Supplementary Material

Mapping histamine H4 receptor-ligand binding modes by molecular dynamics simulations and site-directed mutagenesis studies

Sabine Schultes[1,2*], *Saskia Nijmeijer*[2*], *Harald Engelhardt*[1,2], *Albert Kooistra*[2], *Henry F. Vischer*[2], *Iwan J.P. de Esch*[2], *Eric E.J. Haaksma*[1], *Rob Leurs*[2], *Chris de Graaf*[1]

Table of Contents:

Table S1. Frequency of hydrophobic interactions between the ligand and $L175^{5.39}$ and frequency of H-bonds formed between the ligand and aspartate $D94^{3.32}$ or glutamate $E182^{5.46}$ and combinations thereof in the H_1R-H_4R model which occur during a 1 ns MD-simulation (500 snapshots).

cmps	1a(1+)	1b(1+)	2a(1+)	2b(1+)	2a(2+)	2b(2+)
Pose1						
$L^{5.39} \leq 4$ Å	62.6	98.9	91.6	99.0	88.4	77.4
$D^{3.32}$	91.8	91.0	100.0	95.6	99.4	96.8
$E^{5.46}$	99.6	99.4	99.4	100	99.8	100
$D^{3.32}$+$E^{5.46}$	91.4	90.6	99.4	95.6	99.2	96.8
$D^{3.32}$+$L^{5.39}$	56.6	89.8	91.6	94.6	87.8	75.2
$E^{5.46}$+$L^{5.39}$	62.2	98.2	91.0	99.0	88.2	77.4
$D^{3.32}$+$E^{5.46}$+$L^{5.39}$	56.2	89.4	91.0	94.6	87.6	75.2
Pose 2						
$L^{5.39} \leq 4$ Å	0.0	0.0	21.6	29.4	22.6	31.4
$D^{3.32}$	93.2	82.4	100	100	99.8	99.4
$E^{5.46}$	98.8	99.8	56.6	62.0	100.0	83.6
$D^{3.32}$+$E^{5.46}$	92.0	82.2	56.6	62.0	99.8	83.0
$D^{3.32}$+$L^{5.39}$	0.0	0.0	21.6	29.4	22.6	31.2
$E^{5.46}$+$L^{5.39}$	0.0	0.0	15.4	14.4	22.6	24.2
$D^{3.32}$+$E^{5.46}$+$L^{5.39}$	0.0	0.0	15.4	14.4	22.6	24.0
Pose 3						
$L^{5.39} \leq 4$ Å	0.0	0.0	1.8	43.2	0.0	0.0
$D^{3.32}$	99.2	99.8	79.2	64.6	98.8	91.0
$E^{5.46}$	81.0	50.8	33.0	0.4	96.6	98.4
$D^{3.32}$+$E^{5.46}$	80.4	50.8	12.4	0.4	95.4	89.6
$D^{3.32}$+$L^{5.39}$	0.0	0.0	0.0	9.8	0.0	0.0
$E^{5.46}$+$L^{5.39}$	0.0	0.0	1.8	0.0	0.0	0.0
$D^{3.32}$+$E^{5.46}$+$L^{5.39}$	0.0	0.0	0.0	0.0	0.0	0.0

A hydrophobic interaction is counted if the shortest distance between any heavy atom of the ligand and $L175^{5.39}$ is ≤ 4 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 S2. Frequency of hydrophobic interactions between the ligand and L175$^{5.39}$ and frequency of H-bonds formed between the ligand and aspartate D94$^{3.32}$ or glutamate E182$^{5.46}$ and combinations thereof in the ADBR2-H_4R model which occur during a 1 ns MD-simulation (500 snapshots).

cmps	1a(1+)	1b(1+)	2a(1+)	2b(1+)	2a(2+)	2b(2+)
Pose 1						
$L^{5.39} \leq 4$ Å	21.6	72.8	91.4	82.0	51.2	90.4
$D^{3.32}$	98.2	99.6	99.8	100.0	99.8	98.2
$E^{5.46}$	95.8	97.2	98.8	97.4	100.0	100.0
$D^{3.32}$+$E^{5.46}$	94.0	96.8	98.6	97.4	99.8	98.2
$D^{3.32}$+$L^{5.39}$	21.6	72.6	91.2	82.0	51.2	88.8
$E^{5.46}$+$L^{5.39}$	21.0	70.8	90.4	80.0	51.2	90.4
$D^{3.32}$+$E^{5.46}$+$L^{5.39}$	21.0	70.6	90.2	80.0	51.2	88.8
Pose 2						
$L^{5.39} \leq 4$ Å	0.0	0.0	41.6	0.0	0.2	0.0
$D^{3.32}$	98.2	95.4	95.2	85.0	79.0	87.4
$E^{5.46}$	99.8	98.2	100.0	100.0	100.0	100.0
$D^{3.32}$+$E^{5.46}$	98.0	93.6	95.2	85.0	79.0	87.4
$D^{3.32}$+$L^{5.39}$	0.0	0.0	38.0	0.0	0.2	0.0
$E^{5.46}$+$L^{5.39}$	0.0	0.0	41.6	0.0	0.2	0.0
$D^{3.32}$+$E^{5.46}$+$L^{5.39}$	0.0	0.0	38.0	0.0	0.2	0.0
Pose 3						
$L^{5.39} \leq 4$ Å	0.0	0.0	0.0	0.0	0.0	0.0
$D^{3.32}$	100.0	99.4	57.4	93.2	98.0	96.6
$E^{5.46}$	0.0	11.2	90.6	97.6	84.8	54.0
$D^{3.32}$+$E^{5.46}$	0.0	10.6	51.0	91.0	84.8	53.8
$D^{3.32}$+$L^{5.39}$	0.0	0.0	0.0	0.0	0.0	0.0
$E^{5.46}$+$L^{5.39}$	0.0	0.0	0.0	0.0	0.0	0.0
$D^{3.32}$+$E^{5.46}$+$L^{5.39}$	0.0	0.0	0.0	0.0	0.0	0.0

A hydrophobic interaction is counted if the shortest distance between any heavy atom of the ligand and L175$^{5.39}$ is ≤ 4 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

	Column [a]	Solvent A	Solvent B	flow rat [ml/ min]	Gradient [b]
A1	Phenomenex, Mercury Gemini, C18, 3 □m, 2 × 20 mm, 40 °C	water pH 8 (buffer: NH_3 / NH_4HCO_3)	Acetonitril	1.0	5% → 95%, 2.5 min
P1	Waters, XBridge, C18, 10 µm, 30*100 mm	water pH 8 (buffer: NH3 / NH4CO3)	Methanol	100	10% → 75%, 6 min

[a] company, column name, kind of particle, particle size, column dimension, column temperature; [b] % of solvent B at gradient start → % 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; ^1H 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; ^1H NMR (400 MHz, DMSO) δ (ppm) 11.74 (br, 1H), 7.64 (d, J = 2.0 Hz, 1H), 7.42 (d, J = 8.6 Hz, 1H), 7.17 (dd, J = 2.1, J = 8.7 Hz, 1H), 6.75 (s, 1H), 3.73 (br, 4H), 2.37 (t, J = 5.1, 4H), 2.22 (s, 3H).

(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; ^1H NMR (500 MHz, DMSO) δ (ppm) 11.05 (br, 1H), 7.10 (d, J = 8.9 Hz, 1H), 6.68 (d, J = 2.0 Hz, 1H), 6.60 (dd, J = 2.0 Hz, J = 8.5 Hz, 1H), 6.47 (d, J = 1.9 Hz, 1H), 4.55 (br, 2H), 3.72 (br, 4H), 2.35 (t, J = 5.1 Hz, 4H), 2.21 (s, 3H).

(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; ^1H NMR (500 MHz, DMSO) δ (ppm) 12.32 (br, 1H), 8.64 (d, J = 2.3 Hz, 1H), 8.07 (dd, J = 2.2 Hz, J = 9.1 Hz, 1H), 7.56 (d, J = 9.3 Hz, 1H), 7.08 (s, 1H), 3.74 (br, 4H), 2.38 (t, J = 5.0 Hz, 4H), 2.22 (s, 3H).

(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 274.1547 $[M+H]^+$, C15 H20 N3 O2 requires M^+ 274.1555; ^1H NMR (500 MHz, DMSO) δ (ppm) 11.40 (br, 1H), 7.30 (d, J = 8.7 Hz, 1H), 7.06 (d, J = 2.5 Hz, 1H), 6.83 (dd, J = 2.5 Hz, J

= 8.9 Hz, 1H), 6.68 (d, J = 1.8 Hz, 1H), 3.75 (br, 7H), 2.65 (s, 3H), 2.38 (t, J = 5.0 Hz, 4H), 2.22 (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; ^1H 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; ^1H NMR (500 MHz, DMSO) δ (ppm) 7.76 (dd, J_1 = 8.6 Hz, J_2 = 1.7 Hz, 2H), 6.56 (dd, J_1 = 8.6 Hz, J_2 = 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).

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, 3 H).

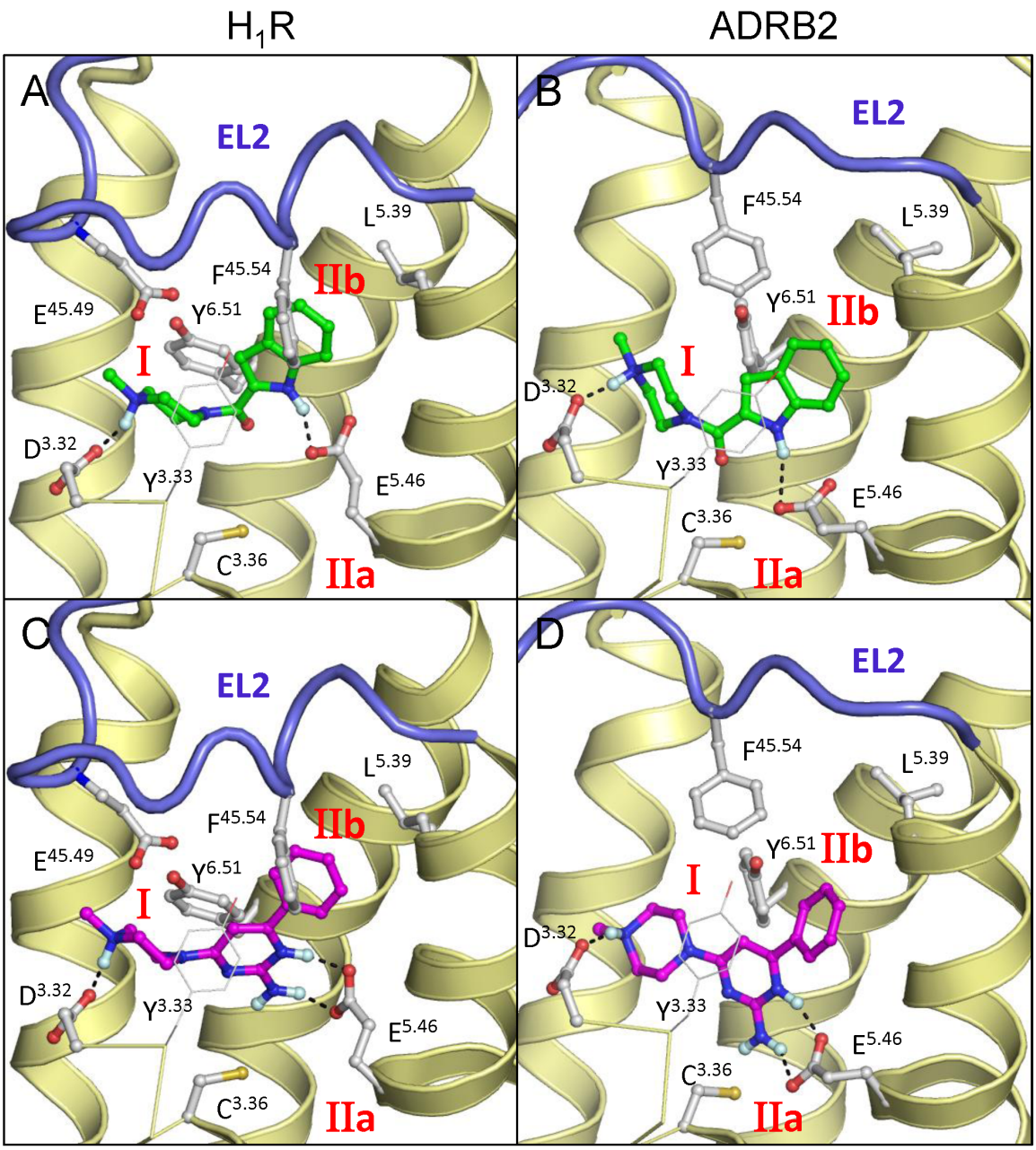

Figure S1. Comparison **1a** and **2a** bound H_4R homology models based on H_1R[1] (**A**, **C**) and ADRB2[2] (**B**, **D**) crystal structures. Compounds and pocket residues are depicted as ball-and-sticks, whereas for clarity $Y95^{3.33}$ is shown as lines. H-bonds between the ligand and pocket residues 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.

REFERENCES:

1. Shimamura, T., Shiroishi, M., Weyand, S., Tsujimoto, H., Winter, G., Katritch, V., Abagyan, R., Cherezov, V., Liu, W., Han, G. W., Kobayashi, T., Stevens, R. C. & Iwata, S. (2011). Structure of the human histamine H1 receptor complex with doxepin. *Nature* **475**, 65-70.

2. Cherezov, V., Rosenbaum, D. M., Hanson, M. A., Rasmussen, S. G., Thian, F. S., Kobilka, T. S., Choi, H. J., Kuhn, P., Weis, W. I., Kobilka, B. K. & Stevens, R. C. (2007). High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. *Science* **318**, 1258-65.