequency of hydrophobic contacts of the ligand with L175\textsuperscript{5.39} in combination with H-bond formation to both D94\textsuperscript{3.32} and E182\textsuperscript{5.46} (Tables S1 and S2 report the individual and combined frequencies of the different interactions).

Table 3. Frequency (%) of hydrophobic interactions\textsuperscript{a} between the ligand and L175\textsuperscript{5.39} in combination with H-bond formation\textsuperscript{b} with D94\textsuperscript{3.32} and E182\textsuperscript{5.46} in H\textsubscript{1}R-based and ADRB2-based H\textsubscript{4}R models that occur during 1ns MD simulations.

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\textsuperscript{a} A hydrophobic interaction is counted if the shortest distance between any heavy atom of the ligand and L175\textsuperscript{5.39} is \textless{} 4Å; \textsuperscript{b} An H-bond is counted if the distance between H-bond donor and acceptor heavy atom is below 3.5Å and the angle between H-bond donor heavy atom, hydrogen and H-bond acceptor heavy atom is between 135° and 225°; \textsuperscript{c} see Figure 5.

Frequent interactions of indolecarboxamides (1a-b) and 2-aminopyrimidines (2a-b) with
L175\textsuperscript{5.39} in combination with stable H-bond formation with D94\textsuperscript{3.32} and E182\textsuperscript{5.46} were only observed in the MD-simulations starting from pose 1 (Table 3, Figure 6). Starting from pose 2, only the MD-simulations of ligands 2a-b showed interactions with L175\textsuperscript{5.39} in combination with H-bond formation with D94\textsuperscript{3.32} and E182\textsuperscript{5.46}, but the frequency of these interactions is much lower than in pose 1 (0-38% vs. 51-95%, Table 3), as demonstrated for ligand 2b in Figure 6B-C. In the ADRB2-H\textsubscript{4}R model only for the mono-protonated ligand 2a interactions with L175\textsuperscript{5.39} (in combination with stable H-bonds with D94\textsuperscript{3.32} and E182\textsuperscript{5.46}) were observed, but with significantly lower frequency (38%) than in pose 1 (90%, Table 3).

Figure 6. Shortest distance between any heavy atom of the L175\textsuperscript{5.39} in the H\textsubscript{4}R-based H\textsubscript{4}R model and 1b (A) 2b single-protonated (1+) (B) and double-protonated (2+) (C) in MD-simulations starting from pose 1 (black), pose 2 (blue), and pose 3 (red) (see Figure 5).

Protonation state elucidation of 2-aminopyrimidines bound to H\textsubscript{4}R

Our mutagenesis studies (Table 2 and Figure 3) showed that the affinities of indolecarboxamides were hardly affected by the E182\textsuperscript{5.46}Q mutation (ΔpK\textsubscript{i}(2a): -0.4, ΔpK\textsubscript{i}(2b): -0.2), while the affinities of the 2-aminopyrimidines were significantly affected by this mutation (ΔpK\textsubscript{i}(2a): -1.1, ΔpK\textsubscript{i}(2b): -2.0). These data indicate that ionic interactions with the negatively ionisable E182\textsuperscript{5.46} residue play a role in binding of 2a-b (but not of 1a-b) and suggest that the basic 2-aminopyrimidines moieties of 2a-b are protonated. To investigate this hypothesis, the mono and double-protonated 2a and 2b were modeled in a E182\textsuperscript{5.46}Q mutated H\textsubscript{4}R model (pose 1) and subsequently subjected to MD simulations. As anticipated and shown in Table 4, only the H\textsubscript{4}R binding models in which ligands 2a and 2b are double-protonated (i.e. both piperazine and aminopyrimidine groups in their positively ionized forms) can explain the observed affinity decrease for 2a and 2b upon E182\textsuperscript{5.46}Q mutation (Table 2). In these models the average number of hydrogen bonding interactions with residue 182\textsuperscript{5.46} observed in the 500 snapshots was decreased in the H\textsubscript{4}R-E182\textsuperscript{5.46}Q mutant model compared to the wt-H\textsubscript{4}R model (difference in H-bond interactions...
formation to residue 182\(^{5.46}\) in the H\(_1\)-H\(_4\) model: 1a(2+): -1.9, 1b(2+): -1.9, ADRB2-H\(_4\) model: 2a(2+): -1.2, 2b(2+): -1.7), while H-bond interactions with D94\(^3.32\) were unaffected. To compare the two ligand classes, also indolecarboxamides 1a and 1b in the H\(_4\)-E182\(^{5.46}\)Q models were subjected to 1ns MD-simulation. In both H\(_4\)-E182\(^{5.46}\)Q models H-bond interactions with 1a-b were hardly affected compared to the H\(_4\)-wt models (Table 4). This is in line with the fact that the E182\(^{5.46}\)Q mutation had no significant effect on binding affinity for these ligands (Table 2, Figure 4).

Table 4. Average H-bond interactions\(^a\) between the ligand and residue 182\(^{5.46}\) in wt and E182\(^{5.46}\)Q-H\(_4\) models during 1 ns MD-simulation starting from pose 1 (see Figure 5).

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\(^a\) An H-bond is counted if the distance between H-bond donor and acceptor heavy atom is below 3.5Å and the angle between H-bond donor heavy atom, hydrogen and H-bond acceptor heavy atom is between 135° and 225°.

Rationalization of differences in ligand structure-activity relationships

The SAR for different substitutions in both ligand series (Table 1) indicated that the larger substituent –OCH\(_3\) is less compatible with the H\(_4\)-H\(_4\) binding site than smaller apolar (-Cl) or polar (-NH\(_2\), -NO\(_2\)) groups. This affinity decrease was much more pronounced for the indolecarboxamides than for 2-aminopyrimidines (Table 1). To explain this effect we monitored the pocket volume around 3.5 Å distance of the chlorine atom for 1b and 2b (Figure 7). We observed that in the H\(_1\)-based H\(_4\) model the chlorine atom of 2b...
(independent of the modeled protonation state) occupies more frequently larger pockets than the chlorine atom of 1b. Average pocket volumes around the chlorine atom in the H₁R-based H₄R model were significantly smaller for ligand 1b (0.6 Å³) than for ligand 2b (8.4 Å³ (mono-protonated) and 3.2 Å³ (double-protonated)) while in the ADRB2-based H₄R model the volume for 1b (11.0 Å³) was significantly larger than for 2b (6.4 Å³ (mono-protonated) and 3.9 Å³ (double-protonated)). The H₁R-based H₄R models therefore explain the fact that 2-aminopyrimidines can accommodate larger substituents than indolecarboxamides.

Figure 7. Representative snapshots of MD simulations starting from pose 1 in the H₁R-based H₄R model showing the pocket volume within 3.5 Å of the chlorine atom of ligand 1b (red, A) and ligand 2b (green, B). The snapshots presented in panels A (red triangle) and B (green circle) are indicated in panel C, showing pocket volumes within 3.5 Å of the chlorine atom of ligand 1b (red) and double-protonated ligand 2b (green) along the full 1ns MD trajectories.
A comparison of representative snapshots at the end of the MD-simulations showed that although the overall orientation of the ligands in the binding pocket is comparable, the chlorine atoms of both ligands are orientated in different pockets (Figure 7A-B). The chlorine atom of 1b is directed towards the extracellular loop (EL2) and TM6 (surrounded by F168<sup>45.54</sup>, F169<sup>45.55</sup>, E163<sup>45.49</sup>, L175<sup>5.39</sup>, T323<sup>6.55</sup>) in a relatively tight binding pocket (Figure 7A). The chlorine atom of 2b occupies a pocket, which is located more towards TM5 (surrounded by I174<sup>5.38</sup>, L175<sup>5.39</sup> and T178<sup>5.42</sup> in TM5, as well as F168<sup>45.54</sup> and backbone atoms of EL2) and has a larger volume (Figure 7B) that allows larger substituents (Table 1).

Discussion

In the current study, ligand SAR and H<sub>4</sub>R SDM studies were combined with molecular docking and MD simulations to elucidate the binding modes of indolecarboxamides and 2-aminopyrimidines in the H<sub>4</sub>R binding pocket. Insights were obtained into the similarities and differences of molecular interactions between H<sub>4</sub>R and these two ligand classes.

Overlap in indolecarboxamides and 2-aminopyrimidines binding modes

Our combined SDM and in silico studies indicate that the binding modes of indolecarboxamides and 2-aminopyrimidines overlap, but also show that there are subtle differences in the importance of different H<sub>4</sub>R interaction features within these two ligand series. Ligands of both ligand classes accommodate their aromatic ring moieties in subpocket IIb that is located in the upper half of the extracellular regions between TM helices 3-6 (pose 1), (Figure 5A and D and Figure S1). Only in this pose all ligands (1a-b and 2a-b) form stable H-bond interactions to D94<sup>3.32</sup> and E182<sup>5.46</sup> in combination with hydrophobic interactions with L175<sup>5.39</sup>. Current (Table 2 and Figure 4) and previous SDM studies show that these interactions are important binding determinants for ligands 1a-b and 2a-b as well as other H<sub>4</sub>R ligands. The overall binding orientation was stable and not influenced by interactions with water molecules.

It should be noted that effects of H<sub>4</sub>R mutations are often ligand specific. While the E182<sup>5.46</sup>Q mutant has decreased affinity for ligands 2a, 2b, 3, and 4, this mutation does not affect binding of ligands 1a, 1b and 7 (Table 2 and Figure 4). The L175<sup>5.39</sup>V mutant that mimics the monkey H<sub>4</sub>R has a decreased affinity for 1 and 2 (Table 2 and Figure 4) but an increased affinity for ligand 7 (Figure 1C), while binding of ligand 3 is not affected by this mutation. This reflects the diversity in H<sub>4</sub>R ligand binding modes (Figure 1 and Figure 7) that can match different parts of the H<sub>4</sub>R ligand pharmacophore.
pharmacophore contains: i) 1 to 2 H-bond donors (e.g. piperazine and indole/aminopyrimidine nitrogen atoms of ligands 1 and 2) that are complementary to D94$^{3.32}$ and E182$^{5.46}$, ii) a central aromatic moiety (e.g., indole (1) and 2-aminopyrimidine (2) moieties) that stacks between Y$^{3.33}$ and Y$^{6.51}$, and iii) 1 to 2 hydrophobic moieties that can accommodate subpockets Ila (e.g., cyclohexyl groups of ligands 6 (Figure 1B) and 8, the chlorinated benzene ring of ligand 7 (Figure 1C)), Iib (benzene rings of ligands 1-2 (Figure 7) and ligands 5-7 (Figure 1B-C) between TM helices 3-6, or alternatively, subpocket I between TM helices 3 and 5-7 (benzene ring of ligand 5).2

Our MD-simulation showed that although the overall orientation of the ligands (1a-b and 2a-b) in the H$_4$R binding pocket is comparable, the chlorine atom of indolecarboxamide and 2-aminopyrimidine ligands are located in subpockets of different size (Figure 7). This size difference explains why larger substituents have a more negative effect on the affinity for indolecarboxamides than for 2-aminopyrimidines (Table 1). Interestingly, SDM studies have shown ligand-dependent effects towards mutations in the same EL2 region that can explain H$_4$R species selectivity differences.5

The role of ligand protonation states in H$_4$R binding

In silico evaluation of different protonation states of 2-aminopyrimidines showed that the H$_4$R binding models in which both the piperazine and 2-aminopyrimidine moieties are positively ionized best explain the affinity decrease of ligands 2a-b (but not 1a-b) for the E182$^{5.46}$Q mutant (Tables 2-4). The pK$_a$ value of the pyrimidine ring of 6.0, however, suggests that under physiological pH 7.4 only a small portion (~2.3%) of ligands 2a-b contains a positively ionized pyrimidine ring. On the other hand, it has been reported that pK$_a$ values of functional groups in ligands can change upon binding, as demonstrated for the pteridine ring of methotrexate that becomes protonated upon binding to dihydrofolate reductase.54,55 Thus, it cannot be excluded that the pK$_a$ value of the 2-aminopyrimidine ring is shifted towards higher values upon binding to H$_4$R.

It should furthermore be noted that also other ligands have been proposed to bind in a double-protonated state to H$_4$R. The two basic groups of ligand 9 have pK$_a$ values of 8.3 and 9.4$^{56}$ indicating that most parts of this ligand will be double-protonated at pH 7.4. Agonist 4$^5$, derived from ligand 9, has two chemically similar basic moieties, while the pK$_a$ values of the amine and imidazole groups of 3 (9.4-9.9 and 5.9-6.5, respectively)$^{58-60}$ suggest that also 3 might bind in a double-protonated form. This is supported by the fact that the E182$^{5.46}$Q H$_4$R mutant has a significantly decreased affinity for small agonists 3 and 4$^3$ that depend on strong H-bond interactions. The effects of the E182$^{5.46}$Q mutation
on H₄R binding affinity are relatively smaller (5 and 6) or not significant (1a-b, 7) for ligands with larger hydrophobic moieties (Table 2, Figure 4).²,³ as the binding of these molecules is more determined by hydrophobic interactions. Interestingly, in this current study we show that also the 2-aminopyrimidines (containing a hydrophobic benzene ring) are to a large extent dependent on ionic interactions with E182⁵,⁴⁶.

**Ligand-binding mode predictions in H₄R homology models were modeling template independent**

To elucidate H₄R-ligand binding modes we considered two H₄R models, one based on the ADRB2 crystal structure³⁴ and another based on the more recent H₁R crystal structure.²³ The ADRB2 and H₁R crystal structures share a similar fold²³ and consequently both models are constructed using the same modeling approach.²,⁴ It is therefore not surprising that the overall structure of the homology models is relatively similar, and gives comparable results in ligand-binding mode prediction² and virtual screening studies.⁵¹ Also in the current study, the conformational ensembles derived from MD simulations of ADRB2- and H₁R-based H₄R models show the same overall binding mode (pose 1) for indolecarboxamides and 2-aminopyrimidines that matches our SDM studies (Table 2 and Figure 4).

In contrast, only the H₁R-based H₄R model is able to explain that larger substituents have a larger negative effect on the affinity for indolecarboxamides than for 2-aminopyrimidines (Table 1) because the chlorine atoms of indolecarboxamide and 2-aminopyrimidine ligands are located in different subpockets close to EL2 (as discussed above). Accurate GPCR loop modeling is still very challenging, as demonstrated by the recent GPCR DOCK competitions to predict the coordinates of GPCR-ligand co-crystal structures⁶¹,⁶², and emphasized by the different extracellular loop structures displayed in the currently available GPCR crystal structures.²¹,⁶³ Despite some recent reports of successful automated retrospective prediction of individual loops of GPCR crystal structures⁶⁴, GPCR loop modeling should be approached with much caution and should be reserved for cases where loop building can be guided and validated by experimental data.⁶⁵ Moreover, structure-based virtual screening studies have shown that loopless TM models of GPCRs can be suitable targets for virtual screening as well.⁶³ Nevertheless, our H₄R modeling studies indicate that the crystal structure of the more closely related H₁R provides a better template for the EL2 region (despite the fact that some parts of this loop were not resolved in the H₁R crystal structure)²³, and to construct H₄R models that can describe subtle differences between ligand binding modes.
Docking in combination with MD simulations explain H₄R mutation studies

In particular the symmetric distribution of two acetic residues (aspartate D94 and glutamate E182) and two hydrophobic subpockets Ila-b that are complementary to basic and several hydrophobic groups in H₄R ligands allow different plausible binding H₄R-ligand modes (Figure 5). Previous and current studies suggest that molecular docking alone is not suitable for accurate determination of relative probabilities of different ligand binding modes. To explain the in vitro SDM data (Table 2, Figure 4), which are an average of different ligand and receptor orientations in time, a dynamic treatment of both ligand and receptor is required (Tables 3-4). MD simulations can account for distributions of protein-ligand interactions and thus can give a more comprehensive explanation of ligand-dependent mutation effects (Tables 2-4) and subtle differences in ligand structure-activity relationships (Table 1, Figure 7). By systematically considering different ligand binding mode hypotheses and protein models, and combining ligand SAR and protein mutagenesis experiments with extensive MD-simulation studies (48 independent MD runs in total) we could identify similarities and differences in the molecular determinants of H₄R binding for both indolecarboxamide and 2-aminopyrimidine ligand classes.

Conclusion

We have successfully combined complementary in silico H₄R structural modeling approaches and ligand SAR with in vitro mutagenesis data to investigate the binding mode of indolecarboxamides and 2-aminopyrimidines in H₄R. By systematically considering different H₄R modeling templates, ligand binding poses, and ligand protonation states in combination with molecular docking and MD simulations we are able to explain ligand-specific mutation effects and subtle differences in ligand SAR. Our results improve the knowledge of H₄R-ligand binding and provide valuable information for optimizing and developing other H₄R-specific ligands. The combined in silico modeling and in vitro SAR and mutagenesis approach presents a promising approach to explore protein-ligand interactions and can be considered as a general method to elucidate protein ligand-binding modes.
Experimental section

Synthetic methods

General remarks

Chemicals and reagents were obtained from commercial suppliers and were used without further purification. Proton and Carbon NMR spectra were obtained on a Bruker Advance 400 FT-NMR or Bruker Advance 500 FT-NMR instrument with chemical shifts (δ) reported relative to tetramethylsilane as an internal standard. High resolution mass spectroscopy data were obtained on a LTQ Orbitrap XL (Thermo Scientific) equipped with a NSI Source (Advion Nanomate) in ESI positive mode. Analytical HPLC-MS analyses were conducted using an Agilent 1100 series LC/MSD system. The analytic method A1 is defined in Table S3. Ligand purities were calculated as the percentage peak area of the analyzed ligand by UV detection at 254 nm. If purity data is not explicitly mentioned the ligand displays a purity > 95%. Flash column chromatography was carried out using hand packed silica gel 60 (230-400 mesh) or pre-packed silica gel columns from Biotage and product was eluted under medium pressure liquid chromatography. Preparative high performance chromatography was carried out on a Gilson system (pump system: 333 & 334 prep-scale HPLC pump; fraction collector: 215 liquid handler; detector: Gilson UV/VIS 155) using pre-packed reversed phase silica gel columns from waters. The method for preparative high performance chromatography P1 are defined in Table S3.

6-Chloro-4-(4-methylpiperazin-1-yl)pyrimidin-2-amine (2h)

4,6-Dichloropyrimidine-2-amine 2f (1.00 g, 5.79 mmol), N-ethyl-diisopropylamine (1.33 g, 11.58 mmol) and 1-Methylpiperazin 2g (0.64 g, 6.37 mmol) were suspended in 30 ml dichloromethane and stirred for 16 h at 20 °C. The solvent was removed under reduced pressure and the crude material is purified using silica gel flash column chromatography with a solvent mixture of dichloromethane, methanol and 25% aqueous ammonia of 90:9:1 for elution. The solvent of the corresponding fractions was evaporated under reduced pressure, yielding 0.9 g (70%) of the title ligand. Purity by method A1: >95%; RT = 1.01 min; MS (ESI') m/z 228/230 [M+H]⁺, Cl distribution; HRMS (ESI') m/z found 228.1015 [M+H]⁺, C9 H15 Cl N5 requires M⁺ 228.1016; ¹H NMR (DMSO) δ (ppm) 6.45 (s, 2H), 6.08 (s, 1H), 3.56 – 3.47 (m, 4H), 2.30 (t, J = 5.1 Hz, 4H), 2.19 (s, 3H).

4-phenyl-6-(4-methylpiperazin-1-yl)pyrimidin-2-amine (2a)

4-Chloro-6-(4-methylpiperazin-1-yl) pyrimidin-2-amine 2h (100 mg, 0.44 mmol), 2-phenylboronic acid (66 mg, 0.53 mmol), caesium carbonate (286 mg, 0.88 mmol) and dichloro[1,1'-bis(diphenylphosphino)ferrochne] palladium (II) dichloromethane adduct (36mg, 0.04mmol) were suspended in 1 ml of a 3:1:1 mixture of tetrahydrofuran, 1-methyl-2-pyrrolidinone and water. The reaction mixture was flushed with argon and stirred for 16h at 100 °C. The crude product was purified using method P1, yielding 76 mg (64%, 0.28 mmol) of the title ligand. Purity by method A1: >95%; RT = 1.35 min; MS (ESI) m/z 270 [M+H]⁺; HRMS (ESI') m/z found 270.1715 [M+H]⁺, C15 H20 N5 requires M⁺ 270.1716; ¹H NMR (500 MHz, DMSO) δ (ppm) 8.05-8.01 (m, 2H), 7.45 – 7.41 (m, 4H), 3.56 – 3.47 (m, 4H), 2.30 (t, J = 5.1 Hz, 4H), 2.19 (s, 3H).
Derivates 2b-e were synthesized according to compound 2a. Analytic data for these ligands are given in the supplementary information.

**Pharmacological assays**

**Cell culture, transfection and membrane preparation**

HEK293T cells were cultured in Dulbecco’s modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 IU/ml penicillin and 50 µg/ml streptomycin at 37 °C / 5% CO₂. One day prior to transfection, the HEK293T cells were seeded at 2E6 cells per 10 cm dish. The polyethylenimine (PEI) transfection method was used to transiently transfect HEK293T cells with H₄R, L175⁵.3³R or E182⁵.4⁶Q cDNA. Briefly, per 10 cm dish 2.5 µg H₄R (mutant) cDNA and 2.5 µg pcDEF3 (empty vector) were incubated with 20 µg 25 kDa linear PEI in a total volume of 500 µl 150 mM NaCl for 30 minutes at room temperature. The transfection mix (cDNA/PEI mix) was subsequently added drop wise to the 10 cm dish with 6ml fresh culture medium. Two days post transfection, cells were washed once with phosphate-buffered saline (PBS) and subsequently scraped from their culture dish in 1ml of PBS. Crude membrane extracts were collected by centrifugation at ~2000 g for 10 min at 4 °C. The crude membrane extract pellets were stored at -20 °C until further use.

**Site-directed mutagenesis**

The construction of the H₄R-L175⁵.3³V and H₄R-E182⁵.4⁶Q mutants has been previously described.³⁴

**[³H]-Radioligand binding assay**

The displacement binding assays were performed using crude membrane extracts from transiently transfected HEK293T cells in 50 mM Tris-HCl binding buffer (pH 7.4 at room temperature). Crude membrane extracts were co-incubated with the 2-aminopyrimidine or indolecarboxamide ligands (Table 1) and ~10 nM radioligand ([³H]-histamine for H₄R-WT and H₄R-L175⁵.3³V or [³H]-JNJ 7777120 for H₄R-E182⁵.4⁶Q) in a total volume of 100 µl/well. The ligand/membrane mixtures were incubated for 1.5 hrs at room temperature on a shaking table (750 rpm). Bound radioligand was separated from free radioligand via rapid filtration over a 0.3% PEI-pre-soaked glass fiber C plate (GF/C, Perkin Elmer). GF/C plates were subsequently washed three times with ice-cold 50 mM Tris-HCl wash buffer (pH 7.4 at 4 °C). The retained radioactivity on the GF/C plates was counted by liquid scintillation counting in a Wallac Microbeta (Perkin Elmer).

**Materials**

[³H]-histamine (10.6-13.4 Ci/mmol) was purchased from Perkin Elmer and [³H]-JNJ 7777120 (56.1 Ci/mmol) was a kind gift of Robin Thurmond (JNJ, La Jolla, CA). PEI was bought from Polysciences and cell cultures media were obtained from PAA (Pasching, Austria).
**pKₐ measurement**

For pKₐ measurement a GLpKa automated pKₐ analyser (Sirius Analytical Instruments Ltd) was used with standard methods described by Allen *et al.*⁶⁹ and Volgyi *et al.*⁷⁰

**Computational methods**

**H₄R model**

The construction of the ADRB2-H₄R model has been described previously.²,⁴ The H₄R-H₄R model was built in the same manner. The mutated H₄R model (E182⁵.⁴⁶Q) was constructed by mutating the corresponding residues of the H₄R model with the mutate function in MOE 2009.¹⁷¹ The H₄R model was used either with the χ₁ torsional angle of C98³.³⁶ in the t-conformations or in the g+ conformation, where an additional pocket is assessable. Ligands were docked into both H₄R models using PLANTS⁴⁹, ⁵⁰ without restraints. As aspartate D94³.³² and glutamate E182⁵.⁴⁶ are reported to be important for binding³⁴, only docking poses where the ligands interact with both of these residues were selected.

**Molecular dynamics simulations**

The protein-ligand complexes were minimized using Amber10⁷², including restraints for the experimentally supported hydrogen bond interactions with aspartate D94³.³² and glutamate E182⁵.⁴⁶ (distance between donor and acceptor atom and angle of donor, hydrogen and acceptor atom). The number of H-bond interactions between ligands and the E182⁵.⁴⁶ side chain were generally low for all ligands in MD simulations in which the carboxylate group of E182⁵.⁴⁶ was considered in the (neutral) protonated state. This is not in line with the ligand-dependent effects of the E⁵.⁴⁶Q mutation and therefore both residues were modeled in the deprotonated (negatively charged) state. The minimized protein-ligand complex was refined by a second minimization without restraints. The minimized complex was embedded in a pre-equilibrated lipid bilayer consisting of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) molecules and solvated with TIP3P water molecules as described by Urizar *et al.*⁷³ The whole complex was again minimized using restraints for the expected hydrogen bonds. Additionally positional harmonic restraints of 10 kcal/mol on Ca atoms of the helical domains of the H₄R receptor were applied during this minimization step. Each minimization was performed using a certain number of steps steepest descent followed by conjugate gradient until the root mean square gradient of the potential energy was lower than 0.0001 kcal/mol. Non-bonded interactions were calculated within a cut-off of 10 Å.

The entire system was subjected to a constant pressure (1 bar) MD-simulation. All bonds involving hydrogen atoms were frozen with the SHAKE algorithm. In the first 100 ps the temperature was increased in stepwise manner to 300 K. During this procedure a positional harmonic restraints on helical Ca atoms of 10 kcal/mol were applied which were reduced to 0.5 kcal/mol for the subsequent 1ns production run (at 300 K). The temperature was controlled using the weak coupling approach⁷⁴ with a coupling constant of 0.2 ps. For calculating the long range electrostatic interactions the Particle Mesh Ewald (PMW) method was applied. Van der Waals interactions were calculated within
a cut-off of 10 Å.

For the protein, the POPC and the water molecules the AMBER03 force field was used. For the ligand atoms the GAFF force field was used. Ligand force field parameters were derived using ANTECHAMBER. These parameters were adjusted and extended for 2-aminopyrimidine ligands to keep the amino group (and in case of double protonation also the additional proton on the pyrimidine ring) in the plane of the pyrimidine ring. Partial charges for the ligand were derived using the AM1-BCC procedure in ANTECHAMBER. The tLEaP module of AMBER10 was used to generate the topology and coordinate files of the protein-ligand complex.

During the MD-simulation trajectories of every 1000th MD-step was saved. This results in 500 snapshots for a 1ns MD-simulation. Using the PTRAJ module of AMBER10 distances and angles could be extracted which were used to analyse H-bond interactions between the protein and the ligand and interactions of the ligand with L175. An H-bond was counted if the distance between H-bond donor and acceptor was below 3.5 Å and the angle between H-bond donor, hydrogen and H-bond acceptor was between 180±45°. Hydrophobic interactions of the ligand with L175 were counted if the distance between any heavy atom of the ligand and L175 was ≤ 4 Å.

**Calculation of the pocket volumes**

The pocket volume around the chlorine atoms of ligands 1b and 2b was determined using POVME. A radius of 3.5 Å was chosen for the calculations. The chlorine atom in the pdb snapshots taken during the MD-simulation was deleted for these calculations. Padding was on standard value of 1.09. This is the radius of a hydrogen atom. Basically, only the volume of the binding site that can be occupied by hydrogen atoms was measured. As the van de Waals radius of the carbon atom (where the chlorine atom is attached to) and the diameter of a hydrogen atom overcomes the diameter of the chlorine atom also pocket volumes of 0 Å³ can be observed.

**Acknowledgments**

This research was financially supported by The Netherlands Organization for Scientific Research [NWO VENI Grant 700.59.408 to C. de G.], and COST Action BM0806. Obbe Zuiderveld is acknowledged for technical assistance.
Supplementary information

Figure S1. Comparison 1a and 2a bound H₄R homology models based on H₁R²³ (A, C) and ADRB2³⁴ (B, D) crystal structures. Compounds and pocket residues are depicted as ball-and-sticks, whereas for clarity Y95¹.₃₃ is shown as lines. H-bonds between the ligand and pocket residues are represented as black dotted lines. The backbone TM helices 5, 6, and 7 (right to left) are presented as yellow helices. Helix 3 is presented by yellow ribbons. The EL2 is colored in blue. Subpockets I, IIa and IIb are labeled in red.
**Table S1.** Frequency of hydrophobic interactions\(^a\) between the ligand and L175\(^{5.39}\) and frequency of H-bonds\(^b\) formed between the ligand and aspartate D94\(^{5.32}\) or glutamate E182\(^{5.46}\) and combinations thereof in the H\(_{1}\)R-H\(_{4}\)R model, which occur during a 1 ns MD-simulation (500 snapshots).

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<td>22.6</td>
<td>24.2</td>
</tr>
<tr>
<td>(D^{3.32}+E^{5.46}+L^{5.39})</td>
<td>0.0</td>
<td>0.0</td>
<td>15.4</td>
<td>14.4</td>
<td>22.6</td>
<td>24.0</td>
</tr>
<tr>
<td><strong>Pose 3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(L^{5.39} \leq 4 \text{ Å})</td>
<td>0.0</td>
<td>0.0</td>
<td>1.8</td>
<td>43.2</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>(D^{3.32})</td>
<td>99.2</td>
<td>99.8</td>
<td>79.2</td>
<td>64.6</td>
<td>98.8</td>
<td>91.0</td>
</tr>
<tr>
<td>(E^{5.46})</td>
<td>81.0</td>
<td>50.8</td>
<td>33.0</td>
<td>0.4</td>
<td>96.6</td>
<td>98.4</td>
</tr>
<tr>
<td>(D^{3.32}+E^{5.46})</td>
<td>80.4</td>
<td>50.8</td>
<td>12.4</td>
<td>0.4</td>
<td>95.4</td>
<td>89.6</td>
</tr>
<tr>
<td>(D^{3.32}+L^{5.39})</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>9.8</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>(E^{5.46}+L^{5.39})</td>
<td>0.0</td>
<td>0.0</td>
<td>1.8</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>(D^{3.32}+E^{5.46}+L^{5.39})</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

\(^a\) A hydrophobic interaction is counted if the shortest distance between any heavy atom of the ligand and L175\(^{5.39}\) is \(\leq 4 \text{ Å}\). \(^b\) An H-bond is counted if the distance between H-bond donor and acceptor heavy atom is below 3.5 Å and the angle between H-bond donor heavy atom, hydrogen and H-bond acceptor heavy atom is between 135° and 225°.
Mapping histamine H\textsubscript{4} receptor–ligand binding modes

Table S2. Frequency of hydrophobic interactions between the ligand and L175\textsuperscript{5.39} and frequency of H-bonds formed between the ligand and aspartate D94\textsuperscript{1.32} or glutamate E182\textsuperscript{5.46} and combinations thereof in the ADBR2-H\textsubscript{4}R model which occur during a 1 ns MD-simulation (500 snapshots).

<table>
<thead>
<tr>
<th>compounds</th>
<th>1a(1+)</th>
<th>1b(1+)</th>
<th>2a(1+)</th>
<th>2b(1+)</th>
<th>2a(2+)</th>
<th>2b(2+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(L\textsuperscript{5.39} \leq 4\ \text{Å})</td>
<td>21.6</td>
<td>72.8</td>
<td>91.4</td>
<td>82.0</td>
<td>51.2</td>
<td>90.4</td>
</tr>
<tr>
<td>(D\textsuperscript{3.32})</td>
<td>98.2</td>
<td>99.6</td>
<td>99.8</td>
<td>100.0</td>
<td>99.8</td>
<td>98.2</td>
</tr>
<tr>
<td>(E\textsuperscript{5.46})</td>
<td>95.8</td>
<td>97.2</td>
<td>98.8</td>
<td>97.4</td>
<td>99.8</td>
<td>98.2</td>
</tr>
<tr>
<td>(D\textsuperscript{3.32}+E\textsuperscript{5.46})</td>
<td>94.0</td>
<td>96.8</td>
<td>98.6</td>
<td>97.4</td>
<td>99.8</td>
<td>98.2</td>
</tr>
<tr>
<td>(D\textsuperscript{3.32}+L\textsuperscript{5.39})</td>
<td>21.6</td>
<td>72.6</td>
<td>91.2</td>
<td>82.0</td>
<td>51.2</td>
<td>88.8</td>
</tr>
<tr>
<td>(E\textsuperscript{5.46}+L\textsuperscript{5.39})</td>
<td>21.0</td>
<td>70.8</td>
<td>90.4</td>
<td>80.0</td>
<td>51.2</td>
<td>90.4</td>
</tr>
<tr>
<td>(D\textsuperscript{3.32}+E\textsuperscript{5.46}+L\textsuperscript{5.39})</td>
<td>21.0</td>
<td>70.6</td>
<td>90.2</td>
<td>80.0</td>
<td>51.2</td>
<td>88.8</td>
</tr>
</tbody>
</table>

A hydrophobic interaction is counted if the shortest distance between any heavy atom of the ligand and L175\textsuperscript{5.39} is \(\leq 4\ \text{Å}\). An H-bond is counted if the distance between H-bond donor and acceptor heavy atom is below 3.5 Å and the angle between H-bond donor heavy atom, hydrogen and H-bond acceptor heavy atom is between 135° and 225°.

Table S3. Chromatography methods

<table>
<thead>
<tr>
<th>Column (^a)</th>
<th>Solvent A</th>
<th>Solvent B</th>
<th>flow rat [ml/ min]</th>
<th>Gradient (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>Phenomenex, Mercury Gemini, C18, 3 µm, 2 × 20 mm, 40 °C</td>
<td>water pH 8 (buffer: NH\textsubscript{3} / NH\textsubscript{4}HCO\textsubscript{3})</td>
<td>Acetonitril</td>
<td>1.0</td>
</tr>
<tr>
<td>P1</td>
<td>Waters, XBridge, C18, 10 µm, 30*100 mm</td>
<td>water pH 8 (buffer: NH\textsubscript{3} / NH\textsubscript{4}CO\textsubscript{3})</td>
<td>Methanol</td>
<td>100</td>
</tr>
</tbody>
</table>

\(^a\) company, column name, kind of particle, particle size, column dimension, column temperature; \(^b\) % of solvent B at gradient start \(\rightarrow\) % of solvent B at gradient end, gradient time;
1H-indol-2-yl-(4-methylpiperazin-1-yl)methanone (1a)
Purity by method A1: >95%; RT = 1.38 min; MS (ESI\(^+\)) m/z 244 [M+H]\(^+\); HRMS (ESI\(^+\)) m/z found 244.1451 [M+H]\(^+\); C14 H18 N3 O requires M\(^+\) 244.1450; \(^1\)H NMR (500 MHz, DMSO) δ (ppm) 11.54 (br, 1H), 7.59 (d, J = 8.0 Hz, 1H), 7.41 (dd, J = 1.0 Hz, J = 8.2 Hz, 1H), 7.20 - 7.15 (m, 1H), 7.06 - 7.01 (m, 1H), 6.77 (d, J = 1.4 Hz, 1H), 3.75 (br, 4H), 2.36 (t, J = 5.2 Hz, 4H), 2.21 (br, 3H).

(5-chloro-1H-indol-2-yl)-(4-methylpiperazin-1-yl)methanone (1b)
Purity by method A1: >95%; RT = 1.62 min; MS (ESI\(^+\)) m/z 278/280 (M+H)\(^+\); Cl distribution; HRMS (ESI\(^+\)) m/z found 278.1057 [M+H]\(^+\); C14 H17 Cl N3 O requires M\(^+\) 278.106;

(5-amino-1H-indol-2-yl)-(4-methylpiperazin-1-yl)methanone (1c)
Purity by method A1: >95%; RT = 0.87 min; MS (ESI\(^+\)) m/z 259 [M+H]\(^+\); HRMS (ESI\(^+\)) m/z found 259.1567 [M+H]\(^+\); C14 H19 N4 O requires M\(^+\) 259.1559;

(4-methylpiperazin-1-yl)-(5-nitro-1H-indol-2-yl)methanone (1d)
Purity by method A1: >95%; RT = 1.33 min; MS (ESI\(^+\)) m/z 289 [M+H]\(^+\); HRMS (ESI\(^+\)) m/z found 289.1302 [M+H]\(^+\); C14 H17 N4 O3 requires M\(^+\) 289.13;

(5-methoxy-1H-indol-2-yl)-(4-methylpiperazin-1-yl)methanone (1e)
Purity by method A1: >95%; RT = 1.28 min; MS (ESI\(^+\)) m/z 274 [M+H]\(^+\); HRMS (ESI\(^+\)) m/z found 274.1322 [M+H]\(^+\); C15 H20 N4 O requires M\(^+\) 274.1329; \(^1\)H NMR (500 MHz, DMSO) δ (ppm) 8.08 (d, 2H, J = 8.5 Hz), 7.49 (d, 2H, J = 8.9 Hz), 6.68 (d, J = 2.5 Hz, 1H), 6.44 (d, J = 1.8 Hz, 1H), 3.66 – 3.57 (m, 4H), 2.38 (t, J = 5.0 Hz, 4H), 2.21 (s, 3H).

4-(4-chlorophenyl)-6-(4-methylpiperazin-1-yl)pyrimidin-2-amine (2b)
Derivate was synthesized according to compound 2a. Purity by method A1: >95%; RT = 1.58 min; MS (ESI\(^+\)) m/z 304 [M+H]\(^+\); HRMS (ESI\(^+\)) m/z found 304.1322 [M+H]\(^+\); C15 H19 Cl N5 requires M\(^+\) 304.1329; \(^1\)H NMR (500 MHz, DMSO) δ (ppm) 8.08 (d, 2H, J = 8.5 Hz), 7.49 (d, 2H, J = 8.9 Hz), 6.61 (s, 1H), 6.11 (s, 2H), 3.66 – 3.57 (m, 4H), 2.38 – 2.31 (m, 4H), 2.21 (s, 3H).

4-(4-aminophenyl)-6-(4-methylpiperazin-1-yl)pyrimidin-2-amine (2c)
Derivate was synthesized according to compound 2a. Purity by method A1: >95%; RT = 1.09 min; MS (ESI\(^+\)) m/z 285 [M+H]\(^+\); HRMS (ESI\(^+\)) m/z found 285.183 [M+H]\(^+\); C15 H21 N6 requires M\(^+\) 285.1827; \(^1\)H NMR (500 MHz, DMSO) δ (ppm) 7.76 (dd, J1 = 8.6 Hz, J2 = 1.7 Hz, 2H), 6.56 (dd, J1 = 8.6 Hz, J2 = 1.7 Hz, 2H), 5.85 (s, 2H), 5.41 (s, 2H), 3.58-3.54 (m, 4H), 2.35-2.32 (m, 4H), 2.20 (s, 3H).
Mapping histamine H₄ receptor–ligand binding modes

4-(4-methylpiperazin-1-yl)-6-(4-nitrophenyl)pyrimidin-2-amine (2d)

Derivate was synthesized according to compound 2a. Purity by method A1: >95%; RT = 1.44 min; MS (ESI⁺) m/z 315[M+H]⁺; HRMS (ESI⁺) m/z found 315.1566 [M+H]⁺, C₁₅ H₁₉ N₆ O₂ requires M⁺ 315.1569; ¹H NMR (500 MHz, DMSO) δ (ppm) 8.34 – 8.27 (m, 4H), 6.75 (s, 1H), 6.23 (s, 2H), 3.65 (t, 4H, J = 4.3 Hz), 2.36 (t, 4H, J = 4.7 Hz), 2.22 (s, 3H).

4-(4-methoxyphenyl)-6-(4-methylpiperazin-1-yl)pyrimidin-2-amine (2e)

Derivate was synthesized according to compound 2a. Purity by method A1: >95%; RT = 1.38 min; MS (ESI⁺) m/z 300 [M+H]⁺; HRMS (ESI⁺) m/z found 300.182 [M+H]⁺, C₁₆ H₂₂ N₅ O requires M⁺ 300.1824; ¹H NMR (500 MHz, DMSO) δ (ppm) 8.01 (d, 2H, J = 8.8 Hz), 6.97 (d, 2H, J = 8.8 Hz), 6.51 (s, 1H), 6.00 (s, 2H), 3.80 (s, 3H), 3.64 – 3.55 (m, 4H), 2.35 (t, 4H, J = 5.1 Hz), 2.21 (s, 3H).

References


Mapping histamine H₄ receptor–ligand binding modes


71. MOE (Molecular Operating Environment) v.2009.10 (http://www.chemcomp.com/software.htm, Montreal, 2009).


