Pharmacological characterization of the zebrafish (Danio rerio) histamine H\(_1\) receptor reveals the involvement of the second extracellular loop in the binding of histamine

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- aa  Amino acid
- AP-1  Activator Protein 1
- Ca\(^{2+}\)  Calcium ion
- COX-2  Cyclooxygenase 2
- CRE  cAMP Response Element
- DAG  Diacylglycerol
- ECL  Extracellular loop
- hH\(_1\)R  human histamine H\(_1\) receptor
- ICL  Intracellular loop
- IP\(_3\)  Inositol 1,4,5 triphosphate
- NFAT  Nuclear Factor of Activated T-cells
- NF\(\kappa\)B  Nuclear Factor kappa-light-chain-enhancer of activated B cells
- PIP\(_2\)  Phosphatidylinositol 4,5 biphosphate
- PKC  Protein Kinase type C
- PLC-\(\beta\)  Phospholipase C-\(\beta\)
- SRE  Serum Response Element
- VEGF  Vascular Endothelial Growth Factor
- WT  wild type
- zfH\(_1\)R  zebrafish histamine H\(_1\) receptor
Abstract

The zebrafish (Danio rerio) histamine H₁ receptor gene (zfH₁R) was cloned in 2007 and reported to be involved in fish locomotion. Yet, no detailed characterization of its pharmacology and signaling properties have so far been reported. In this study, we pharmacologically characterized the zfH₁R expressed in HEK-293T cells by means of [³H]-mepyramine binding and G protein signaling assays. The zfH₁R (Kᵋ 0.7 nM) displayed similar affinity for the antagonist [³H]-mepyramine as the hH₁R (Kᵋ 1.5 nM), whereas the affinity for histamine is 100-fold higher than for the human H₁R. The zfH₁R couples to Gα₉/₁₁ proteins and activates several reporter genes, i.e. NFAT, NFκB, CRE, VEGF, COX-2, SRE and AP-1, and zfH₁R-mediated signaling is prevented by the Gα₉/₁₁ inhibitor YM-254890 and the antagonist mepyramine. Molecular modeling of the zfH₁R and human H₁R shows that the binding pockets are identical, implying that variations along the ligand binding pathway could underlie the differences in histamine affinity instead. Targeting differentially charged residues in extracellular loop 2 (ECL2) using site-directed mutagenesis revealed that Arg210 is most likely involved in the binding process of histamine in zfH₁R. This study aids the understanding of the pharmacological differences between H₁R orthologs and the role of ECL2 in histamine binding and provides fundamental information for the understanding of the histaminergic system in the zebrafish.
Significance statement

The use of the zebrafish as *in vivo* model in neuroscience is growing exponentially, which asks for detailed characterization of the aminergic neurotransmitter systems in this model. This study is the first to pharmacologically characterize the zebrafish histamine H₁ receptor after expression in HEK-293T cells. Our results show a high pharmacological and functional resemblance with the human ortholog but also reveal interesting structural differences and unveils an important role of the second extracellular loop in histamine binding.
Introduction

The zebrafish *Danio rerio* has emerged as an alternative for *in vivo* animal studies, because of its easy handling and the fast-offspring production, making it suitable for a broad spectrum of *in vivo* pharmacological and toxicological assays (Goldsmith, 2004). Although the zebrafish is phylogenetically distant from mammals, ~71% of its proteome has an ortholog in humans, with a 50-90% identity (MacRae and Peterson, 2015). This level of identity between the mammals and zebrafish proteomes indicates a conservation of the function and structures of the proteins, supporting the notion that the zebrafish is a good model for drug discovery, target finding and toxicology studies (Guo, 2004; Bayés *et al.*, 2017);

The zebrafish conserves the main organs present in mammals (Kari *et al.*, 2007). Additionally, the central nervous system (CNS) of the zebrafish conserves the main brain structures and neurotransmission systems, such as the adrenergic, histaminergic, serotonergic and cannabinoid systems (Kaslin and Panula, 2001). In mammals, the histaminergic system is formed by histamine-producing neurons located in the tuberomamillary nucleus in the posterior hypothalamus (Haas and Panula, 2003), and a cluster of neurons with similar properties is present at the same brain region in the zebrafish (Sundvik and Panula, 2012; Panula *et al.*, 2022). The histaminergic system exerts its function via four G protein-coupled receptors, H1R, H2R, H3R and H4R (Panula *et al.*, 2015, 2022). Interestingly, only genes encoding the three first receptors are found in the zebrafish (Peitsaro *et al.*, 2007).

Up to now, the human H1R has therapeutically been exploited most intensively, with first- and second generation H1R antagonist being among the most used therapeutics worldwide (Nieto *et al.*, 2021). In mammalian cells, the H1 receptor (H1R) couples to the Gαq/11 protein family (Burghi *et al.*, 2021; Verweij *et al.*, 2022), whose activation is linked to the production of IP3 and DAG via the hydrolysis of the phospholipid PIP2 by membrane associated PLC-β. DAG activates PKC resulting in a variety of physiological responses. IP3 binds to specific receptors in the cell endoplasmic reticulum triggering the mobilization of Ca2+ from the intracellular reservoir. The mobilized Ca2+ will modulate the function of several proteins via PKC or Ca2+ dependent proteins. (Matsubara *et al.*, 2005b; Zappia *et al.*, 2015; Verweij *et al.*, 2022)

The zfH1R ortholog was identified and its coding sequence cloned in 2007 along with the zfH2R and zfH3R cDNAs from the zebrafish brain (Peitsaro *et al.*, 2007). The intronless zfH1R gene is similar to its mammalian counterpart, located at chromosome 8 of the zebrafish and, encodes for a 534-aa protein. The percentage of overall protein identity among the H1R of vertebrates is ~40-46% as determined by protein sequence analysis (Peitsaro *et
al., 2007). In mammals, the H₁R is expressed in CNS, smooth muscle, mast cells, cardiac tissue, and endothelium. In the CNS, the H₁R has been detected in the cerebral cortex, hippocampus, amygdala, hypothalamus, thalamus, striatum and cerebellum. The zfH₁R transcripts are expressed in similar brain regions and organs and the zfH₁R is reported to be involved in locomotive behavior, sleep-wake cycle and the response to changes in the environment (Peitsaro et al., 2007; O’Mahony et al., 2011; Parmentier et al., 2016; Sundvik et al., 2016; Chen et al., 2017; Panula et al., 2022). The zfH₁R is expressed after three hours post-fertilization in the embryo and its expression is conserved in adult fishes (Sundvik et al., 2016). Additionally, during its development zfH₁R induces the differentiation of cardiomyocytes (Zhu et al., 2020).

Despite insights in potential physiological roles of the zfH₁R, no information is currently available about the pharmacological and signaling properties of the zfH₁R. In order to develop detailed understanding of the histaminergic system in the zebrafish Danio rerio, in this study we recombinantly expressed the cloned zfH₁R in HEK-293T cells and analyzed its pharmacological profile and signaling properties of a large set of histaminergic tools by [³H]-mepyramine radioligand binding assays and a diverse set of cellular signaling assays.
Materials and Methods

Protein sequence analysis

The protein sequence similarity matrix and protein alignment were obtained from the translated DNA sequences available at the National Center for Biotechnology Information (NCBI, Genbank) with accession numbers: human (h) hrh1 NM_001098213.1, hrh2 NM_001131055.1, hrh3 NM_007232.2, hrh4 NM_021624.3; rat (r) hrh1 NM_017018.1; mouse (m) hrh1 D50095.1; chicken (ch) hrh1 XM_004944510.2; Xenopus tropicalis (xt) hrh1_XM_002944349.3 and zebrafish (zf) hrh1 NM_001042731.1. The protein sequences were aligned employing MUSCLE algorithm in Uniprot UGENE open-source software (Okonechnikov et al., 2012; Golosova et al., 2014; Rose et al., 2019). The transmembrane domains (TM) were delimited comparing the zfH1R protein sequence with the predicted hH1R TMs structures available at the GPCRdb database (PDB-3RZE).

Cell culture

HEK-293T cells were cultured in DMEM supplemented with 10% Fetal Bovine Serum, 100 U/ml penicillin and 100 μg/ml streptomycin in humid atmosphere at 37°C and 5% CO2.

Transfection of HEK-293T cells

Cells (2x10^6) were seeded in 10 cm cell culture dishes the day prior transfection. The day of transfection, cells were transfected with 5 μg DNA by the polyethyleneimine method (PEI) in a DNA:PEI ratio 1:4 (Kooistra et al., 2016). Briefly, 0.5 μg of cDNA encoding the human or zebrafish H1 receptor contained in the pcDEF3 or pcDNA3.1 plasmid, respectively, were complemented to 5 μg with empty plasmid vector and mixed with 20 μg of 25 kDa linear PEI in 500 μl of a sterile 150 mM NaCl solution. The transfection mixture was incubated at 22°C for 30 min. The medium of the cells was replaced by fresh supplemented DMEM and the transfection mixture was added dropwise to the cells and incubated for 48 h at the conditions mentioned before. For the reporter gene screening, 0.5 μg of receptor plasmid was co-transfected with 4 μg of NFAT-RE, NFκB, CRE, VEGF, COX-2, SRE and AP1 reporter genes (Bakker et al., 2001a; Casarosa et al., 2001; Gruijthuijsen et al., 2002; Maussang et al., 2006, 2009) in the conditions described previously. In selected experiments, the transfected receptor DNA was adjusted to 0.01 μg or 0.05 μg for hH1R or zfH1R, respectively, in order to achieve similar receptor levels, and co-transfected with 4 μg of NFAT reporter gene and complemented with empty vector plasmid to a total of 5 μg.
Membrane preparation

Crude cell membranes were obtained 48 h post transfection. Briefly, hH1R or zfH1R expressing HEK-293T cells were rinsed with ice-cold phosphate buffered saline (PBS), scrapped from the plastic dish and the homogenate centrifuged at ~2000xg for 10 min at 4°C. The supernatant was discarded, and the cell pellet was resuspended in 1 ml PBS and centrifuged again under the conditions described before and membranes were frozen at -20°C until further use.

Radioligand binding assays

The day of the assay, frozen membrane pellets were resuspended in cold phosphate buffer (50 mM Na2HPO4, 50 mM KH2PO4, pH 7.4 at 25°C), homogenized by sonication (40 Watt Labsonic 1510) for 3-5 s, and kept on ice until further use. Binding assays were performed by incubating the membranes with [3H]-mepyramine in absence or presence of excess non-labeled mepyramine (10 μM) to determine total and non-specific binding, respectively. For saturation assays, the concentration of [3H]-mepyramine varied from 0-15 nM. Initial screening of histaminergic ligands was performed in the presence of ~1.5 nM [3H]-mepyramine and 10 μM of ligands for the four histamine receptors. For competition binding, the membranes were incubated with increasing concentrations of non-labeled H1 receptor ligands (10⁻¹² to 10⁻³ M) and ~1.5 nM [3H]-mepyramine. Incubations for saturation assays and ligand screening were for 2 h at 25°C on a shaking table (600 rpm). Because some of the H1R antagonists are known to bind slowly to the hH1R (Bosma et al., 2016, 2018), incubations for competition assays were performed for 5 h under the same conditions. The reaction was terminated by rapid filtration through glass fiber-C plates (GF/C plates Perkin Elmer) previously soaked in a solution of 0.5% polyethyleneimine, followed by 3 washes with 50 mM Tris-HCl buffer (pH 7.4, 4°C). The plates were dried for 1 h at 50°C and scintillation liquid was added to each well. Retained radioactivity was measured by liquid scintillation counting in a Wallac Microbeta (Perking Elmer). Protein determination was performed with the Pierce BCA protein assay kit and measured by spectrophotometry using a Power Wave X340 (Biotek Instruments Inc.).

Luciferase Reporter Gene assays
The day prior to the assay, HEK-293T cells transfected with receptor and the reporter gene of interest were collected and transferred to a white flat-bottomed 384-well plate (GREINIER Bio-One) at a density of 15,000 cells per well in supplemented phenol red-free DMEM medium, culturing overnight. The day of the assay, cells were stimulated with ligands in serum- and phenol red-free DMEM at 37°C and 5% CO₂. After 6 h, 10 μl of luciferase assay reagent (LAR, 0.83 mM ATP, 0.83 mM D-luciferine, 18.7 mM MgCl₂, 0.78 μM Na₂HPO₄, 38.9 mM Tris-H₃PO₄, pH 7.8, 0.39% glycerol, 0.03% Triton X-100 and 2.6 μM dithiotreitol) was added to each well. Luminescence (0.5 s per well) was measured in a CLARIOstar plate reader (BMG LabTech) after 20 min of incubation at 37°C and 5% CO₂.

For the competitive antagonism assays, 3 μl of the different antagonists was added to each well 10 min before the stimulation with the agonist. The Gα₉/11 protein inhibitor YM-254890 (1 μM) was dissolved in supplemented phenol red-free DMEM and added 4 h after the cells were seeded in the 384-well plate to ensure proper cell membrane passage and subsequent effective G protein inhibition (Takasaki et al., 2004).

**Molecular modeling and phylogenetic analysis**

The experimentally determined structure of hH1R in complex with Doxepin (PDB-code 3RZE) was obtained from the Protein Data Bank and processed using MOE, version 2018.01, from the Chemical Computing Group. Since the 7DFL structure was not yet available at the time of this analysis, the hH1R-doxepin structure was used. As part of the ECL2 loop was missing and some atoms were missing, the hH1R-doxepin structure was modeled using MOE and the model was subsequently refined using MD simulations using as described earlier (Stoddart et al., 2018). The final MD snapshot was then used to create a homology model of zfH1R using MOE. Based earlier models of the postulated binding mode of histamine (Kooistra et al., 2013; Panula et al., 2015), histamine was placed in the snapshots and subsequently minimized, equilibrated and simulated for 100 ns for both hH1R and zfH1R.

For the phylogenetic analysis, all sequences for hH1R orthologs were obtained from the OrthoDB (Kriventseva et al., 2019). The sequences were subsequently aligned using ClustalW (Thompson et al., 1994) and manually refined using MOE. Using Seq2Logo (Thomsen and Nielsen, 2012), sequence logos were generated for the overall alignment of ECL2 as well as for the mammals, ray-finned fishes and birds.

An exploratory analysis of potential ligand entrance and egress tunnels of hH1R and zfH1R was performed using CAVER 3.0 (Chovancova et al., 2012). A CAVER analysis was performed on the protein structure of both
histamine-bound hH,R and the zfH,R homology model. Tunnels intersecting with the histamine binding pocket connected with the extracellular vestibule were selected for inspection.

**Cloning of ECL2 chimeras and mutagenesis of ECL2 residues of human and zebrafish H,Rs**

To generate the chimeric receptors and the single point mutants of hH,R extracellular loops, genes fragments were synthesized by Eurofins Genomics, Germany. The gene fragments of hH,R were flanked by the restriction sites AflII and PpuMI comprising a fragment of 292 bp from the ICL2 to the ICL3 of the receptor. The gene fragments for the chimeric receptors exchanges the ECL2 nucleotide sequence of hH,R (nucleotide position 496 to 558) for the zfH,R (nucleotide position 568 to 633) and vice versa. For the hH,R single-point mutants, the changes were performed on codons in the positions 496-498 (ATT (N) → AGG (R)); 523-525 (CGC (R) → GAG (E)); 535-537 (AAG (K) → CAG (Q)) and 553-555 (TAT (Y) → AGA (R)). The gene fragments were digested with the fast digest AflII and PpuMI restriction enzymes for 15 min at 37°C and the enzymes were subsequently inactivated at the temperatures indicated by the supplier. Following the digestion, the gene fragments were ligated using T4 ligase in a 3:1 molar ratio into the dam/dcm demethylated, digested and alkaline phosphatase-treated pUC57-hH,R. The ligation reaction was performed at 25°C for 1 h and the solution was added to chemically competent bacteria (DH5α), incubating the mixture on ice for 30 min followed by a heat shock at 42°C for 1 min and cooling on ice for 5 min. Cells were allowed to recover in Luria-Bertani (LB) broth for 1 h at 37°C with shaking (250 rpm) and plated afterwards in LB agar plates supplemented with ampicillin (100 μg/ml) incubating overnight at 37°C. The colonies obtained were picked and inoculated into LB broth with ampicillin for 24 h at 37°C. The resulting culture was centrifuged at ~3000g for 10 min (4°C) and plasmid DNA was extracted employing the GeneJet Miniprep Kit (ThermoFisher Scientific). DNA concentration was quantified by spectrophotometry (Nanodrop).

The resulting pUC57-hH,R plasmids containing the mutations and the pcDEF3-hH,R plasmid were digested with fast digest KpnI and BamHI for 15 min at 37°C and the gene fragment containing the mutation was ligated into the dephosphorylated pcDEF3-hH,R to subsequently transform bacteria. Plasmid DNA was amplified and extracted as described earlier. Since the original DNA sequence of hH,R has a KpnI restriction site in the position 401-406, a silent mutation was introduced in pUC57-hH,R (GGTACC → GATACC) to eliminate the endogenous KpnI restriction site. For the zfH,R chimera, the gene fragment synthesized was flanked by AauI and PpuMI sites, comprising a fragment of 497 bp (TM2 to the ICL3 of the receptor). The gene fragment was inserted into the
plasmid pEX-128. To facilitate digestion by PpuMI restriction enzyme, dam/dcm+ chemically competent cells (NEB) were transformed with pcDNA3.1-zfH1R or obtained pEX-128 plasmids. Both plasmids were digested with the corresponding restriction enzymes and the gene fragment was ligated into the dephosphorylated pcDNA3.1-zfH1R plasmid as described. Bacterial transformation, plasmid DNA amplification and extraction were performed as described before.

The mutagenesis of the ECL2 residues of zfH1R was performed by a modified version of the QuikChange One Site-directed mutagenesis protocol using the following primers including the mutated codon: R190454N forward_ACTATTCCTATATTGGGTTGGAACATGTTTGCTAGCGTGTATAAG and reverse_CTTATCGACGCTAGCAACATGTTCCAACCCCAATATAGGAAATGT; E200454R forward_GCTAGCGTGTATAAGAAGACGAGATTGGAAAATCAATGCGATACT and reverse_AGGGTCGACAATCGACGCTAGC; Q204454K forward_AAAGAAGACGAGTTGGAATATTCAATGCGATACTGATTTTCGCTTT and reverse_AAAGCGAAAATCAATCGACGCTAGC; R210455Y forward_AATCAATCGACGCTAGCATTATTATTTCCACTCCGCTCTTCTTTT and reverse_R210455Y forward_CACATTTTGAACCACGTCAACAAAAATCAATCGACGCTAGCATT. The PCR reactions were performed using Phusion High-Fidelity DNA polymerase (NEB). The PCR program consisted of a single 98°C denaturation for 30 s, followed by 18 cycles of denaturation at 98°C for 10 s, annealing at 65°C for 30 s and extension at 72°C for 5 min and a final extension at 72°C for 10 min. The expected PCR products were digested with DpnI for 3 h at 37°C and DH5α competent cells were transformed employing the digested PCR products under the same conditions described before. The cells were recovered with LB broth for 1 h at 37°C and seeded in LB agar plates supplemented with ampicillin at 37°C overnight. The next day, the bacterial colonies were picked and amplified in ampicillin-supplemented LB broth for 24 h at 37°C. The DNA was extracted using the Miniprep Kit from Thermo Fischer Scientific. The DNA concentration was measured by spectrophotometry. All mutations resulting from the different methods described were confirmed by DNA sequencing (Eurofins Genomics, Germany).

Materials

Phosphate Buffered Saline (PBS), Trizma-Base, Adenine Triphosphate (ATP), Triton X-100, Glycerol, dithiotreitol, promethazine, R-α-methylhistamine (RαMH), diphenhydramine, polyethyleneimine solution (50%, PEI), Na2HPO4,
KH₂PO₄ and 1x trypsin solution were purchased from Sigma Aldrich. Fetal Bovine Serum and Penicillin/Streptomycin (streptomycin 10,000 IU·mL⁻¹; penicillin and 10,000 µg·mL⁻¹; were purchased from GE, Healthcare. Dulbecco’s Modified Eagles Medium (DMEM), Phenol Red-free DMEM, GeneJet Plasmid Miniprep Kit, Fast Alkaline Phosphatase, Fast Digest restriction enzymes BamHI, KpnI, PpUMI, AflIII, AauI, BCA protein assay kit, 100x L-Glutamine solution and 100x Sodium Pyruvate solution were obtained from Thermo Fischer Scientific. Phusion High-Fidelity DNA polymerase and dam/dcm chemically competent cells, NEB bio labs. Linear 25 kDa polyethyleneimine (PEI) were from Polysciences, Warrington, PA, U.S. [³H]-mepyramine (specific activity 20 Ci/mmol) was obtained from Perkin Elmer. Thioperamide, famotidine and ciproxifan were bought from Abcam, while clozapine was from Duchefa. R-lisuride was obtained from Santa Cruz Biotechnology. 2-Pyridylethilamine, ranitidine, tiotidine, Nα-methylhistamine [NαMH], S-α-methylhistamine [SαMH], methimepip, proxyfan, impentamine, doxepin, triprolidine were purchased from Tocris. D-luciferine was bought from Promega and histamine from TCI. YM-254890 was purchased from Wako Chemicals Europe (GMBH). Mepyramine was bought from Research Biochemical International and acrivastine from BOC Sciences. Cetirizine was purchased from Biotrend. Cyclizine was bought from Toronto Research Chemicals (TRC). Dimaprit, amthamine, aminopotentidine, burimamide, immepip, imetit, VUF8621, VUF5524, iodophenpropit [IPP], VUF8430, 4-methylhistamine [4MH], JNJ7777120, VUF6002 and VUF4904 were (re)synthesized at Vrije Universiteit Amsterdam.

Data Analysis

Data were analyzed with GraphPad Prism 7.a. For the analysis of saturation and competition binding experiments, data were fitted by non-linear regression according to a one-site specific binding model. Kᵢ values were obtained using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). For the functional assays the data were fitted according to a three-parameter sigmoidal dose-response curve model. pA₂ values and slopes from Schild analysis were obtained with the linear regression function of the software plotting the dose-ratios and the logarithm of the concentration of competitors.
Results

Identification of the zebrafish histamine H₁ receptor as member of the histamine receptor subfamily

The zfH₁R is a member of the rhodopsin-like GPCR family and belongs to the histamine receptor subfamily by comparison of the amino acid sequences of the identified zebrafish protein with the sequences of the histamine H₁ receptor vertebrate orthologs and the four known human histamine receptor subtypes (Table 1, Suppl. Fig. 1). This analysis indicates that the putative zfH₁R shows the highest sequence identity (41% - 43%) with other H₁R orthologs. The zfH₁R is only distantly related (21% – 23% sequence identity) to the three other human histamine receptors (Table 1, Suppl. Fig. 1).

Within the transmembrane domains, the class of rhodopsin-like GPCRs conserves a number of amino acid motifs, that are considered to be involved in the activation and folding of the receptors (i.e. gN (TM1), LaXaD (TM2), DRX (TM3), WXaP (TM4), FX2P (TM5), WXP (TM6) and NPX₂Y (TM7)) (Isberg et al., 2015). Furthermore, the residues suggested to be involved in histamine binding to the human H₁R are W₁₀₃^3x28, D₁₀₇^3x32, T₁₁₂^3x37, K₁₉₁^5.39x40, N₁₉₈^{5.46x461}, Y₄₃₁^6x51, F₄₃₅^6x55 and Y₄₅₈^7.4₃x4₂ (Structure-based generic numbering (Isberg et al., 2015);(Ohta et al., 1994; Nonaka et al., 1998; Bruysters et al., 2004; Shimamura et al., 2011; Cordova-Sintjago et al., 2012; Xia et al., 2021). Sequence alignment of the zfH₁R and human H₁R (Suppl. fig. 1) indicates that all the GPCR motifs involved in folding and activation and the amino acids supposed to be involved in histamine binding are conserved in the zfH₁R.

Pharmacological characterization of zebrafish H₁ receptor heterologously expressed in HEK-293T cells

Saturation binding of the H₁R radioligand [³H]-mepyramine to cell homogenates of HEK-293T expressing either the human (h) or zfH₁Rs revealed comparable equilibrium dissociation constants (Kᵢ) 1.5 ± 0.4 nM and 0.7 ± 0.1 nM for the saturable binding of [³H]-mepyramine to the human and zebrafish H₁Rs, respectively. The maximum number of binding sites (B_max) was significantly higher (p<0.05 Student t test) for the hH₁R (11.5 ± 2.8 pmol/mg protein) compared to the zfH₁R (4.6 ± 1.5 pmol/mg of protein) under similar transfection conditions (figure 1A).

To pharmacologically characterize the zfH₁R, a screening with 55 well-known reference ligands of the four human histamine receptors was performed. The screening showed that the zfH₁R is able to bind all the standard H₁R ligands (Suppl. figure 2). Interestingly, histamine (10 µM) was able to displace 78% of [³H]-mepyramine (1.5 nM)
bound to the zfH₁R. In contrast the same concentration histamine only reduced [³H]-mepyramine to the hH₁R by 22%. Furthermore, at 10 µM some H₂R and H₃R/H₄R ligands (H₂R, dimaprit, impromidine, and zolantidine; H₃R/H₄R, immepip, methimepip, iodophenpropit, clobenpropit, A-331440, A-349821, Pitolisant, ABT-239 and JNJ7777120), were also able to displace more than 50% of the specific [³H]-mepyramine binding to both receptors. On average, the inhibition profiles of the H₂-H₄ compounds were similar for both receptors. Next, the inhibition constants (pKᵢ) were obtained for histamine and seven selective H₁R compounds (Table 2, figure 1B-1E). The zfH₁R affinities for histamine and 2-PEA were significantly higher (~100-fold and ~40-fold, respectively, p<0.05 Student t test, Table 2, figure 1B and 1C) than those of the hH₁R. Interestingly, the affinities for the H₁R antagonists fexofenadine, mepyramine, cetirizine (figure 1E) and cyclizine were similar (pKᵢ ~7-9, Table 2) between the hH₁R and the zfH₁R. However, the affinity for acrivastine for the zfH₁R was ~6-fold lower compared to the hH₁R (p<0.05 Student t test, figure 1D). The correlation of the pKᵢ values from the zfH₁R and hH₁R (figure 1F) showed an R² value of 0.95 for the tested antagonists. Yet, the observed large differences in the affinities calculated for the agonists suggests important differences in the binding sites of the hH₁R and the zfH₁R.

Ligand-induced signaling of the zebrafish H₁ receptor

Mammalian H₁Rs signal via Gq/11 proteins and subsequently activate several transcription factors upon agonist activation (Bakker, Stefan B.J. Schoonus, et al., 2001; Matsubara et al., 2005a; Burghi et al., 2021; Verweij et al., 2022) To analyze if the zfH₁R was also able to activate specific signaling routes, HEK-293T cells were co-transfected with either the hH₁R or zfH₁R and different reporter genes, in which NFAT, NFκB, CRE, VEGF, COX-2, SRE and AP-1 response elements are linked to firefly luciferase as reporter protein. In our initial experiments, co-transfected cells were stimulated with histamine (10 µM). Using the reporter gene readout, stimulation of the zfH₁R with histamine led to activation of NFAT, NFκB, CRE, COX-2, SRE and AP-1 reporter genes (figure 2A). Interestingly, the zfH₁R-mediated activation of these reporter genes was generally lower compared to the hH₁R. Especially, CRE activation by zfH₁R was less pronounced than the hH₁R-mediated activation of this transcription factor. We observed a modest stimulation of the VEGF reporter gene after hH₁R activation, but this response was not detected for the zfH₁R under the chosen experimental conditions (Fig. 2A). It is in this respect important to mention that the expression levels of the hH₁R were 2.5-fold higher than for the zfH₁R. To continue with the characterization of the zfH₁R, the NFAT-RE reporter gene was employed as readout of activation of the hH₁R and
zfH₁R. Pre-treatment of HEK-293T cells with the Gαq/11 protein inhibitor YM-254890 (1 μM) (Takasaki et al., 2004), or the H₁R antagonist mepyramine completely abolished the histamine-induced activation of NFAT by both the hH₁R and zfH₁R (Fig. 2B). These results indicate that the histamine-stimulated activation of NFAT is mediated by a mepyramine-sensitive zfH₁R and the consequent activation of Gαq/11 proteins.

Functional characterization of histamine H₁ receptor ligands

In order to functionally profile agonists and antagonists at the hH₁R and zfH₁R, the expression levels of the receptors were titrated to 390 and 690 fmol/mg of protein for the hH₁R and zfH₁R, respectively (Supl. figure 3, no statistical difference, two-way ANOVA, Tukey’s multiple comparison test P>0.05), as such expression levels are within the normal physiological expression range (Hill et al., 1978). Concentration-response curves of histamine and 2-PEA were subsequently measured for the activation of the NFAT-luciferase reporter gene. At both the hH₁R and the zfH₁R both agonists increased the activity of NFAT in a concentration-dependent manner with no differences in the intrinsic activities (α) (Table 2). The synthetic agonist 2-PEA was at both receptors slightly less effective (α = 0.8) compared to histamine. The potencies (pEC₅₀) of histamine and 2-PEA were ~8- to 12-fold higher for the zfH₁R compared to the hH₁R (p<0.05 Student t test, figure 3A and 3B, Table 2). This finding is in accordance with the observed higher affinity for histamine and 2-PEA at the zfH₁R. Remarkable, the pEC₅₀ values of the 2 agonists almost match their affinities at the zfH₁R, whereas at the hH₁R a clear receptor reserve is detected for both agonist responses (Table 2).

To evaluate the functional effect of H₁R antagonists on histamine-induced NFAT activity at zfH₁R and hH₁R, competitive antagonism assays were performed (figure 3C, 3D and 3E, Suppl. figure 4, Table 2). The H₁R antagonists fexofenadine, mepyramine, acrivastine and cyclizine induced a rightward shift of the histamine concentration-response curve for the zfH₁R, while cetirizine presented insurmountable antagonism as shown by a reduction in the Eₘₐₓ value for histamine (Suppl. figure 4). Acrivastine is known to present insurmountable antagonism at the hH₁R (Bosma, Witt, et al., 2017); however, this effect was not observed for the zfH₁R since the Eₘₐₓ of each curve reached the same level as the control condition. Interestingly, cyclizine and especially acrivastine increased the Eₘₐₓ of histamine at the zfH₁R at higher concentrations in a concentration-dependent manner. Schild analysis of the antagonist effect yielded pA₂ values similar to the pKᵢ values of the antagonists at the zfH₁R and
slope values close to the unit (Table 2). These results indicate that the H$_1$R antagonists compete with histamine for the orthosteric site of the zfH$_1$R, and conserve the antagonistic profile observed at the hH$_1$R. However, the change from insurmountable to surmountable antagonism between the hH$_1$R and zfH$_1$R for several antagonists suggests differences in the binding kinetic of these antagonists, since slow binding kinetics has previously been linked to unsurmountable antagonists (Bosma, Mocking, et al., 2017).

Molecular modeling of histamine binding to the zebrafish H$_1$ receptor

As mentioned before, histamine shows a 100-fold higher affinity for the zfH$_1$R than for the hH$_1$R (Table 2). However, the alignment of the protein sequences of the hH$_1$R and zfH$_1$R (figure 1 and 4) indicates that the residues involved in the binding of histamine W103$^{3x28}$, D107$^{3x32}$, T112$^{7x37}$, K191$^{5.39x40}$, N198$^{5.46x461}$, Y431$^{6x51}$, F435$^{6x55}$ and Y458$^{7.43x42}$ are conserved in both receptors (Ohta et al., 1994; Nonaka et al., 1998; Bruysters et al., 2004; Shimamura et al., 2011; Cordova-Sintjago et al., 2012; Xia et al., 2021). It is thus possible that the 3D structure of the binding pocket differs despite having all the binding partners present. To analyze the conformational behaviour of the binding pocket of the zfH$_1$R, molecular dynamics (MD) simulations were performed with a homology model of the zfH$_1$R based on the crystal structure of the hH$_1$R and the hH$_1$R crystal structure itself. During these MD simulations, the binding of histamine to the zfH1R and the hH$_1$R binding site was (almost) identical and the interactions of histamine with the binding site residues were conserved.

The molecular dynamics studies revealed no substantial differences between the two binding pockets, suggesting that the increased affinity of histamine for the zfH1R likely stems from another source. Alternatively, this discrepancy in histamine affinity might be attributed to energetic barriers along the ligand entrance and egress pathways (Dror et al., 2011) Notably, the second extracellular loop (ECL2) has been reported to be a pivotal component in governing ligand binding to GPCRs. The ECL2 of class A GPCRs forms a disulphide bond between the conserved Cys$^{45x50}$ present in the loop and Cys$^{3x25}$ from TM3, creating a ‘lid’ over the binding pocket and presumably regulating the entry of the ligands to the orthosteric site of the receptor or directly participating in the binding of ligands in the orthosteric pocket (Karnik et al., 2003; Lim et al., 2008; Unal et al., 2010). The ECL2 of the zfH$_1$R also contains the C205$^{45x50}$ residue and the zfH$_1$R model shows that this amino acid residue probably interacts with C124$^{3x25}$ also creating a ‘lid’ on top of the binding pocket (figure 5). In addition, an exploratory
analysis using CAVER (Chovancova et al., 2012) shows tunnels that could be potential ligand binding and unbinding pathways (Supplementary Figure S5), all of which are passing ECL2 at one point.

Comparing the protein sequences of the ECL2 from the zfH₁R and hH₁R, the ECL2 displays some important differences in amino acid composition between the different vertebrate classes (figure 5). While the zfH₁R possesses E200, Q204 and R210, the corresponding hH₁R residues are R175, K179 and Y185. The extracellular 3D view of the receptors (figure 5) shows a quite different charge distribution in ECL2 of both H₁Rs. Interestingly, along the Ray-finned fishes, the E45x45 and R45x55 residues are fully conserved in 48 species analyzed (figure 5). For mammals and birds, the residue in the position 45x45 is much more variable than K45x49, indicative that this residue has a species-specific function in ray-finned fishes. At position 45x55 there is a low overall conservation, but a high clade conservation: tyrosine (99%) for mammals, arginine (100%) for ray-finned fishes, and serine (82%) for birds. This suggests that the residue at position 45x55 has an important function in all H₁ receptors as well as a species-specific task.

The zfH₁R shows a more negative environment before C205 and a more positively charged region after the conserved Cys residue, whereas the hH₁R presents a more positive charged region before C180 and a neutral region after this residue. Based on these data, coupled with the knowledge that histamine carries a positive charge under physiological conditions, we hypothesize that these variations in ECL2 might be the determining factor for the discrepancies in histamine binding.

Pharmacological characterization of zebrafish H₁ receptor ECL2 mutants

To analyze the role of ECL2 in histamine binding, first two reciprocal ECL2 chimeras were constructed and expressed in HEK-293T cells. Saturation [³H]-mepyramine binding showed that both chimeric receptors were properly expressed and bound the radioligand with high affinity (Table 3). The zfH₁R-hECL2 chimera exhibited an increase in Bₘₐₓ (~ 2-fold) and a slight increase in Kᵢ (~ 0.3 nM) compared to the WT receptor, while the hH₁R-zfECL2 chimera showed the opposite effect, with a reduction in Bₘₐₓ (~ 3-fold) and a ~50% decrease in Kᵢ compared to the WT receptor (Table 3). Displacement of [³H]-mepyramine binding to the ECL2 chimeric receptors by histamine revealed a shift in the pKᵢ value of histamine, either 15-fold increase or a 25-fold reduction for the hH₁R-zfECL2 and zfH₁R-hECL2, respectively (figure 6A and B, Table 3). These data corroborate the hypothesis that the high affinity for histamine for the zfH₁R is indeed due to involvement of the ECL2.
Subsequently, we generated single point mutations to exchange the E200\textsuperscript{45x45}, Q204\textsuperscript{45x49} and R210\textsuperscript{45x55} residues in the ECL2 of the zfH\textsubscript{1}R to the corresponding residues R175\textsuperscript{45x45}, K179\textsuperscript{45x49} and Y185\textsuperscript{45x55} in the hH\textsubscript{1}R and vice versa (Table 3). Upon expression in HEK-293T cells all mutant receptors were again analyzed by \textsuperscript{3}H-mepyramine binding studies. The hH\textsubscript{1}R R45x45E and Q45x49K mutants showed similar B\textsubscript{max} but reduced K\textsubscript{D}-values for \textsuperscript{3}H-mepyramine binding when compared to the WT receptor. For the zfH\textsubscript{1}R, the E200\textsuperscript{45x45}R and Q210\textsuperscript{45x55}K mutants exhibited B\textsubscript{max}- and K\textsubscript{D} values values for \textsuperscript{3}H-mepyramine binding similar to the WT receptor (Table 3). The hH\textsubscript{1}R Y185\textsuperscript{45x55}R mutant increased the B\textsubscript{max}- value and displayed a similar K\textsubscript{D} value for \textsuperscript{3}H-mepyramine binding as the hH\textsubscript{1}R WT (Table 3). The zfH\textsubscript{1}R R210\textsuperscript{45x55}Y mutant exhibited a modest decrease in B\textsubscript{max}-value and a minor increase in the K\textsubscript{D} value for \textsuperscript{3}H-mepyramine binding (Table 3). These results indicate that the mutants are all properly expressed and bind \textsuperscript{3}H-mepyramine with high affinity.

Next, we performed \textsuperscript{3}H-mepyramine competition binding studies with the agonist histamine (Table 3). The zfH\textsubscript{1}R E200\textsuperscript{45x45}R mutant showed a modest decrease of ~ 4-fold in the affinity for histamine compared to the WT receptor, whereas the hH\textsubscript{1}R R175\textsuperscript{45x45}E mutant showed no significant effect on the affinity of histamine. There was also no change in affinity of histamine for the zfH\textsubscript{1}R Q204\textsuperscript{45x49}K mutant and the hH\textsubscript{1}R K179\textsuperscript{45x49}Q only showed a small (~3-fold) and non-significant increase in the affinity for histamine. Finally, the zfH\textsubscript{1}R R210\textsuperscript{45x55}Y mutant displayed a ~30-fold decrease in the affinity for histamine, resulting in a pK\textsubscript{i} value similar to that of the hH\textsubscript{1}R and zfH\textsubscript{1}R ECL2 chimera. Yet, the reciprocal hH\textsubscript{1}R Y185\textsuperscript{45x54}R mutant showed a modest decrease (~4-fold) in its affinity for histamine compared with the WT receptor.
Discussion

Following the cloning and expression analysis of the histamine H₁ receptor in the zebrafish (Peitsaro et al., 2007), this study investigated the pharmacological properties of this H₁R ortholog. Despite the moderate sequence similarity (41-43%) with other H₁R orthologs, the zfH₁R contains all the conserved GPCR motifs and all key residues proposed to be involved in histamine binding (Leurs et al., 1994; Ohta et al., 1994; Nonaka et al., 1998; Bruysters et al., 2004; Bruysters, Jongejan, Gillard, Manakker, et al., 2005; Bruysters, Jongejan, Gillard, van de Manakker, et al., 2005; Shimamura et al., 2011; Cordova-Sintjago et al., 2012; Xia et al., 2021).

To study the pharmacology of the zfH₁R, the human and zebrafish H₁Rs were expressed in HEK-293T cells and compared in radioligand binding studies. The observed Kᵩ values of the H₁R radioligand [³H]-mepyramine for the zfH₁R and hH₁R, and its B_max values are within the range reported for hH₁R and H₁R orthologs (Strasser et al., 2013; Bosma et al., 2016). Although the main residues for histamine binding are conserved between zfH₁R and hH₁R, striking differences are observed for the affinities of the agonists histamine and 2-PEA. The zfH₁R shows higher affinities for histamine (~100-fold) and 2-PEA (~40-fold) compared with the values obtained for the hH₁R, suggesting differences in the agonist binding pocket of zfH₁R. For the tested H₁R antagonists no major differences in affinity for the zfH₁R and hH₁R were observed, except for acrivastine, which shows a lower affinity for the zfH₁R.

Upon histamine binding, the H₁R-activated Gαq/11 proteins lead to signaling cascades stimulating several reporter genes, based on transcription of NFAT, NFκB, CRE, VEGF, COX-2, SRE and AP-1 (Boss et al., 1998; Bakker, Stefan B. J. Schoonus, et al., 2001; Roumestan et al., 2008; Notcovich et al., 2009; Kordulewska et al., 2017; Laakkonen et al., 2017). The zebrafish also expresses these proteins (ZFIN database: accession date July 2019; (Howe et al., 2013) and our reporter gene assays showed that all but the VEGF-based transcription are activated upon histamine stimulation of zfH₁R. Furthermore, histamine-induced NFAT activation by the zfH₁R was blocked by an H₁R antagonist and a Gαq/11 protein inhibitor.

In the functional NFAT reporter gene assay histamine and 2-PEA showed ~10-fold higher potencies in HEK-293T cells expressing the zfH₁R compared to cells expressing the hH₁R. Interestingly, a comparison of the pEC₅₀ and the pKᵩ values of histamine indicates that the hH₁R requires a ~30-fold lower agonist concentration to produce 50% of response in comparison to 50% of hH₁R occupation. For the zfH₁R, the pEC₅₀ of histamine in only ~2.5-fold leftward shifted compared to its pKᵩ value. Such so-called receptor reserve, i.e. only a fraction of the receptor
population needs to be occupied for full response, has been reported before for the hH1R in both native and recombinant systems (Christophe et al., 2003; Bosma et al., 2016) and indicates an intrinsic more efficient signaling via hH1R.

Functional analysis of the H1R antagonists also revealed some interesting differences. Schild analysis was used to determine surmountable or insurmountable antagonism (Kenakin et al., 2006). Currently, insurmountable antagonism is a hot topic in the field of antihistamines due to the clinical implications of this phenomenon (Copeland, 2021; Ren et al., 2022). Our data show that fexofenadine, mepyramine, acrivastine and cetirizine displayed a different degree of insurmountable antagonism of the hH1R responses. Surprisingly, the insurmountable effect of these antagonists was markedly reduced at the zfH1R to the extent of behaving as surmountable antagonists. Slow binding kinetic leading to a long residence time (RT, 1/K_{off}) of ligands, is known to show up as insurmountable antagonism. The RT of fexofenadine and cetirizine at hH1R have been reported to be over 60 min (Chen, 2008; Bosma et al., 2018). In our analysis only cetirizine displayed an insurmountable blockade of the histamine action at both receptors, suggesting that its RT is similarly long for both receptors. Interestingly, fexofenadine and mepyramine did not show insurmountable antagonism of histamine activation of the zfH1R, whereas insurmountable antagonism was observed for the hH1R. This difference suggests a change in the RT of fexofenadine and mepyramine for the zfH1R without markedly affecting their pK_{i} values, like previously e.g. also was described for the muscarinic M3 receptor (Kruse et al., 2012).

Since these data suggest differences in the binding pocket of the zfH1R compared to the hH1R, the zfH1R binding pocket was investigated. Computational models, structural data and mutagenesis studies on the hH1R binding pocket have reported seven residues (D1073x32, K17945x49, K1913x39x40, H4507x35x34, W1584.56x57, F4326x52 and F4346x54) potentially involved in H1R antagonist binding (Wieland et al., 1999; Kiss et al., 2004; Shimamura et al., 2011; Shiroishi and Kobayashi, 2016; Xia et al., 2021). All these residues, but K17945x49, are conserved in the zfH1R. The residue K17945x49 in ECL2 is highly conserved between the vertebrate classes including the ray-finned fishes, but not in the zebrafish, which possess a Gln residue at this position. Our radioligand binding studies show that the affinity of the zfH1R and hH1R for cetirizine is identical (pK_{i} 8.1), indicating that, this particular residue, is not involved in cetirizine binding. However, acrivastine appears to interact with K17945x49 forming a salt bridge between the carboxyl group of acrivastine and the amine group of Lys179 (Wieland et al., 1999; Shimamura et al., 2011; Akimoto et al., 2021). The present study shows that the affinity of acrivastine is ~10-fold reduced for the
zfH₁R compared to the hH₁R, indicating that the H₁R affinity of acrivastine is partly determined by the presence of a positive charge in the ECL2.

Since all the residues involved in histamine binding are conserved between the zfH₁R and hH₁R (Figure 4), a determinant of the high affinity of histamine for zfH₁R could also be ECL2. Several studies have addressed the role of ECL2 in ligand binding to GPCRs, including the H₁R and H₁R (Lim et al., 2008; Shimamura et al., 2011). Structurally, the GPCR classes A, B₁, B₂ and C conserve a disulphide bridge between two cysteines found in the ECL2 and TM3, as confirmed by crystal structures of several GPCRs including the H₁R (Shiroishi and Kobayashi, 2016; Woolley and Conner, 2017). This disulphide bridge restricts the flexibility of the loop keeping it close to the extracellular part of the binding pocket and in several cases also determining ligand binding and selectivity (Audoly and Breyer, 1997; Cao et al., 2018; Clement et al., 2018).

Our data obtained with chimeric receptors following the exchange of ECL2 demonstrate an important role of ECL2 for the high affinity of histamine for the zfH₁R. Furthermore, the point mutants E200R and Q204K at the zfH₁R, changing the electrostatics of the ECL2, do not substantially affect the affinity of histamine, whereas the mutation R210Y markedly reduced the affinity of histamine (30-fold) to levels similar to the hH₁R WT and the zfH₁R-hECL2 chimera. Remarkably, the reciprocal mutant of hH₁R did not show the opposing effect. Sequence alignment of H₁R orthologs from 235 different species in the OrthoDB (Kriventseva et al., 2019) show that solely the residue at position 45x55 is conserved in a species class (i.e. mammals, birds or ray-finned fishes), but differs between the classes. This could indicate a species class-specific role for the residue at this position.

An aromatic cluster (Phe-Phe, Phe-Tyr, Tyr-Phe or Tyr-Tyr) within ECL2 has been reported to form part of the binding pocket of some GPCRs, including the hH₁R (Scarselli et al., 2007; de Graaf et al., 2008; Lim et al., 2008), establishing lipophilic interactions with various residues within the receptors. Noteworthy, the charge of the amino acid residues found after Cys⁴⁵x⁵⁰ is conserved except for the last three residues of the ECL2. Within this region of the loop, the aromatic cluster is not present in the WT zfH₁R; however, the R210⁴⁵x⁵⁵Y mutation restores this cluster and results in decreased affinity for histamine. Surprisingly, the reciprocal hH₁R Y45x55R mutation does not increase the histamine affinity, but modestly reduced binding of histamine. Examining the amino acids in close proximity to this cluster identifies an D186⁴⁵x⁵⁶ next to the aromatic residues in hH₁R WT. We speculate that upon exchanging the hydrophobic Tyr for the positively charged Arg in the hH₁R Y185⁴⁵x⁵⁵R mutant a salt bridge is formed between these residues, sterically hindering the binding of histamine.
In conclusion, the zfH1R shares ~40% of homology with the hH1R and [3H]mepyramine radioligand binding studies establish an H1R-like pharmacological profile using a large and diverse set of H1R-H4R agonists and antagonists. In signaling studies the zfH1R also closely resembles the hH1R and couples to a wide array of signal transduction pathways, most likely via activation of Goq/11 proteins. While conserving within the transmembrane domains the main amino acid residues involved in histamine binding, striking differences are observed regarding the affinity of agonists, like histamine for the zfH1R and hH1R. The use of chimeric H1Rs with swapped ECL2 loops between human and zebrafish H1Rs resulted in the observation that the ECL2 loop determines the high affinity of histamine for the zfH1R. Introducing single point mutations allowed the final identification of residue R210 in the zfH1R being responsible for the 100-fold increase in agonist affinity.
Data Availability Statement

The authors declare that all the data supporting the findings of this study are contained within the paper.
Authorship contributions

Research design. McNaught-Flores, Kooistra, Leurs, Panula, Chen

Conducted experiments: McNaught-Flores, Kooistra, Chen

Performed data analysis: McNaught-Flores, Kooistra, Leurs, Panula, Chen, Arias-Montaño

Wrote or contributed with the writing of manuscript: McNaught-Flores, Kooistra, Leurs, Panula, Arias-Montaño
References


Cheng Y, and Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* **22**:3099–108.


receptor, COX-2, NF-κB, CCR1, chemokine CCL5/RANTES and interleukin-1β in PBMC allergic and non-allergic patients. Immunobiology 222:571–581.


O’Mahony L, Akdis M, and Akdis CA (2011) Regulation of the immune response and inflammation by histamine and histamine receptors.


Footnotes

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**Figure legends**

**Figure 1.** Pharmacological characterization of zebrafish and human H1 receptors after heterologous expression of zebrafish H1 receptors in HEK-293T cells. **A** Representative graph of specific [3H]-mepyramine saturation binding to cell homogenates expressing human (black circles) or zebrafish (red squares) H1Rs. Specific binding (SB) was obtained from subtracting the total and non-specific [3H]-mepyramine binding. **B, C, D and E.** Competition binding of histamine, 2-pyridylethilamine (2-PEA), acrivastine and cetirizine respectively, displacing ~1.5 nM of [3H]-mepyramine. **F** Correlation of pKᵢ values from zebrafish and human H1R. The dotted line represents a correlation of 1. In blue are shown the H1R agonists and in green the H1R antagonists. Values shown as means ± SD of 3 independent experiments.

**Figure 2.** Histamine-induced signaling of human and zebrafish H1 receptors. HEK-293T cells were transfected with human or zebrafish H1R and luciferase-based reporter genes and stimulated with histamine (10 µM for 6 h at 37°C). Results were normalized as fold-stimulation over basal levels. **A** Activity of the reporter genes in histamine-stimulated cells expressing human or zebrafish H1 receptors. Means of 3 experiments **B** Inhibition of receptor-mediated or Gαq/11 protein signaling in zebrafish H1 receptors by YM-254890 (1 µM) or mepyramine (10 µM). Means ± SD of 3 experiments. a,b = P < 0.0001 for comparison of YM-254890 or mepyramine treatment compared to control (maximal histamine response) following Two-way ANOVA and Tukey’s multicomparison test.

NFAT, Nuclear Factor of Activated T cells; NFκB, Nuclear Factor kappa-light-chain-enhancer of activated B cells; CRE, cAMP Response Element; VEGF, Vascular Endothelial Growth Factor; COX-2, Cyclooxygenase-2; SRE, Serum Response Element; API, Adaptor Protein 1.

**Figure 3.** Functional characterization of histamine H1 receptor ligands. HEK-293T cells expressing the human and zebrafish H1Rs and NFAT reporter genes were incubated with different concentrations of H1R agonists (10⁻¹¹ – 10⁻⁴ M) or H1R antagonist mepyramine in presence of histamine (10⁻¹⁰ – 10⁻³ M) for 6 h at 37°C. Luminescence was measured 20 min after addition of Luciferase Assay Reagent in a Victor³ plate reader. **A and B** H1R agonists-induced NFAT activity in human and zebrafish H1 receptors. **C, D and E** Competitive antagonism of mepyramine on histamine-induced NFAT activation in human and zebrafish H1 receptors. Receptor expression
levels were optimized to $\sim 690$ and $390$ fmol/mg protein for hH$R$ and zfH$R$ respectively. Representative graphs of at least 3 independent experiments. **A and B** Values were normalized against maximal response of histamine or **C, D and E** folds over the basal (FOB). Black and red squares represent hH$R$ and zfH$R$ activity respectively. All values are shown as mean ± SD of 3 independent experiments.

**Figure 4.** The orthosteric binding site of the human and the zebrafish histamine H$_1$ receptors are identical. The experimentally determined binding modes of histamine (Kooistra *et al.*, 2013; Panula *et al.*, 2015) (orange) and of antihistamine Doxepin (Shimamura *et al.*, 2011) (magenta) are shown based on the cryo-EM hH1R-Histamine complex (PDB-code 7DFL). The postulated binding mode of histamine (Kooistra *et al.*, 2013; Panula *et al.*, 2015) that was used for the modeling and MD simulations is shown in transparent yellow sticks. All residues within 4.5Å of histamine and Doxepin are shown in blue and green respectively in the structure ($Y^{333}$, $I^{340}$ and $F^{644}$ not shown for clarity purposes) and the highlighted in the alignment.

**Figure 5.** The structures of human and zebrafish H$_1$ receptors with the second extracellular loop (ECL2) and mutated positions highlighted together with an orthologue sequence analysis across 235 species. The ECL2 region swapped in the chimeras is shown in gold with the Cα atom of the three mutated residue positions highlighted with spheres (blue = basic residue, red = acidic residue, orange = neutral residue). The human histamine receptor in complex with histamine (Shimamura *et al.*, 2011) in orange sticks is shown on the left and in the middle and on the right side the zebrafish homology model is shown. On the bottom an alignment of human and zebrafish H$_1$R sequences for ECL2 is shown together with the tips of the TM4 and TM5. The sequence logos (Thomsen and Nielsen, 2012) show what the conservation of this region is across all 235 species, for the mammals, the ray-finned fishes and the birds. This analysis highlights that the residue at position 45x55 is not conserved across the species but is conserved for each individual species class (i.e. mammals, birds, and ray-finned fishes).

**Figure 6.** Displacement of [${}^3$H]-mepyramine by histamine in the **A** human and **B** zebrafish H$_1$ receptor WT and ECL2 chimeras. Representative graphs of 4 independent experiments. Values are expressed as percentage of specific binding (\%SB). In black is shown the wild type receptor and in red the ECL2 chimera. WT, wild type. Mean ± SD
Tables

**Table 1.** Protein sequence similarity of zfH₁ receptors as members of the histamine G protein coupled H₁ receptor subfamily. The DNA sequences of human (h), mouse (m), rat (r), chicken (ch), *Xenopus tropicalis* (xt) and zebrafish (zf) histamine receptors were aligned by Muscle algorithm by Unipro UGENE software. Protein sequences were obtained from translation of the DNA sequences available in Genbank. The protein sequence similarity of H₁ receptors orthologs is given as percentage of identity.

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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>zfH₁R</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>41</td>
<td>43</td>
<td>100</td>
<td></td>
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</tr>
<tr>
<td>hH₂R</td>
<td>24</td>
<td>23</td>
<td>23</td>
<td>23</td>
<td>23</td>
<td>23</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hH₃R</td>
<td>22</td>
<td>23</td>
<td>23</td>
<td>21</td>
<td>24</td>
<td>21</td>
<td>21</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>hH₄R</td>
<td>22</td>
<td>22</td>
<td>22</td>
<td>23</td>
<td>23</td>
<td>21</td>
<td>19</td>
<td>38</td>
<td>100</td>
</tr>
</tbody>
</table>
Table 2. pKᵢ, pEC₅₀ and pA₂ values of H₁R ligands for human and zebrafish H₁ receptors

<table>
<thead>
<tr>
<th></th>
<th>hH₁R</th>
<th></th>
<th>zfH₁R</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pKᵢ</td>
<td>pEC₅₀ / pA₂</td>
<td>α / Schild slope</td>
<td>pKᵢ</td>
</tr>
<tr>
<td>Histamine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.7 ± 0.1 *</td>
<td>6.2 ± 0.2 b</td>
<td>1</td>
<td>6.7 ± 0.1 *</td>
<td>7.1 ± 0.1</td>
</tr>
<tr>
<td>2-pyridyl ethylamine (2-PEA)</td>
<td>4.2 ± 0.5 a</td>
<td>5.1 ± 0.2 b</td>
<td>0.8 ± 0.0</td>
<td>5.8 ± 0.2 a</td>
</tr>
<tr>
<td>Fexofenadine*</td>
<td>7.6 ± 0.1</td>
<td>7.8 ± 0.4</td>
<td>0.8 ± 0.2</td>
<td>7.1 ± 0.1</td>
</tr>
<tr>
<td>Mepyramine</td>
<td>9.0 ± 0.1</td>
<td>9.4 ± 0.3</td>
<td>0.8 ± 0.1</td>
<td>9.3 ± 0.1</td>
</tr>
<tr>
<td>Acrivastine</td>
<td>7.6 ± 0.0 a</td>
<td>8.2 ± 0.2</td>
<td>0.7 ± 0.0</td>
<td>6.8 ± 0.1 a</td>
</tr>
<tr>
<td>Cetirizine</td>
<td>8.1 ± 0.1</td>
<td>Insurmountable</td>
<td>Insurmountable</td>
<td>8.1 ± 0.1</td>
</tr>
<tr>
<td>Cyclizine</td>
<td>8.4 ± 0.2</td>
<td>8.1 ± 0.2</td>
<td>0.9 ± 0.1</td>
<td>8.8 ± 0.3</td>
</tr>
</tbody>
</table>

pKᵢ values were determined in competition binding experiments with [³H]-mepyramine. Values shown are mean ± SD of 3 independent experiments with triplicate determinations.

* p<0.05 Student t test. Statistically different between human and zebrafish H₁Rs affinities (pKᵢ)

b p<0.05 Student t test. Statistically different between human and zebrafish H₁Rs potencies for agonists (pEC₅₀)

pA₂ values were obtained from competitive antagonism assays and Schild analysis. Means ± SEM of 3 experiments.

* Fexofenadine and acrivastine binding on hH₁R display an insurmountable effect at 1 and 10 µM concentrations.
Table 3. Dissociation constants ($K_D$), maximal binding sites ($B_{max}$) and inhibition constants ($pK_i$) of human and $zfH_1R$ mutants.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>$K_D$ (nM)</th>
<th>$B_{max}$ (pmol/mg protein)</th>
<th>$pK_i$</th>
<th>$\Delta pK_i$</th>
<th>$\Delta pK_i$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>hH$_1$R</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>1.5 ± 0.4</td>
<td>11.5 ± 2.8</td>
<td>4.8 ± 0.2</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>zfECL2</td>
<td>0.6 ± 0.2</td>
<td>4.0 ± 0.4</td>
<td>6.2 ± 0.3</td>
<td>-1.4</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>R45x45E</td>
<td>0.7 ± 0.2</td>
<td>9.6 ± 3.3</td>
<td>4.9 ± 0.5</td>
<td>-0.1</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>K45x49Q</td>
<td>0.8 ± 0.3</td>
<td>10.1 ± 0.3</td>
<td>5.2 ± 0.4</td>
<td>-0.5</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Y45x55R</td>
<td>1.5 ± 0.6</td>
<td>16.2 ± 3.3</td>
<td>4.1 ± 0.2</td>
<td>0.6</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>zfH$_1$R</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>0.7 ± 0.1</td>
<td>4.6 ± 1.5</td>
<td>6.5 ± 0.2</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>hECL2</td>
<td>1.0 ± 0.4</td>
<td>7.9 ± 2.1</td>
<td>5.3 ± 0.2</td>
<td>1.2</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>E45x45R</td>
<td>0.8 ± 0.1</td>
<td>6.1 ± 0.5</td>
<td>5.9 ± 0.2</td>
<td>0.6</td>
<td>0.0072</td>
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</tr>
<tr>
<td>Q45x49K</td>
<td>0.6 ± 0.1</td>
<td>5.7 ± 0.2</td>
<td>6.2 ± 0.2</td>
<td>0.3</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>R45x55Y</td>
<td>1.2 ± 0.2</td>
<td>3.5 ± 0.4</td>
<td>5.0 ± 0.4</td>
<td>1.5</td>
<td>0.0001</td>
<td></td>
</tr>
</tbody>
</table>

$K_D$ and $B_{max}$ values were determined by $[^3]$H-mepyramine saturation analysis to homogenates of HEK-293T cells recombinantly expressing the various receptor proteins. Values are means ± SD from 3 or 4 experiments.

$pK_i$ constants were determined by displacement of $[^3]$H-mepyramine by histamine in HEK-293T cells expressing the H$_1$R mutants or wild type (WT) receptors. Values are means ± SEM from 4 experiments and are expressed as the absolute value of the negative logarithm of the inhibition constant ($pK_i$). $\Delta pK_i$ was determined as the difference between the $pK_i$ values of the WT and the mutant receptors. When $\Delta pK_i < 0$ the affinity for histamine is higher than the WT receptor, whereas $\Delta pK_i > 0$ the affinity for histamine is lower than for the WT receptor. Statistical analysis was determined by one-way ANOVA and Dunnett’s multicomparison test. ND, not determined; ns, not significant ($P>0.05$).
Fig. 1
Fig. 2

A

B

Graph 1: Radar chart showing the activity of different regulatory elements (NFAT, NFκB, AP1, SRE, CRE, COX-2, VEGF) under control conditions.

Graph 2: Bar graph showing the NFAT activity (%) control for control, YM-254890, and Mepyramine treatments. The graphs compare hH1R and zfH1R.
Fig. 3
Fig. 4
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Fig. 5
Fig. 6

- [H]-mepyramine (% SB)

Log [histamine] M

- hH₁R WT
- hH₁R-zfECL2

- [H]-mepyramine (% SB)

Log [histamine] M

- zfH₁R WT
- zfH₁R-hECL2