Luciferase Reporter Gene Assay on Human, Murine and Rat Histamine H4 Receptor Orthologs: Correlations and Discrepancies between Distal and Proximal Readouts

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Abstract

The investigation of the (patho)physiological role of the histamine H4 receptor (H4R) and its validation as a possible drug target in translational animal models are compromised by distinct species-dependent discrepancies regarding potencies and receptor subtype selectivities of the pharmacological tools. Such differences were extremely pronounced in case of proximal readouts, e.g. [³²P]GTPase or [³⁵S]GTPγS binding assays. To improve the predictability of in vitro investigations, the aim of this study was to establish a reporter gene assay for human, murine and rat H4Rs, using bioluminescence as a more distal readout. For this purpose a cAMP responsive element (CRE) controlled luciferase reporter gene assay was established in HEK293T cells, stably expressing the human (h), the mouse (m) or the rat (r) H4R. The potencies and efficacies of 23 selected ligands (agonists, inverse agonists and antagonists) were determined and compared with the results obtained from proximal readouts. The potencies of the examined ligands at the human H4R were consistent with reported data from [³²P]GTPase or [³⁵S]GTPγS binding assays, despite a tendency toward increased intrinsic efficacies of partial agonists. The differences in potencies of individual agonists at the three H4R orthologs were generally less pronounced compared to more proximal readouts. In conclusion, the established reporter gene assay is highly sensitive and reliable. Regarding discrepancies compared to data from functional assays such as [³²P]GTPase and [³⁵S]GTPγS binding, the readout may reflect multifactorial causes downstream from G-protein activation, e.g. activation/amplification of or cross-talk between different signaling pathways.

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Introduction

The histamine H4 receptor (H4R) [1–5] is preferably expressed on cells of hematopoietic origin such as eosinophils and mast cells and supposed to be involved in inflammatory diseases, e.g. asthma, and pruritis [6–10]. To investigate the (patho)physiological role of the H4R translational animal models for allergic asthma and conjunctivitis [16,17] were used. Most of the allergic contact dermatitis in mice [11–15] or rat models for acute inflammation and conjunctivitis [16,17] were used. Most of the studies confirmed the pro-inflammatory role of the H4R by blocking the H4R-mediated response with JNJ 7777120 (1-[(5-N2-propionylguanidine) and UR-PI376 (2-cyano-1-[(4-(1H-imidazol-4-yl)butyl]-3-[(2-phenylthio)ethyl]guanidine) [24,25] displayed considerably lower potencies and efficacies (UR-PI376) in the [³²P]GTPase and [³⁵S]GTPγS binding assays on membrane preparations of Sf9 insect cells expressing the mouse or rat H4R [23]. Most strikingly, JNJ 7777120 exhibited stimulatory effects at
the mouse and rat H4R in functional assays on Sf9 cell membranes [23]. Moreover, the use of JNJ 7777120 as standard antagonist in animal models was questioned due to stimulation of G-protein independent β-arrestin recruitment [26]. Biased signaling of the hH4R has also been shown for other H4R ligands [27].

The aforementioned controversial findings underline the necessity to evaluate pharmacological tools at the H4R species orthologs of interest using different assay systems. For this purpose, a cAMP response element (CRE) controlled luciferase reporter gene assay in HEK293T cells, stably expressing the human, the mouse or the rat H4R, was established. The H4Ri sGαi/o-coupled and reduces forskolin stimulated cyclic adenosine monophosphate (cAMP) formation after agonist binding [2]. The optimal concentration of forskolin used for pre-stimulation depends on the cell type [28] and should correspond to the EC50 of forskolin in the assay system [29]. Therefore, the potency of forskolin was determined, and the effect of the phosphodiesterase (PDE) inhibitor isobutylmethylxanthine (IBMX) was evaluated to optimize the sensitivity of the procedure. Due to the delayed onset of gene expression, incubation periods of four to six hours are required [30], increasing the risk of agonist mediated receptor desensitization, which can lead to a decrease in agonist potencies [30]. Therefore, the time course of the luciferase expression was determined to find the minimum incubation period required for appropriate signal strength. For validation, potencies and efficacies of 23 selected H4R ligands, comprising agonists, inverse agonists and antagonists, were determined (Figure 1).

Materials and Methods

Ethics Statement

Human embryonal kidney (HEK293T) cells were purchased from the German Collection of Microorganism and Cell Cultures (DSMZ, Braunschweig, Germany).

Histamine Receptor Ligands

Histamine (HA, 1) was purchased from Alfa Aesar (Karlsruhe, Germany). (R)-α-methylhistamine (2), (S)-α-methylhistamine (3), Nα-methylhistamine (4), 5(4)-methylhistamine (5), immepip (6), immethridine (7), imetit (8), clobenpropit (9), iodophenpropit (10), proxyfan (PRO, 11), ciproxifan (CIP, 12), clozapine (17), VUF 5681 (18), A 943931 (22) and A 987306 (23) were from Tocris Bioscience (Ellisville, MO, USA), UR-PI294 (13), UR-PI376 (14), VUF 8430 (15), ST-1006 (16), JNJ 7777120 (19), thioperamide (THIO, 20).
(THIO, 20) and ST-1012 (21) were synthesized in our laboratories. Chemical structures of the ligands are depicted in Figure 1. Except for 14, 16, 17, 21 and 23 all stock solutions (10 mM) were prepared in Millipore water. Stock solution of 17 and 23 were made in 20 mM HCl, whereas 14, 16 and 21 were dissolved in 50% (v/v) dimethyl sulfoxide (DMSO). Stock solutions of 17 and 23 and those ligands dissolved in water were diluted with Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS). The stock solutions of 14, 16 and 21 were diluted with DMEM supplemented with 10% (v/v) FCS and 10% (v/v) DMSO.

Subcloning of FLAG Epitope- and Hexahistidine-tagged mH4R cDNA into the Shuttle Vector pcDNA3.1(+)

The FLAG epitope (F)- and the hexahistidine (His6)-tagged mH4R cDNA cloned in pGEM-3Z [23] was subcloned at HindIII and XbaI restriction sites into pcDNA3.1(+) encoding G418 resistance. Double digestion with HindIII (Fermentas GmbH, St. Leon-Rot, Germany) and XbaI (Fermentas) restriction enzymes was performed in reaction buffer Tango (Fermentas) with a twofold excess of HindIII at 37°C for 3 h. The DNA bands of the SF- mH4R-His6 (1336 bp) (S stands for the cleavable signal peptide from influenza hemagglutinin, F for flag) insert as well as the linearized pcDNA3.1(+) vector (5352 bp) were extracted from the

Figure 2. Stimulation of luciferase activity by forskolin. (A) Representative time course of the luciferase expression in HEK293T-CRE-Luc cells, stably expressing the CRE-controlled luciferase, upon stimulation with 10 μM of forskolin. The luciferase activity was determined after the indicated incubation periods (mean values ± SEM; n = 9). (B) Representative “bell-shaped” concentration-response curve obtained with HEK293T-SF-hH4R-His6-CRE-Luc cells, stably expressing the hH4R and the CRE-controlled luciferase. (C) Concentration response curves covering the ascending region of the signal obtained with different transfectants.

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Figure 3. Inhibition of luciferase activity by histamine in rH4R expressing cells. Gαi/o mediated inhibition of forskolin (0.5μM–5.0 μM) stimulated luciferase activities by histamine (HA) in HEK293T-SF-rH4R-His6-CRE-Luc cells, stably expressing the rH4R and the CRE-controlled luciferase. (A) Representative luciferase reporter gene with RLU values as readout. (B) Normalized inhibition of forskolin stimulated luciferase activity (100%) by histamine (HA), with the maximum inhibitory effect of which set at 0%. Data points shown are the mean ± SEM of at least three independent experiments performed in triplicate.

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Figure 4. Effect of histamine and thioperamide on the luciferase activity in hH4R expressing cells. Concentration-response curves of histamine (HA) and thioperamide (THIO) on HEK293T-SF-hH4R-His6-CRE-Luc cells, stably co-expressing the CRE-controlled luciferase and the hH4R. The cells were pre-stimulated with 500 nM of forskolin alone or in combination with IBMX (50 μM). The effect of forskolin or that of forskolin plus IBMX was defined as 100% luciferase activity. Data points shown are the mean ± SEM of two independent experiments performed in triplicate. doi:10.1371/journal.pone.0073961.g004

1% (m/v) agarose (pegGOLD Universal-Agarose, Peqlab, Erlangen, Germany) gel using the QIAquick Gel Extraction Kit

| Table 1. Potencies and efficacies of H4R ligands in the luciferase reporter gene assay at the hH4R, the mH4R and the rH4R.

| Ligand | hH4R pEC50 or (pKb) | α | N | mH4R pEC50 or (pKb) | α | N | rH4R pEC50 or (pKb) | α | N |
|--------|---------------------|---|---|---------------------|---|---|---------------------|---|---|
| Histamine (1) | 7.77±0.12 | 1.00 | 6 | 7.06±0.13 | 1.00 | 4 | 6.53±0.04 | 1.00 | 6 |
| (R)-α-Methylhistamine (2) | 6.47±0.09 | 1.03±0.04 | 5 | 6.16±0.07 | 0.98±0.01 | 6 | 5.60±0.12 | 0.96±0.02 | 3 |
| (S)-α-Methylhistamine (3) | 5.22±0.09 | 0.90±0.04 | 5 | 4.72±0.16 | 0.82±0.07 | 3 | 4.26±0.04 | 0.69±0.03 | 3 |
| N3-Methylhistamine (4) | 6.74±0.12 | 0.98±0.03 | 4 | 6.24±0.13 | 0.97±0.02 | 3 | 6.23±0.09 | 0.98±0.04 | 3 |
| S(4)-Methylhistamine (5) | 7.25±0.05 | 0.97±0.03 | 3 | 6.87±0.05 | 0.97±0.02 | 4 | 6.03±0.05 | 1.00±0.03 | 3 |
| Immepip (6) | 7.64±0.12 | 0.98±0.02 | 5 | 6.85±0.17 | 0.95±0.03 | 3 | 7.17±0.06 | 0.93±0.05 | 3 |
| Immethidine (7) | 6.12±0.20 | 0.65±0.02 | 3 | 5.95±0.03 | 0.87±0.02 | 3 | 5.80±0.13 | 0.94±0.01 | 3 |
| Clopenprop (9) | 7.87±0.07 | 0.97±0.03 | 3 | 6.73±0.08 | 0.55±0.05 | 3 | 6.80±0.11 | 0.37±0.03 | 3 |
| Iodopenprop (10) | 7.30±0.14 | 0.73±0.02 | 4 | (6.66±0.03) | 0.01±0.05 | 3 | (6.49±0.11) | –0.01±0.06 | 3 |
| Proxyfan (11) | 6.93±0.06 | 0.68±0.02 | 4 | 6.10±0.07 | 0.88±0.04 | 3 | 5.67±0.13 | 0.76±0.03 | 3 |
| UR-P294 (13) | 8.74±0.11 | 0.98±0.02 | 6 | 8.29±0.18 | 0.97±0.02 | 5 | 8.16±0.03 | 1.03±0.02 | 3 |
| UR-P1376 (14) | 7.70±0.07 | 1.02±0.02 | 4 | 6.61±0.25 | 0.51±0.05 | 3 | (5.15±0.05) | 0.08±0.10 | 3 |
| VUF 8430 (15) | 7.04±0.10 | 0.97±0.04 | 3 | 6.83±0.03 | 0.96±0.02 | 3 | 6.06±0.06 | 0.98±0.02 | 3 |
| ST-1006 (16) | 8.05±0.05 | 0.91±0.01 | 3 | 7.76±0.11 | 0.37±0.04 | 4 | 6.08±0.17 | –0.55±0.12 | 3 |
| Clozapine (17) | 6.96±0.14 | 1.30±0.05 | 8 | 5.44±0.06 | 0.99±0.01 | 3 | 5.70±0.11 | 1.12±0.05 | 4 |
| VUF 5681 (18) | (6.16±0.20) | 0.09±0.00 | 3 | 5.20±0.15 | 0.42±0.02 | 3 | n.d. | – | – |
| JNU 777120 (19) | (7.81±0.19) | –0.31±0.06 | 3 | (7.58±0.13) | –0.23±0.03 | 4 | 6.21±0.10 | 0.49±0.05 | 5 |
| Thioperamide (20) | 6.92±0.10 | –0.32±0.04 | 6 | 6.52±0.13 | –0.44±0.02 | 4 | (6.89±0.14) | –0.20±0.02 | 4 |
| ST-1012 (21) | 7.26±0.05 | –0.39±0.03 | 3 | 7.49±0.09 | 0.24±0.05 | 4 | 8.12±0.08 | 0.24±0.07 | 4 |
| A 943931 (22) | 7.58±0.12 | –0.63±0.07 | 6 | n.d. | – | (6.79±0.11) | –0.06±0.00 | 6 |
| A 987306 (23) | 7.17±0.07 | –0.62±0.07 | 4 | n.d. | – | (7.85±0.13) | –0.08±0.00 | 6 |

Data are represented as mean values ± SEM of N independent experiments performed in triplicate. α: intrinsic activity, referred to histamine = 1.00; n.d.: not determined. doi:10.1371/journal.pone.0073961.t001

Cell Culture and Transfection

HEK293T cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Sigma) containing L-glutamine, 4500 mg/L glucose, 3.7 g/L NaHCO3 (Merck, Darmstadt, Germany), 110 mg/L sodium pyruvate (Serva, Heidelberg, Germany) and 10% (v/v) fetal calf serum (FCS) (Biochrom, Berlin, Germany). The HEK293T cells, stably expressing the tagged human H4 receptor (HEK293T-SF-hH4R-His6), were cultured in the above-mentioned medium supplemented with 600 μg/mL genetin (G418) (Biochrom). Cells were maintained at 37°C and 5% CO2 in a water-saturated atmosphere in 75-cm2 culture flasks (Sarstedt, Numbrecht, Germany) and diluted (1:10) twice a week with fresh medium.
HEK293T-SF-hH4R-His6 cells were stably co-transfected with plG4.29[ lucP2/CRC/ Hygro] (Promega, Mannheim, Germany) encoding hygromycin resistance (Hygro) and the firefly luciferase (lucP2), the transcription of which is controlled by the cAMP responsive element (HEK293T-SF-hH4R-His6-CRE-Luc cells). HEK293T cells were stably co-transfected with plG4.29[lucP2/CRC/ Hygro] (HEK293T-CRE-Luc cells) and pcDNA3.1(+)/SF-mH4R-His6 (HEK293T-SF-mH4R-His6-CRE-Luc) or pcDNA3.1(+)/SF-rH4R-His6 (HEK293T-SF-rH4R-His6-CRE-Luc) cells, respectively. For transfection, the cells were seeded into a 24 well-plate (Becton Dickinson, Heidelberg, Germany), so that they reached 60–70% confluency on the next day. The transfection mixture containing 0.5 μg of the DNA and each 1 μL (4:2 ratio), 1.5 μL (6:2 ratio) or 2 μL (8:2 ratio) of FuGene HD transfection reagent (Roche Diagnostics, Mannheim, Germany) was prepared according to the manufacturer’s protocol and added to the cells, followed by an incubation period of 36–48 h at 37°C and 5% CO2 in a water-saturated atmosphere. Co-transfected cells were cultured in DMEM supplemented with 10% (v/v) FCS, 600 μg/mL of G418 and 200 μg/mL of hygromycin B (A.G. Scientific, San Diego, USA).

### Table 2. Reference data of H4R ligands determined in the [35S]GTPγS binding assay at the hH4R, the mH4R and the rH4R and reported in literature.

| Ligand | hH4R | mH4R | rH4R |
|--------|------|------|------|
| Histamine (1) | 7.1–8.2, 1.5 | 5.2–7.5, 1.1 | 4.3–7.1, 1.0 |
| (R)-α-Methylhistamine (2) | 6.2–7.0 | 6.0 | 5.1 |
| (S)-α-Methylhistamine (3) | 4.9 | 1.1 | -- |
| Nα-Methylhistamine (4) | 6.1–7.4 | 0.6–1.0 | -- |
| S(4)-Methylhistamine (5) | 7.2–7.8 | 6.02 | 1.1 |
| Imipenem (6) | 7.7–7.9 | 5.27 | 5.0 |
| Imethidine (7) | 6.0 | -- | -- |
| Imetit (8) | 7.9–8.5 | 8.1 | 8.1 |
| Clobenpropit (9) | 7.7–8.3 | 6.1 | 6.3 |
| Iloprost (10) | 7.7–8.0 | 6.4 | 6.0 |
| Proxan (11) | 7.2 | -- | -- |
| U73781 (13) | 8.3–8.6 | 6.1–6.5 | 4.6–5.5 |
| U73772 (14) | 7.5–7.8 | 6.1–6.9 | 5.5–4.5 |
| VUF 8430 (15) | 7.3–8.2 | 5.1 | 4.5 |
| ST-1006 (16) | 8.9 | -- | -- |
| Clozapine (17) | 5.8–6.8 | <4 | -- |
| VUF 5681 (18) | <5 | -- | -- |
| JNJ 7777120 (19) | 7.6–7.5 | 6.1–6.7 | 6.1–6.5 |
| Thiorperamide (20) | 6.4–7.0 | 6.9–6.7 | 6.1–6.5 |
| ST-1012 (21) | 7.4 | -- | -- |
| A 943931 (22) | 8.2–7.3 | 6.2–8.0 | 0.0 |
| A 987306 (23) | 8.3–7.1 | 7.1–8.3 | 0.0 |

Reference data are taken from (unless otherwise noted, α values referred to histamine = 1.0):

- Functional [35]S-GTPγS-binding assay on SF9 cell membranes co-expressing the hH4R, mH4R or rH4R and GαS+[βγJ] (α value of ST-1012 referred to thiorperamide = -1.0, [39]);
- Steady-state [35]S binding assay on SF9 cell membranes co-expressing: hH4R-GαS+ [βγJ] ([43], hH4R-GAlp+GαS+ [βγJ] ([46], hH4R or mH4R+GαS+ [βγJ] ([46], Gαi ([23], Giαi+GαS+ [βγJ] ([47]);
- Calcium mobilization in 293-EBNA cells transiently co-expressing the hH4R, mH4R or rH4R with Gαq ([20], [46], [45], [47]);
- [35]S binding assay at the hH4R, the mH4R and the rH4R and reported in literature.

Luciferase Reporter Gene Assay

Approximately 2·10⁴ transfected cells, suspended in DMEM supplemented with 10% (v/v) FCS, were seeded per well into flat-bottomed 96-well plates (Greiner, Frickenhausen, Germany). The cells were allowed to attach for 17 h at 37°C, 5% CO2 in a water-saturated atmosphere. A stock solution (10 mM) of forskolin (Sigma) in DMSO was used to prepare feed solutions in DMEM containing 10% (v/v) FCS (final DMSO concentration in the assay was ≤1%). For experiments in the presence of a PDE inhibitor, the feed solution of forskolin contained 500 μM of IBMX (Sigma).

After addition of forskolin (0.4 μM for the cells expressing the human H4R and 1 μM for the rat and mouse H4R expressing cells) alone (to determine forskolin potency) or in combination with histaminergic ligands, the cells were incubated for 5 h. In antagonist mode, the forskolin solution was supplemented with 0.10, 0.15 or 1.00 μM of histamine as the agonist for the human, mouse and rat H4R expressing cells, respectively. Thereafter, the medium was discarded, the cells were washed once with 100 μL of phosphate buffered saline (PBS, pH 7.4) (KCl 2.7 mM; KH2PO4 1.5 mM; NaCl 137 mM; Na2HPO4 5.6 mM; NaH2PO4 1.1 mM in Millipore water; all chemicals were from Merck, Darmstadt,
Germany) and lysed in 40 μL of lysis buffer (pH 7.8) (N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine (Tricine) 25 mM (Sigma); glycerol 10% (v/v) (Merck); ethyleneglycoltetraacetic acid (EGTA) 2 mM (Sigma); MgSO₄·7H₂O, 5 mM (Merck); dithiotreitol (DTT) 1 mM (Sigma)) for 45–60 min under shaking (180 rpm). For luminescence measurement, 20 μL of lysate were transferred into a white flat-bottomed 96-well plate (Greiner) and the GENios Pro microplate reader (Tecan, Salzburg, Austria) was primed with the luciferase assay buffer (glycyl-glycine (Gly-Gly) 25 mM (Sigma); MgSO₄·7H₂O, 5 mM (Merck); dithiotreitol (DTT) 1 mM (Sigma)) for 45–60 min under shaking (180 rpm). For luminescence measurement, 20 μL of lysate were transferred into a white flat-bottomed 96-well plate (Greiner) and the GENios Pro microplate reader (Tecan, Salzburg, Austria) was primed with the luciferase assay buffer (glycyl-glycine (Gly-Gly) 25 mM; MgSO₄·7H₂O, 15 mM; KH₂PO₄, 15 mM (Merck); EGTA, 4 mM; adenosine 5′-triphosphate (ATP) disodium salt, 2 mM (Sigma); DTT 2 mM; D-luciferin potassium salt 0.2 mg/mL (Synchem, Felsberg, Germany)) [31]. Light emission was induced by the injection of 80 μL of the luciferase assay buffer into each well. Luminescence, expressed as RLUs (relative light units), was measured for 10 s. The basal luciferase activity was subtracted from each signal. EC₅₀ and IC₅₀ values were analyzed by nonlinear regression and best fitted to sigmoidal concentration-response curves with GraphPad Prism 5.04 (Graph Pad, San Diego (CA), USA). IC₅₀ values were converted to Kᵦ values using the Cheng-Prussoff equation [32]. The intrinsic activity of ligands

Figure 5. Effect of selected standard ligands on H₄R orthologs. (A) Potencies and efficacies of histamine (HA), thioperamide (THIO), UR-PI294 and JNJ 7777120 at the hH₄R, (B) the mH₄R and (C) the rH₄R (agonist mode). (D) Reversal of the HA (100–150 nM) mediated inhibition of the forskolin-stimulated luciferase activity by JNJ 7777120 at the hH₄R and the mH₄R (antagonist mode), in the luciferase reporter gene assay in HEK293T cells. Reaction mixtures contained ligands at the concentrations indicated on the abscissa to achieve saturated concentration response curves. Data points shown are the mean ± SEM of at least three independent experiments performed in triplicate. Data points connected by dashed lines reflect H₄R-independent increase in luciferase activity at high ligand concentrations. The corresponding values were therefore excluded from non-linear correlations (D).

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Figure 6. H₄R-independent cellular effects of selected ligand. Representative H₄R-independent increase in the forskolin (1 μM) stimulated luciferase activity by ciproxyfan (CIP), proxyfan (PRO), JNJ 7777120 and thioperamide (THIO) in HEK293T-CRE-Luc cells, stably expressing the CRE-controlled luciferase and devoid of the H₄R.

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was referred to the maximal response to histamine (HA), defined as $\alpha = 1$ (full agonist). Agonist potencies are given as $pEC_{50}$ values and antagonist activities were calculated as $pKB$ values. Measured RLUs were converted to percentual values referred to the span between the maximum effect induced by forskolin and the residual luciferase activity in the presence of histamine at the highest tested concentration. All data are means ± SEM of $N$ independent experiments, each performed in triplicate. For monitoring the time course of the luciferase expression, transcription was stimulated with 10 μM of forskolin, and the cells were lysed after various incubation periods. For analysis, the respective basal RLUs were subtracted from each value and plotted against the time. For Schild analysis, concentration ratios ($r$) were obtained by dividing the $EC_{50}$ concentrations of agonist in the presence of JNJ 7777120 (antagonist) by the $EC_{50}$ concentration of agonist in the absence of JNJ 777120. The log ($r - 1$) values were plotted against the corresponding log [JNJ 7777120] values according to the Schild equation [33] and analyzed by linear regression with GraphPad Prism 5.04. The $pA_2$ values were obtained from the intercept of the Schild plot with the $x$-axis.

Figure 7. Inhibition of the response to histamine and clozapine by JNJ7777120. Concentration response curves of histamine (A) and clozapine (B) alone and in the presence of JNJ777120 at increasing concentrations, determined on hH4R expressing HEK293T-CRE-Luc cells in the luciferase reporter gene assay, and corresponding Schild plots (C). The $pA_2$ values determined for JNJ 7777120 from Schild regression were 8.39 (slope: 0.83 ± 0.02) and 8.17 (slope: 0.45 ± 0.01) versus histamine and clozapine, respectively. Data points shown are the mean ± SEM of at least three (histamine) or five (clozapine) independent experiments performed in triplicate. doi:10.1371/journal.pone.0073961.g007
H2R Luciferase Assay: Distal vs. Proximal Readout

Optimization of the Assay Conditions

In order to detect a Gz-mediated inhibitory effect on the adenylyl cyclase (AC) activity, the reporter gene assay was performed in the presence of the AC stimulator forskolin. The time course of the luciferase expression upon stimulation with 10 μM forskolin is shown in Figure 2A. After a latency period of 0.5–1 h, the enzyme activity steeply increased, and a maximum was reached after 8 h. An incubation period of 5 h was sufficient to obtain 76–94% of the maximum expression. To optimize assay performance, the pEC50 value of forskolin in the respective cAMP reporter gene assay system [29] was determined (Figure 2). As the concentration-response curve shows an optimum (Figure 2B), only the ascending part of the curve was considered up to a forskolin concentration of 10 μM (Figure 2C). Interestingly, the potency of forskolin was significantly different; pEC50 values were 6.41 ± 0.05 and 5.95 ± 0.04 in the hH4R and mH4R co-transfected cells, respectively, and 5.50 ± 0.11 in the HEK293T-CRE-Luc cells (Figure 2C). Forskolin concentration-dependently increased the luciferase expression in HEK293T-SF-rH4R-His6-CRE-Luc cells, which was inhibited by histamine (1) (Figure 3A) with pEC50 values of 6.81 ± 0.11, 6.53 ± 0.04, 6.29 ± 0.07 and 5.91 ± 0.04 (Figure 3B) at forskolin concentrations of 0.5, 1.0, 2.5 and 5 μM, respectively. Therefore, a concentration of 0.4 μM forskolin was used for pre-stimulating the hH4R expressing cells, whereas 1 μM forskolin was considered optimal for AC stimulation in mH4R and rH4R expressing cells. With respect to comparability of concentration-response curves of H4R ligands at H4R orthologs, the difference between maximum forskolin concentration was significant (Figure 3B) at forskolin concentrations of 0.5, 1.0, 2.5 and 5 μM, respectively. 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Functional Activity of H4R Ligands at the Human, Mouse and Rat H4R

A set of ligands (Figure 1), generally accepted as agonists (1–17), neutral antagonists or inverse agonists (18–23) at the human H4R was selected for functional investigations. The results from the reporter gene assays performed with the H4R species orthologs are summarized in Table 1 and compared to functional data from the [3H]GTPγS binding assay and the literature in Table 2.

H4R agonists (compounds 1–17).

The endogenous agonist histamine (1) inhibited forskolin stimulated luciferase activity with pEC50 values of 7.77, 7.06 and 6.53 in the hH4R, mH4R and rH4R expressing reporter cells, respectively (Table 1). The methyl-substituted analogs of histamine (2–5) acted, with the exception of 3, as full agonists at the three H4R orthologs. Compared to the hH4R, a trend towards decreased potency was detected at the rodent receptors for compounds 1–5 (Figure 5A, B, C). Among the enantiomers 2 and 3, (S)-α-methylhistamine (2) was the eutomer at all species orthologs. Compared to immepip (6), the pyridine analog Immetnil (7) showed significantly reduced potency and intrinsic activity at the hH4R. By contrast, immetnil (7) exhibited almost full agonist activity at both, the mouse and rat H4R, with similar moderate potency compared to the hH4R. Imetit (8) exhibited almost the same potency and efficacy at the three H4R orthologs. In contrast, clofenopropr (9) and iodophenpropr (10), which can be considered as analogs of imetit (8) with an increased distance between the basic moieties and a large lipophilic group in the side chain, displayed a clear decrease in potency and maximal response at the mouse and rat H4R compared to the hH4R. Clofenopropr (9) was a potent full agonist at the hH4R and only a moderate partial agonist at the mouse and rat H4R, whereas iodophenpropr (10) acted as a partial agonist at the hH4R and a neutral agonist at both, the mouse and the rat H4R. Proxynan (11) partially activated the three H4R orthologs with significantly lower potencies on the rodent receptors. Whereas H4R-independent effects of 11 were negligible at concentrations >10 μM, the structural analog ciproxifan (12) induced a strong increase (by up to 250%) in luciferase activity at concentrations from 1 to 100 μM in HEK293-CRE-Luc cells devoid of H4R expression (Figure 6). Therefore, functional activities of 12 on H4R orthologs were not determined in the luciferase assay. The non-selective acylguanidine-type H4R agonist UR-PI294 (13) fully activated the human, mouse and rat H4R (Figure 5A, B, C), being the most potent agonist at all three H4R orthologs (Table 1). In contrast, the selective cyanoanidine-type H4R agonist UR-PI376 (14) acted as a potent full hH4R agonist, exhibited only partial agonistic activity at the mH4R and was devoid of agonism at the rH4R (Table 1). VUF 8430 (15) had about the same potency at both, the mH4R and the rH4R, whereas the potency at the rH4R was distinctly lower. At all three H4R species orthologs, VUF8430 (15) was almost as efficacious as histamine (α = 0.96–0.98). The aminopyrimidine-type compound ST-1006 (16) exhibited pronounced differences in the quality of action at the H4R orthologs with nearly full agonism at the hH4R, partial agonism at the mH4R and inverse agonism at the rH4R. The antipsychotic drug clozapine (17) exhibited only moderate agonistic potency at the hH4R. However, with an α value of 1.30, clozapine was even more efficacious than histamine (1). Furthermore, clozapine (17) fully activated both, the mouse and the rat H4R, though with low pEC50 values (Table 1).

hH4R agonists and inverse agonists (18–23).

Interestingly, VUF 5681 (18), with a spacer extended by two carbon atoms compared to the H4R agonist immepip (6), displayed no agonistic activity at the hH4R and only partial agonism at the mH4R. In the antagonist mode at the hH4R, VUF 5681 (19) inhibited the histamine-induced decrease in luciferase reporter with a pKb value of 6.16±0.20. JNJ 777120 (19) behaved as neutral antagonist at the human and mouse H4R in the luciferase reporter gene assay with comparable pKb values of 7.01±0.19 and 7.58±0.13, respectively (Figure 5A, B, D). In contrast, at the rH4R [JNJ 777120 (19) acted as a partial agonist (α = 0.49±0.05) with a pEC50 value of 8.21±0.10 (Figure 5C). By analogy with ciproxifan, but much less pronounced, JNJ 777120 (19) and thioperamide (20) produced receptor-independent increases in luciferase activity at concentrations ≥10 μM in control experiments using cells devoid of H4R expression (Figure 6). The corresponding values were therefore omitted in the construction of concentration-response curves of 19 and 20, when studied in the antagonist mode (shown for JNJ 777120 (19) in Figure 5D). Thioperamide (20) acted as an inverse agonist, achieving comparable pEC50 values at the human and mouse H4R (Figure 5A, B, Table 1), and revealed moderate antagonist activity at the rH4R with a pKb value of 6.89±0.14. The aminopyrimidine ST-1012 (21) acted as an inverse agonist at the hH4R, but revealed partial agonistic activity at the mouse and the rat H4R. The conformationally constrained aminopyrimidines A 943931 (22) and A 907306 (23) were inverse agonists at the hH4R and neutral antagonists at the rH4R.

Discussion and Conclusions

Assay Optimization

The pEC50 value of forskolin varied among the different transfectants probably due to different expression levels of the CRE-controlled luciferase. The concentration-response curve revealed a decline at forskolin concentrations higher than 10 μM. This decline of the forskolin effect became already obvious at concentrations >3.2 μM in the presence of 50 μM of the PDE inhibitor IBMX (data not shown), as already described for a CRE-directed luciferase reporter gene assay in Chinese hamster ovary cells (CHO) [37]. By analogy with a report by Kemp et al. [38] an activation of the inducible cAMP early repressor (ICER) may counteract the luciferase expression in HEK293T cells. Gαi-protein mediated inhibition of the cAMP synthesis as well as the signal-to-noise ratio was lowered by increasing concentrations of forskolin and IBMX. This was reflected by smaller relative effects and potencies of histamine (1) in the presence of increasing forskolin concentrations (Figure 3) and 50 μM of IBMX (Figure 4). Thus, high forskolin concentrations should be avoided and the altered potency of forskolin, when used in combination with IBMX, must be considered in this assay.

The co-expression of a CRE-controlled luciferase reporter gene with the human, mouse and rat H4R, respectively, in HEK293T cells enabled the functional analysis of H4R ligands. A set of 23 imidazole and non-imidazole ligands comprising agonists, inverse agonists and antagonists was investigated for ability to effect forskolin stimulated luciferase activity. The obtained pEC50 values or pKb values were compared with ligand activities from different functional assay systems reported in literature.
Off-target Effects

The luciferase stimulation becoming obvious at concentrations greater than 1 μM of JNJ7777120 (19) and thioperamide (20) in cells expressing the H4R orthologs (cf. dashed lines in the concentration-response curves of 19 and 20 in Figure 5A-C) suggest inverse agonism. However, the investigation of selected compounds on HEK293T-CRE-Luc cells lacking the H4R (cf. Figure 6) revealed H4R-independent increase in luciferase activity. This effect was most prominent in case of cipofloxacin (12), but also pronounced for 19 and 20. Therefore, off-target effects should be taken into account to avoid misinterpretation of biological responses to such compounds at concentrations ≥10 μM.

Activities at the Human H4 Receptor

Except for ST-1006 (16) [39], all determined H4R ligand activities at the hH4R were in agreement with results reported in literature [20,23,39-42]. However, a tendency toward elevated intrinsic activities was observed. Contrary to partial agonistic activity of immepp (6) and clobenpropit (9) in the [35S]GTPγS binding assay on membrane preparations of H4R-expressing SF9 cells (≥0.81 and 0.45, respectively) (Table 2), full agonism at the hH4R was determined in the luciferase assay. Iodophenpropit (10), described as a neutral antagonist [40], exerted strong partial agonistic activity at the hH4R in the present study. Partial agonistic activity was also determined for iodophenpropit (10) in a Ca2+ mobilization assay in HEK293 cells, co-transfected with the hH4R and the chimeric G-protein Goε5 [5]. ST-1006 (16) had low intrinsic activity in the [35S]GTPγS binding assay at the hH4R [39], but was an almost full agonist in the luciferase assay. The increased intrinsic activity was accompanied with a decrease in potency of about one order of magnitude. In case of clozapine (17), the maximal agonistic response surpassed that of histamine by 30%. In control experiments on HEK293T-CRE-Luc cells devoid of the H4R, clozapine (17) at concentrations as high as 100 μM caused an increase in CRE activity by up to 17% (data not shown). The effect of clozapine on hH4R-expressing cells was antagonized by JNJ 7777120 in a concentration-dependent manner, indicating that the (super)agonistic effect was receptor mediated (Figure 7). Using histamine or clozapine as H4R agonists revealed approximately the same pA2 value for JNJ 7777120 (pA2 values: 8.39 and 8.17). However, compared to the concentration response curve of histamine in the presence of JNJ 7777120 (Figure 7A), the extent of rightward shift was smaller in case of clozapine (Figure 7B), resulting in different slopes (0.83 compared to 0.45) of the corresponding Schild plots (Figure 7C). This may be taken as a hint that histamine and clozapine activate the H4R not exactly in the same way. However, due to the pleiotropic character of clozapine (17), effects mediated by targets other than the H4R must be taken into account. Most probably, increased intrinsic activities in the luciferase assay compared to more proximal readouts are caused by amplifications in signaling downstream from G-protein activation [30,37]. For instance, in functional assays on SF9 cell membranes, ST-1006 (16) [39] and clozapine (17) [43] showed only partial agonism (Table 2).

The constitutive activity of the hH4R, obvious from inverse agonism of thioperamide (20), was rather low compared to functional assays on SF9 cell membranes [23,34]. In accordance with reported data ST-1012 (21) acted as an inverse hH4R agonist in the [35S]GTPγS assay [39], and JNJ 7777120 (19) behaved as a neutral hH4R agonist [18,40]. Inverse agonism was also found for A 943931 (22) and A 987306 (23) in the luciferase assay (Table 1) and the GTPγS assay (Table 2), whereas neutral antagonism was observed in Ca2+ (FLIPR) assays [44,45].

Activities at Rodent H4 Receptors

Comparing the results from the luciferase assay on mouse and rat H4R with data from other functional assays revealed marked differences. The potencies of histamine (1), 5-(4)-methylhistamine (5), immepip (6), UR-P1294 (13), VUF 8430 (15) and clozapine (17) were significantly higher compared to the [32P]GTPase [23] and [35S]GTPγS binding assay (Table 2). By contrast, the agonist potencies of histamine (1), (R)-α-methylhistamine (2), N2-methylhistamine (4) and imetit (8) were consistent or lower compared to results from a Ca2+ assay using HEK293 cells, co-expressing the mouse or the rat H4R with Goε5 [2,46]. For example, in the luciferase assay the pEC50 values of histamine (1) were in good agreement with results from the Ca2+ assay at the mouse and rat H4R (7.23 and 6.49, respectively) [46], but distinctly higher compared to pEC50 values from the [32P]GTPase assay (5.81 and 5.25, respectively) [23]. UR-P1294 (13) achieved pEC50 values ≥8 at the hH4R, mH4R and rH4R in the luciferase assay, whereas the [32P]GTPase assay revealed dramatic differences in pEC50 values (5.52, 6.50 and 4.64, respectively) [23]. The potency of imetit (8) was lower compared to the Ca2+ assay in HEK293 cells (pEC50 values: 7.4 and 7.2 vs. 8.1 at both receptors) [20]. Whereas being full agonists in the luciferase assay, (R)-α-methylhistamine (2), N2-methylhistamine (4) and imetit (8) only reached 75–80% of the maximal Ca2+ response at the mH4R and 30–50% at the rH4R [20].

The pKB values of neutral antagonists, such as iodophenpropit (10) at the mouse and rat H4R as well as thioperamide (20) and UR-P1376 (14) at the rH4R were comparable to those determined in the [35S]GTPγS binding assay (Table 2). Mouse and rat H4R-mediated inhibition of forskolin-stimulated luciferase activity in HEK293T-CRE-Luc cells resulted in higher potencies compared to functional assays using Gα-protein activation as readout. This suggests that signal amplification or concomitant activation of different signaling pathways potentiates the inhibition of the luciferase activity. For example, the cAMP pathway may be modulated by a cross-talk with Ca2+ signaling elicited by activation of phospholipase C (PLC) [47]. Ca2+ is an inhibitor of (forkolin) stimulated and Ca2+ sensitive adenylyl cyclases type V/VI [48-50], which are endogenously expressed in HEK293T cells [51] and interact with the Gαi protein [52]. Furthermore, the relevance of this crosstalk with regard to the cAMP signaling pathway of G-protein coupled receptors (GPCRs) was demonstrated by the inhibitory effect of the activated Gαi coupled histamine H1R on the cAMP level in U373 MG cells [53] and, more importantly, by a crosstalk between the Gαi coupled M2 mACh receptor and the Gαi coupled 32-mACH receptor. In the latter case the inhibition of forskolin-stimulated cyclic AMP accumulation was facilitated at low agonist concentrations [54]. Further studies on the influence of Ca2+ are needed to clarify, whether only the rodent H4Rs are concerned, since agonist potencies at the hH4R were consistent with data from the [32P]GTPase and [35S]GTPγS binding assay (Table 2). Very recently, investigations on human eosinophils revealed a lower Ca2+ response to stimulation by histamine (1) and UR-P1376 (14) compared to the chemokine eotaxin via the CCR3 receptor [55]. This may be interpreted as a hint to minor contribution of Ca2+ signaling to the overall H4R mediated response, at least in native human cells. The presence of a range of alternative signaling pathways for the H4R in living cells was underlined recently by the Gxi independent β-arrestin recruitment of several H4R ligands [26,27].

The results for the standard antagonist JNJ 7777120 (19) at the mouse and rat H4R compared with data reported for other functional assays revealed discrepancies, too. In the luciferase assay, JNJ 7777120 (19) acted as a neutral antagonist at the mH4R,
both receptors was found in a CRE-driven \( {^{32}}P \)GTPase [23] and \( {^{35}}S \)GTP binding assay (Table 2). The \( K_D \) value at the mH4R in the luciferase assay is consistent with the \( K_D \) value in the Ca\(^{2+} \) assay [46], whereas the agonistic potency at the rH4R is about two orders of magnitude higher compared to the \( {^{32}}P \)GTase assay [23]. Discrepancies between the H4R orthologs in the different assay systems may result from differential equilibria between the active and inactive states of the H4R in the different assay systems as described recently [56]. In the luciferase assay, the constitutive activity, reflected by the inverse agonism of compounds 20–23, was considerably higher for the mH4R than for the rH4R. At the latter, JNJ 7777120 shifted the equilibrium toward the active state, becoming obvious as agonistic activity. Inversely, ST1006 (latter JNJ 7777120 shifted the equilibrium toward the active state, becoming obvious as agonistic activity. Inversely, ST1006, however, the potencies are.

This also holds for the rank order of agonists at the mouse and rat H4R (Table 1, Table 2, Figure 8B,C), however, the potencies are.

up to 100-fold higher in the luciferase assay. In vitro data with respect to translational animal studies and their clinical relevance.

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Author Contributions

Conceived and designed the experiments: UN DW DS. Analyzed the data: UN DW DS GB RS AB. Contributed reagents/materials/analysis tools: HS RS. Wrote the paper: UN DW DS AB.
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