Review

Molecular and cellular analysis of human histamine receptor subtypes

Roland Seifert[1], Andrea Strasser[2], Erich H. Schneider[1], Detlef Neumann[1], Stefan Dove[2], and Armin Buschauer[2]

[1] Institute of Pharmacology, Medical School of Hannover, Carl-Neuberg-Straße 1, D-30625, Germany
[2] Department of Pharmaceutical/Medicinal Chemistry II, University of Regensburg, Regensburg, Germany

The human histamine receptors hH_1R and hH_2R constitute important drug targets, and hH_3R and hH_4R have substantial potential in this area. Considering the species-specificity of pharmacology of H_xR orthologs, it is important to analyze hH_xRs. Here, we summarize current knowledge of hH_xRs endogenously expressed in human cells and hH_xRs recombinantly expressed in mammalian and insect cells. We present the advantages and disadvantages of the various systems. We also discuss problems associated with the use of hH_xR antibodies, an issue of general relevance for G-protein-coupled receptors (GPCRs). There is much greater overlap in activity of 'selective' ligands for other hH_xRs than the cognate receptor subtype than generally appreciated. Studies with native and recombinant systems support the concept of ligand-specific receptor conformations, encompassing agonists and antagonists. It is emerging that for characterization of hH_xR ligands, one cannot rely on a single test system and a single parameter. Rather, multiple systems and parameters have to be studied. Although such studies are time-consuming and expensive, ultimately, they will increase drug safety and efficacy.

Clinical relevance of drugs targeting human histamine receptors

Histamine plays an important role in diverse human diseases. In immediate-type (type I) allergies, massive IgE-triggered release of histamine from mast cells takes place; this results in activation of the H_1 receptor (H_1R) and contributes to the development of conjunctivitis and rhinitis with the lead symptoms pruritus (itching), erythema (reddening of the skin), and edema (accumulation of fluid in the skin) [1,2]. Accordingly, H_1R antagonists, specifically compounds of the second generation with low penetration into the central nervous system (CNS), are used for the local and systemic treatment of these ailments [1,2]. In human bronchial asthma, H_1R antagonists are ineffective, but the results of mouse studies suggest that H_4R antagonists could be useful in the treatment of asthma [3,4]. However, peer-reviewed clinical studies of H_4R antagonists in patients with asthma have not yet been published.

First-generation H_1R antagonists penetrate well through the blood–brain barrier (BBB) and are used for the treatment of sleep disorders and pruritus [5,6]. In a mouse pruritus model, the combination of a first-generation H_1R antagonist and a H_4R antagonist was more effective than either drug alone [7], but corresponding studies in humans have not yet been published. Recently, the first H_3R antagonist, pitolisant, has been introduced as an orphan drug for the treatment of narcolepsy [8]. H_3R antagonists have also therapeutic potential for other CNS diseases such as Alzheimer's disease (AD) and attention deficit hyperactivity disorder (ADHD) [8].

H_2R antagonists were developed in the 1960s by Sir James Black, who has recently been honored by a series of articles in *Trends in Pharmacological Sciences* [9]. H_2R antagonists block H^+ secretion in parietal cells of the stomach and provided the first effective drug for the treatment of gastroduodenal ulcer and gastroesophageal reflux disease [10]. These drugs have now been largely substituted by the irreversibly acting proton pump inhibitors that are more effective because of their longer duration of action and the fact that the proton pump constitutes the converging point of several GPCRs beyond H_2R that stimulate H^+ secretion (i.e., muscarinic acetycholine receptors and cholecystokinin/gastrin receptors) [10]. In myeloid cells, H_2R mediates inhibition of the superoxide anion (O_2^-)-producing NADPH oxidase [11,12]. Through this effect, histamine facilitates T cell-mediated killing of tumor cells in acute myeloid leukemia (AML), specifically in monocytic forms M4/M5 (FAB classification) [13]. In conjunction with interleukin 2, histamine has been approved as an orphan drug for the maintenance treatment of AML [14]. H_2R agonists have also potential as positive inotropic drugs for the treatment of acute heart failure, but following some promising publications in the 1990s, this avenue of research has

Glossary

Anti-peptide antibody: antibody, which is generated by immunization of the host organism with a peptide, usually conjugated to a larger carrier protein such as keyhole limpet hemocyanin.
Epitope: also known as antigen determinant, part of an antigen, which is recognized by molecules of the immune system.
Fc receptor: receptor that recognizes the Fc part of the heavy chain of an antibody.
Monoclonal antibody (mAb): preparations of antibodies with identical epitope-directed specificity because all antibody-producing cells (usually hybridoma cells) are derivatives of a single precursor cell.
Nanobody: antigen-binding domain derived from an antibody that naturally occurs in *Camelidae* (camel and llama) and consists of only a heavy chain without light chain.
Polyclonal antibody (pAb): preparations of antibodies recognizing the same antigen but at several epitopes, because antibody-producing cells are not derived from a single precursor cell.

Corresponding author: Seifert, R. (seifert.roland@mh-hannover.de).

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Table 1. Summary of representative publications on human cells endogenously expressing hH_xRs[a]

hH_xRs studied	Biological system	Parameters determined	Most important findings	Comments	Refs
hH_1R	HeLa cervix carcinoma cells	Increase in $[Ca^{2+}]_i$, [^{3}H]inositol phosphate accumulation, cell proliferation assays, cell migration, gene expression, phosphorylation cascades (e.g., protein kinase C, β-catenin) receptor desensitization studies	hH_1R couples positively to the phospholipase C–Ca^{2+} pathway and activates protein kinase C and the β-catenin pathway. As a result, migration, proliferation, and gene expression are activated. Noncompetitive effects of H_1R antagonists are explained by nonequilibrium conditions.	HeLa cells are a classic and widely used cell culture model to study the pharmacology, signal transduction, and function of hH_1R. In this system, hH_1R can be studied at many different levels. The cells have been used for hH_1R analysis for three decades and are still popular.	[103–108]
hH_1R	U373 MG astrocytoma cells	Increase in $[Ca^{2+}]_i$, [^{3}H]inositol phosphate accumulation, [^{3}H]mepyramine binding, cAMP accumulation, cell proliferation, endocytosis, and internalization	hH_1R couples positively to the phospholipase C–Ca^{2+} signaling pathway and inhibits cAMP accumulation. hH_1R is mitogenic in U373 cells. hH_1R internalization can be well studied. The cells have been used as a model to predict the sedative and nonsedative properties of H_1R antagonists.	U373 MG cells are a classic cell culture model to study the pharmacology and signal transduction of hH_1R. The cells have been used for hH_1R analysis for more than two decades.	[109–114]
hH_1R	Differentiated HL-60 promyelocytes	Increase in $[Ca^{2+}]_i$, [^{3}H]inositol phosphate accumulation	hH_1R couples positively to the phospholipase C–Ca^{2+} signaling pathway. Effects are partially or completely mediated via G_i proteins. Coupling of hH_1R to G_i proteins is not commonly observed in other systems. Rather, coupling to G_q is typical. The functional role of hH_1R in HL-60 cells is unknown. Classic phagocyte effector functions are not activated via hH_1R, i.e., histamine is only an incomplete activator of these cells.	HL-60 cells have to be differentiated with dibutyryl-cAMP towards neutrophils or with 1α,25-dihydroxycholecalciferol towards monocytes to reveal good hH_1R responses. In undifferentiated cells, H_1R responses are small compared with H_2R responses. However, human neutrophils do not express functional hH_1R. HL-60 cells have been rarely used as an hH_1R model during the past years. A reason for this may be the fact that HL-60 cell clones are not stable and differ among various laboratories, rendering data reproduction difficult.	[25,26,36]
hH_1R	Human coronary artery endothelial cells (HCAECs)	FACS analysis of hH_1R expression with antibody, RT-PCR, [^{3}H]mepyramine binding, synthesis of prostaglandins and IL-6, siRNA studies	Lipopolysaccharide (LPS) enhances expression and function of hH_1R in HCAECs. LPS has no effect on hH_2R mRNA expression, but hH_2R antibody was not tested.	Combination of expression studies and functional studies. Specificity of commercial hH_1R antibody is a concern (Box 1).	[115]
hH_1R?	Pulmonary mucoepidermoid carcinoma cell NCI-H292 and colon adenocarcinoma HM3-MUC2 cells	Mucin gene expression	Histamine differentially regulates gene expression in a partially fexofenadine-sensitive manner. High ligand concentrations and only partial blockade of histamine signals by fexofenadine are of concern so that off-target effects cannot be excluded.	No antagonists for other H_xRs were studied. Evidence for hH_1R not fully convincing.	[116]
hH_1R?	Archived paraffin-embedded tissue specimen from patients with oral squamous cell carcinoma (OSCC), OSCC cell lines BICR56, and BICR3	Immunohistochemical analysis of hH_1R expression	Immunohistochemical reactivity with hH_1R antibodies is enhanced in several specimens of patients with advanced OSCC. Thus, high hH_1R expression is suggested to point to poorer prognosis.	Validation of hH_1R and hH_2R antibodies is a concern; nonspecific binding effects cannot be ruled out (Box 1). No functional experiments showing, e.g., increased proliferation of malignant cells in the presence of histamine were performed.	[117]
hH_1R, hH_2R	THP-1 monocytic leukemia cells	RT-PCR, TNF-α release, phagocytosis, cytotoxicity assay	2-Pyridylethylamine (classified by authors as H_1R agonist) and dimaprit (classified by authors as H_2R agonist) downregulate a key ligand for natural killer cell activation (NKG2D) via the ubiquitin–proteasome pathway.	Studies with H_xR antagonists were not performed; hH_4R was not studied. Thus, contribution of individual receptors to the response remains ambiguous, specifically in light of the fact that these cells were also reported to express hH_4R.	[118]

Table 1 (*Continued*)

hH_xRs studied	Biological system	Parameters determined	Most important findings	Comments	Refs
hH_1R, hH_2R?	Monocytes, monocyte-derived macrophages (MDMs), and dendritic cells (DCs)	RT-PCR, FACS and western blot analysis of hH_1R expression with antibody, increase in $[Ca^{2+}]_i$, IL-8 release	No evidence for functional expression of hH_1R in monocytes. However, in MDMs, hH_1R increases $[Ca^{2+}]_i$ and stimulates IL-8 release. hH_2R is present at mRNA level but apparently with no functional role.	Validation of commercial hH_1R antibodies is a concern; nonspecific binding effects cannot be ruled out. Bands in western blots do not have the appearance of glycosylated proteins. Control western blots with recombinant hH_xRs would have been useful to assess band appearance and occurrence of possible dimers and atypically migrating receptors (Box 1 and Table 1).	
hH_2R, (hH_1R)	HL-60 promyelocytes	cAMP accumulation, increase in $[Ca^{2+}]_i$, myeloid (neutrophilic) differentiation	hH_2R mediates both increases in cAMP and $[Ca^{2+}]_i$. In promyelocytes and retinoic acid-differentiated cells, a minor component of the increase in $[Ca^{2+}]_i$ is hH_1R-mediated. There is evidence for functional selectivity of hH_2R in this system. In promyelocytes, H_2R activation induces functional and morphological differentiation.	HL-60 cells are a classic cell culture model system for hH_2R analysis. However, results regarding hH_2R expression and function with different differentiation procedures vary between laboratories, probably due to cell clone variability.	[35,36,42, 120–122]
hH_1R, hH_2R, hH_3R?	Monocyte-derived DCs	Detection of hH_xRs by RT-PCR, increase in $[Ca^{2+}]_i$, actin polymerization, chemotaxis, IL-10 and IL-12 secretion, cAMP accumulation	Histamine differentially regulates various functional parameters during DC maturation. In immature cells, increases in $[Ca^{2+}]_i$, actin polymerization, and chemotaxis are seen. In mature cells, cAMP increases and modulation of interleukin release are observed.	H_3R is claimed to be functionally expressed, but selective H_4R antagonists were not studied, only the dual H_3R/H_4R antagonist thioperamide. Concentrations of *R*-α-methylhistamine and betahistine in the calcium assay are very high so that specificity of effects is a concern. Expression of hH_3R in blood cells is highly unlikely. Probably, hH_4R is present but RT-PCR for hH_4R was not performed.	[123]
hH_1R, hH_2R?	$CD4^+$ cells, $CD8^+$ cells, peripheral blood mononuclear cells (PBMCs) from healthy subjects and patients with allergic asthma or rhinitis	FACS analysis of hH_1R and hH_2R expression with antibody, IL-4, IL-13, and IFN-γ secretion	House dust mite, one of the most important allergens, supposedly increases hH_1R expression in patients with allergic asthma and rhinitis. In patients with rhinitis, the H_1R mediates an increase in Th2 cytokine production via the H_1R, whereas in control subjects and patients with asthma, histamine exerts an inhibitory effect on cytokine production, most likely via the H_2R.	Specificity of commercial hH_1R and hH_2R antibodies is a concern; nonspecific binding effects cannot be ruled out (Box 1). Substantial interindividual variability in cytokine responses.	[124]
hH_1R, hH_2R?, hH_4R?	Bone marrow stromal cells (BMSCs) from healthy volunteers	Immunofluorescence staining with hH_xR antibodies, RT-PCR analysis of hH_xR expression, IL-6 secretion, neutrophil function assays	Histamine stimulates IL-6 production via hH_1R and exhibits antiapoptotic effects in neutrophils. No evidence for functional role of hH_2R and hH_4R.	Specificity of commercial hH_1R and hH_2R antibodies is a concern; nonspecific binding effects cannot be ruled out (Box 1). Subcellular localization of immunostaining of hH_xRs apparently not confined to plasma membrane.	[125]
hH_1R?, hH_2R?	Formalin-fixed tissue from patients with complicated sigmoid diverticulitis	Immunohistochemical analysis of hH_1R and hH_2R expression, immunodetection of histamine	Complicated diverticulitis is associated with increased immunoreactivity against hH_1R, hH_2R, and histamine.	Specificity of commercial hH_1R and hH_2R antibodies is a concern; nonspecific binding effects cannot be ruled out (Box 1). Histamine would be expected to be washed out by the staining procedure, also pointing to methodological problems.	[126]
hH_2R	Neutrophils	cAMP accumulation, superoxide (O_2^-) formation, lysosomal enzyme release	hH_2R mediates inhibition of formyl peptide-stimulated O_2^- formation and lysosomal enzyme release. In bronchial asthma, hH_2R function is decreased and may contribute to inflammation. There is evidence for functional selectivity of hH_2R. It is discussed whether or not H_2R responses are cAMP-mediated.	The human neutrophil is a classic model system for the analysis of hH_2R. Large numbers of cells can be easily obtained from peripheral blood or buffy coat preparations. There is no evidence for functionally active hH_1R and hH_4R in human neutrophils. Early clinical studies on changes in H_2R function in asthma were not followed up.	[11,12,32, 34,127–129]

Table 1 (*Continued*)

hH_xRs studied	Biological system	Parameters determined	Most important findings	Comments	Refs
hH_2R	U937 promonocytes	cAMP accumulation, cAMP export via MRP4, [^{3}H]tiotidine binding, c-fos gene expression, monocytic differentiation	hH_2R induces robust cAMP accumulation and monocytic differentiation. Because of the robust responses, the cells have been used to study receptor models and desensitization in a native system.	U937 cells serve as a classic model system for M4/M5 AML in which histamine is used as an orphan drug for remission therapy.	[37–42]
hH_2R	M4/M5 AML cells	Analysis of hH_2R expression with antibody in FACS, reactive oxygen species (ROS) formation, apoptosis of natural killer cells	In M4/M5 cells, hH_2R inhibits ROS formation and, thereby, promotes survival of natural killer cells which then destroy tumor cells. This mechanism is suggested to contribute to the clinical effects of histamine in AML. Substantial inter-individual differences in ROS formation responses are noted.	Validation of commercial hH_2R antibody is a concern, and nonspecific binding effects cannot be ruled out (Box 1). Probably, owing to limited availability of AML cells for functional experiments, hH_2R in these cells has so far only been characterized to a very small extent. However, this is a very promising model for translational research. AML is the only disease in which histamine has been used as a drug.	[13,14]
hH_2R	PBMCs	Accumulation of mRNA for IL-1 and IL-6 and synthesis of interleukins	Histamine stimulates IL-1- and IL-6 expression in a cimetidine- but not diphenhydramine-sensitive manner, indicating H_2R involvement.	The precise cell type mediating the histamine response was not defined. At the time when the study was performed, hH_4R was not yet known, but the studies provide no evidence for H_4R involvement as the inhibition by cimetidine is complete.	[130,131]
hH_2R	PBMCs	Production of IL-18, IFN-γ, IL-2, and IL-10	Histamine activates production of IL-18 and IFN-γ and inhibits production of IL-2 and IL-10 in a H_2R antagonist sensitive manner. Chlorpheniramine and thioperamide are without effect, ruling out hH_1R, hH_3R and hH_4R involvement.	Dimaprit and 5-methylhistamine mimic the effects of histamine. This is a classic study showing that 5-methylhistamine cannot be considered as selective H_4R agonist but is also a potent H_2R agonist.	[132]
hH_2R	HGT-1 gastric cancer cells	cAMP accumulation, adenylyl cyclase activity in membranes	hH_2R increases cAMP in intact cells and activates adenylyl cyclase in membranes. The cells have been extensively used to characterize hH_2R antagonists and hH_2R desensitization.	Since the early 1990s, the cell line has not been used very much anymore for hH_2R analysis. However, it cannot be excluded that the cells are useful for analysis of functional selectivity if hH_2R, like in other cells such as HL-60 cells, also activates other signaling pathways. This remains to be studied.	[133–137]
hH_2R, hH_4R	Eosinophils	Increase in $[Ca^{2+}]_i$, chemotaxis, cAMP accumulation, superoxide anion (O_2^-) formation, cell shape change, adhesion molecule regulation, actin polymerization, eosinophil peroxidase (EPO) release	hH_4R induces only incomplete eosinophil activation compared with other activators such as eotaxin, i.e., there are only moderate increases in $[Ca^{2+}]_i$ and moderate chemotaxis, but no O_2^- formation and EPO release. hH_2R inhibits chemotaxis, EPO release, and O_2^- formation. There is evidence for functional selectivity of hH_2R and hH_4R.	Purity of eosinophil preparations varies considerably in various studies. Eosinophils are very difficult to obtain in large numbers and high purity from healthy volunteers. Nonetheless, eosinophils are the best-studied human cell system for hH_4R. Despite some data variability, the presence of hH_4R in eosinophils has been confirmed by several groups. We have been unable to purify viable eosinophils from buffy coat preparations so that the low number of available cells constitutes a major obstacle for extensive pharmacological studies.	[32,33, 45–48, 138–140]
hH_1R, hH_4R	DCs (obtained by differentiation with GM-CSF and IL-4)	Increase in $[Ca^{2+}]_i$, CD86 expression, polarization of naïve $CD4^+$ cells to TH2 cells	Histamine increases $[Ca^{2+}]_i$, and CD86 expression in DCs and TH2 polarization both via H_1R and H_4R as assessed by the inhibitory effects of mepyramine and JNJ7777120.	The effects of histamine on $[Ca^{2+}]_i$ is mediated both via Ca^{2+} mobilization from intracellular stores and Ca^{2+} influx from the extracellular space via store-operated Ca^{2+} entry that is inhibited by imidazole SK&F 96365.	[141]

Table 1 (*Continued*)

hH_xRs studied	Biological system	Parameters determined	Most important findings	Comments	Refs
hH_2R, hH_4R	$CD8^+$ T cells	Analysis of hH_xR expression by RT-PCR, IL-6 secretion	Histamine inhibits IL-6 release in a partially thioperamide- and cimetidine-sensitive manner. (R)-α-Methylhistamine, dimaprit, and clobenpropit mimic the effects of histamine. At a concentration of 10 μM, all ligands are equi-effective, but they differ from each other in potency. Pertussis toxin partially inhibits the effects of histamine, (*R*)-α-methylhistamine, and clobenpropit but not of dimaprit.	The study provides complete concentration–response curves for relevant H_xR agonists and antagonists. The study shows that even at a time when highly selective H_4R ligands were not yet available, a comprehensive characterization of H_xR subtypes could be accomplished. The effect of pertussis toxin points to the involvement of G_i proteins in the signaling pathway of H_4R.	[142]
hH_2R, hH_4R, hH_3R?	Monocyte-derived DCs (obtained by incubation with GM-CSF and IL-4)	Analysis of xH_xR expression with RT-PCR and BODIPY-FL-histamine FACS analysis, cAMP accumulation, IL-12p70 production, AP-1 transcription factor activation, actin polymerization, chemotaxis	Activation of hH_2R increases cAMP formation and decreases IL-12p70 production. Clobenpropit inhibits IL-12p70 production in a partially JNJ7777120-sensitive manner, pointing to H_4R involvement. The H_4R signaling pathway remains unclear and does not involve cAMP, Ca^{2+}, or ERK activation. The paper also discusses controversies in the literature regarding the function of H_4R in monocytes and monocyte-derived DCs. Heterogeneity in cell culture conditions in various studies could account for discrepancies.	FACS analysis with BODIPY-FL-histamine is ambiguous because high concentrations of the fluorescent ligand and competing nonfluorescent ligands were used, and because the ligands used have considerable affinities for various hH_xRs. Suitability of BODIPY-FL-histamine as fluorescent ligand for hH_xRs remains to be validated. Functional experiments are not easy to interpret with respect to differentiation between H_3R and H_4R. The authors encountered difficulties with the use of commercial antibodies against H_3R and H_4R in western blot and FACS experiments and, therefore, did not include data with these tools into their paper (Box 1).	[143]
hH_4R	TH2 cells	Analysis of hH_4R expression by RT-PCR and FACS with antibody, activation of transcription factor AP-1, expression of IL-31 mRNA	Histamine, 5-methylhistamine, and clobenpropit (10 μM each) activate AP-1 in a JNJ7777120-sensitive manner. Histamine and 5-methylhistamine also increase IL-31 mRNA. In some assays, clobenpropit and 5-methylhistamine far exceed the effects of histamine.	Specificity of the commercial hH_4R antibody is a concern; nonspecific binding effects cannot be ruled out (Box 1). Other hH_xRs cannot be ruled out because antagonists for H_1R and H_2R were not studied. The molecular basis for the 'superagonism' of clobenpropit and 5-methylhistamine in some assays remains to be determined. It could reflect that histamine but not the former ligands also activate inhibitory hH_xRs. Accordingly, H_1R and/or H_2R antagonists should enhance the effect of histamine. This remains to be studied.	[87]
hH_4R	Natural killer cells	Analysis of hH_xR expression with antibodies by FACS, chemotaxis, increase in $[Ca^{2+}]_i$, and cytotoxicity assays	Histamine induces chemotaxis in a thioperamide-sensitive manner, but does not increase $[Ca^{2+}]_i$ or induce cytotoxicity. Lack of effect of histamine on $[Ca^{2+}]_i$ in the case of H_4R involvement is rather unexpected. In fact, Ca^{2+} is a classic intracellular mediator of hH_4R effects. The basis for this striking negative result remains to be determined as holds for the actual signal mediating activation of chemotaxis.	Specificity of commercial antibodies is a concern (Box 1). The lack of effects of histamine on $[Ca^{2+}]_i$ indicates that calcium is not an essential signal for activation of chemotaxis. Other hH_xRs cannot be ruled out because H_1R and H_2R antagonists were not studied. The study also examined monocytes and dendritic cells. In these cells, chemotaxis was induced as well, but a Ca^{2+} influx was only seen in dendritic cells.	[144]
hH_4R	TH17 cells	Analysis of hH_4R expression by RT-PCR, FACS with antibody and immunohistochemistry, IL-17 production (mRNA and secretion), activation of transcription factor AP-1	Histamine and 5-methylhistamine (10 μM each) induce IL-17 mRNA production, IL-17 secretion, and AP-1 activation. The effects of histamine on IL-17 mRNA (and to a lesser extent on IL-17 secretion) are inhibited by JNJ7777120. H_1R and H_2R antagonists are not inhibitory.	Specificity of the commercial hH_4R antibody is of concern; nonspecific binding effects cannot be ruled out (Box 1). Large variability between various experiments is noted too. hH_4R may enhance the inflammatory response in psoriasis via Th17 cells.	[145]

Table 1 (*Continued*)

hH_xRs studied	Biological system	Parameters determined	Most important findings	Comments	Refs
hH_4R?	Colorectal carcinoma and adjacent control tissue	Analysis of hH_4R expression by western blotting, immunohistochemistry, and RT-PCR	Decreased signals in western blot, immunohistochemistry, and RT-PCR signals in colorectal carcinoma.	Specificity of commercial hH_4R antibodies is a concern; nonspecific binding effects cannot be ruled out (Box 1). Molecular mass and specificity of immunoreactive bands in western blot remain to be clarified.	[146]
hH_4R?	CD34-positive umbilical cord hematopoietic progenitor cells	Analysis of hH_4R expression with antibody in FACS, cell cycle analysis	Inhibitory effect of clobenpropit at a single concentration (10 μM) on cell proliferation; data for JNJ7777120 not shown.	Specificity of commercial hH_4R antibody is a concern; nonspecific binding effects cannot be ruled out (Box 1). However, some effort was undertaken to document specificity of the antibody. Pharmacological characterization of hH_4R is very preliminary, probably due to the limited availability of umbilical cord progenitor cells. Involvement of other hH_xRs cannot be excluded.	[147]
Unknown H_xR?, off-target? (other GPCR or non-GPCR target)	PBMCs	Inhibition of IFN-γ release in the presence of a *Mycobacterium tuberculosis* protein	Histamine, dimaprit, clobenpropit, and clozapine (100 μM each) inhibit IFN-γ release in the presence of a combination of chlorpheniramine + famotidine + thioperamide. These data indicate that known hH_xRs are not involved in the response.	The data are difficult to interpret. The concentrations of histamine, dimaprit, clobenpropit, and clozapine used are very high so that off-target effects of these ligands cannot be ruled out. It is important to assess the effects of hH_xR antagonists in the presence of histamine, dimaprit, clobenpropit, and clozapine at low micromolar concentrations.	[148]

[a]Table 1 summarizes representative studies on hH_xRs in native systems. Some data on hH_xRs expressed in mammalian cells, with special emphasis on pharmacological key parameters are presented in Table 2, and a comprehensive analysis of hH_xRs expressed in Sf9 insect cells is presented in Tables 3 and 4. The aim of Table 1 is to summarize key properties and unresolved problems of native hH_xR systems ranging from cell culture systems to isolated blood cells and human tissues. Studies with blood cells and tissues have become a focus of recent research and often entail the extensive use of antibodies [13,51,117,119,124–126,87,144–147]. However, the proper use of antibodies against biogenic amine GPCRs in general and hH_xR antibodies in particular is not trivial and requires extensive and rigorous validation of antibody quality [52–54,149–152]. Hence, caution must be exerted when interpreting studies with hH_xR antibodies (for more details, see Box 1). The best approach to study hH_xRs in native human cells and tissues is to combine well-validated antibodies fulfilling all of the criteria listed in Box 1, RT-PCR studies, and extensive pharmacological studies that entail complete concentration–response curves for multiple ligands and not only single concentrations of supposedly hH_xR-selective ligands. It should also be kept in mind that mRNA expression studies involving RT-PCR are prone to false-positive results due to the presence of contaminating mRNA from other cells than the cells of particular interest. Table 1 does not list all studies on hH_xRs in native human systems but rather provides representative research of various groups across basic and clinical science disciplines. An emerging field is the analysis of hH_xRs in human diseases [13,117,124,146]. There are only a few systems in which a single hH_xR is expressed functionally, for example, HeLa cells, U373 cells, human neutrophils, and HGT-1 cells. In most cells, multiple hH_xRs are expressed, complicating analysis, specifically because of the overlap in pharmacology (Figure 1 and Table 4). Specifically, the use of agonists can be fairly ambiguous (Figure 1). The use of first-generation H_1R antagonists at reasonably low concentrations (up to 1–10 μM), a potent H_2R antagonist such as famotidine or tiotidine (up to 1–10 μM), and the use of dual H_3/H_4R antagonist thioperamide (up to 1–10 μM), and H_4R antagonist JNJ7777120 (up to 1–10 μM), all being commercially available, is the best approach to assign hH_xR subtypes to specific signaling pathways and cell functions. Table 1 lists studies covering receptor pharmacology, signaling pathways, cell functions, and diseases. The signaling pathways of hH_1R and hH_2R are well defined, although new details are still emerging, specifically for hH_1R [107]. In case of hH_4R, there are still several unresolved and controversial issues with respect to signaling pathways [143,144]. With regard to expression and functions of hH_xRs in PBMCs and cell populations derived therefrom, there is substantial controversy [119,123,124,130–132,141–143,87,144,145]. This is, at least in part, due to the use of different cell populations, their purity and the different activation states of the cells. Also, the presence of multiple hH_xRs in these cells renders analysis difficult. The situation regarding PBMCs is further complicated because in some studies, expression of hH_3R has been postulated [123,143]. However, one should keep in mind that hH_3R and hH_4R are pharmacologically similar [16,18,20] (Figure 1 and Table 4). Although well-defined native human cell systems for hH_1R, hH_2R, and hH_4R are available, this is not the case for hH_3R. In some cases, specifically when data for a given cell system are coherent, multiple papers were summarized in one entry. However, in other cases, it was more appropriate to discuss papers individually. Some limitations of papers include unavailability of tools or limited knowledge about specificity of pharmacological and immunological tools at the time of study performance. We hope that Table 1 will assist future studies in the hH_xR field to use the available pharmacological and immunological tools critically and to be aware of potential pitfalls. With respect to hH_xR ligands, one should always aim at constructing complete concentration–response curves for multiple ligands and check agonist effects with appropriate antagonists. High ligand concentrations (10 μM or higher) should be generally avoided, specifically in the case of lipophilic ligands. Such ligands are prone to off-target effects. Off-target effects have been observed for several hH_xR ligands at high concentrations including clozapine, clobenpropit, first-generation H_1R antagonists, guanidine-type H_2R agonists, and phenylhistamines [27,148,153–155]. A further complication in the pharmacological analysis of hH_xRs in native systems is functional selectivity, that is, potencies and efficacies of ligands may be parameter-dependent [32–36]. Even for ligands that are exceedingly well characterized in the literature such as H_2R antagonist cimetidine, surprising findings can be obtained. Specifically, this compound behaves as a partial agonist in human monocytes and dendritic cells with respect to IL-18 production [156]. Accordingly, it is also very important to examine even 'inactive' control compounds such as classic H_xR antagonists by themselves in native cells.

not been further pursued [15]. Numerous excellent reviews on the medicinal chemistry, pharmacology, and (patho)-physiology of H_xRs are available [8,16–22]. Considering the fact that there is substantial variability in the effects of H_xR ligands among H_xR species orthologs [23], it is particularly important for the treatment of human diseases to possess broad knowledge on the properties of hH_xRs. The purpose of this review is to fill this important gap in the

literature and to provide strategies for productive and critical research on hH_xRs.

Challenges to the analysis of hH_xR subtypes in native human cells: the H_1 receptor

From an experimental point of view, it is not easy to comprehensively characterize H_xR ligands in human cells endogenously expressing hH_xRs. Table 1 summarizes the results of selected studies dealing with the characterization of hH_xRs in native human cells and critically analyzes these studies. We list several classic studies in the field, but clearly focus on the most recent data. Papers comprise studies on pharmacology, signal transduction, cell biology, and clinical relevance of hH_xRs. We aimed at showcasing the research of numerous groups and many experimental systems, some being popular, some having come out of fashion. Intentionally, we also included into Table 1 several papers that yielded controversial results to stimulate further research in these areas so that discrepancies will be ultimately resolved. Recently, a large number of papers dealing with hH_xR antibodies have been published. Representative studies dealing with hH_xR antibodies are listed in Table 1 because studies with these tools often made far-reaching conclusions regarding the possible pathophysiological roles of hH_xR subtypes in human diseases and corresponding therapeutic implications. Unfortunately, the use of hH_xR antibodies in particular and of GPCR antibodies in general is much more problematic than the community of histamine researchers often appreciates. To encourage a more critical use of hH_xR antibodies in future

Box 1. Problems associated with the use of GPCR antibodies

Antibodies are the most widely used tools for the analysis of proteins. Since the establishment of hybridoma technology, honored by the Nobel Prize in physiology or medicine to Milstein, Köhler, and Jerne in 1984, antibodies have become most valuable for the highly specific detection of a given protein. The portion of a protein recognized by an antibody, the epitope (see Glossary), may consist of only approximately 15–20 linear amino acids of the primary structure of the protein [157]. For optimal reactivity of the antibody, the epitope-determining sequence should be of high hydrophilicity and possibly in a linear conformation [158,159]. A very well-established technique to develop specific antibodies is the immunization of animals with a carrier protein-coupled peptide composed of the amino acid sequence deduced from the protein of interest [160]. When designing such peptides, considerations such as the predicted peptide solubility, antigenicity, surface exposure, and homologies have to be taken into account. Because this method generates antibodies that typically recognize a linear peptide, a differing secondary structure in the target protein could, at worst, prevent any interaction between the antibody and the protein.

Currently, antibodies are generated against virtually every known protein, including GPCRs such as H_XRs. Because H_XRs do not bear extensive homologies, specifically in the extracellular and intracellular domains (see Figure 2a in main text), the identification of specific, nonhomologous sequences is not difficult. Indeed, the sequences with highest homology are located in the membrane-integral portions of the receptors (see Figure 2a in main text), and the designated epitopes are in either the extracellular or the intracellular loops. This, however, also constitutes a problem, because these loops, with the exception of the third intracellular loop, of the H_XRs are rather short, reducing the probability of finding linear peptides with sufficient solubility and antigenicity. Nonetheless, anti-peptide antibodies using peptides comprising sequences deduced from H_XR sequences have been generated and are available, commercially or noncommercially, to the histamine research community. Because of the above-mentioned reasons, such antibodies, although they perfectly recognize the immunizing peptide, have to be carefully evaluated for their ability to recognize the holo-protein. Concerns regarding the specificity of GPCR antibodies are the subject of current discussion [52–54], going beyond H_XRs [150,161–163]. Therefore, four different criteria, of which at least one has to be fulfilled to document the specificity of a GPCR antibody, have been published recently (Table I) [151,152]. Moreover, this issue is also of higher complexity, because the specificity of an antibody is not only determined by the antigen–antibody combination but also depends on the method used to detect the protein [149]; most basically by whether a protein is detected in its native form (e.g., by flow cytometry) or in a denatured form (e.g., by western blot). In addition, for GPCRs, there is good evidence that these receptors do not exist in a single conformation, but that their conformation changes, tightly regulated by interaction with a specific ligand [30,31,43,164]. Thus, identifying a feasible sequence for peptide antibody generation is even more difficult. A strategy to overcome these difficulties is not to use a peptide from the receptor sequence for immunization but to use the entire protein inserted in the plasma membrane (e.g., as in genomic immunization) [165]. Alternatively, the generation of GPCR-specific nanobodies in the llama may become a useful strategy to obtain truly selective GPCR antibodies [166].

In considering the above-mentioned criteria for antibody specificity, at the time being, none of the available H_XR antibodies, polyclonal or monoclonal ones, can be recommended. All antibodies that we have studied were raised as 'anti-peptide antibodies'. If reported at all, mostly they were validated by the supplier for their reactivity against the immunizing peptide only. This, however, as discussed above, does not prove their reactivity against the holo-protein. Also, isotype-matched antibodies are commonly used to demonstrate specificity of an antibody. However, this control only excludes unspecific binding mediated via Fc receptors, at best providing a hint for specificity, but certainly not conclusively proving it. A notable exception from this generally unsatisfying situation is the publication by Petit-Bertron *et al.* [147], who evaluated an H_4R antibody by small interfering RNA (siRNA)-mediated knockdown and applied at least one of the criteria required for antibody specificity.

Table I. Four criteria to evaluate the specificity of an antibody

Criterion	Description
Knockout cells	Reactivity of a specific antibody, observed in wild type cells, must be lost upon analysis of cells obtained from animals genetically deficient in expression of the receptor of interest. This method, however, is not applicable to antibodies recognizing human antigens.
siRNA	Reactivity of a specific antibody must distinctly decrease after genetic knockdown of the expression of the receptor of interest.
Related GPCR subtypes	Reactivity of a specific antibody must be present when analyzing cells recombinantly expressing the receptor of interest, but must be absent when analyzing closely related receptor subtypes.
Additional antibodies	Reactivity of a specific antibody must be comparable to that of other antibodies recognizing different epitopes of the same receptor or that of an epitope-tag antibody following recombinant expression of the tagged receptor protein.

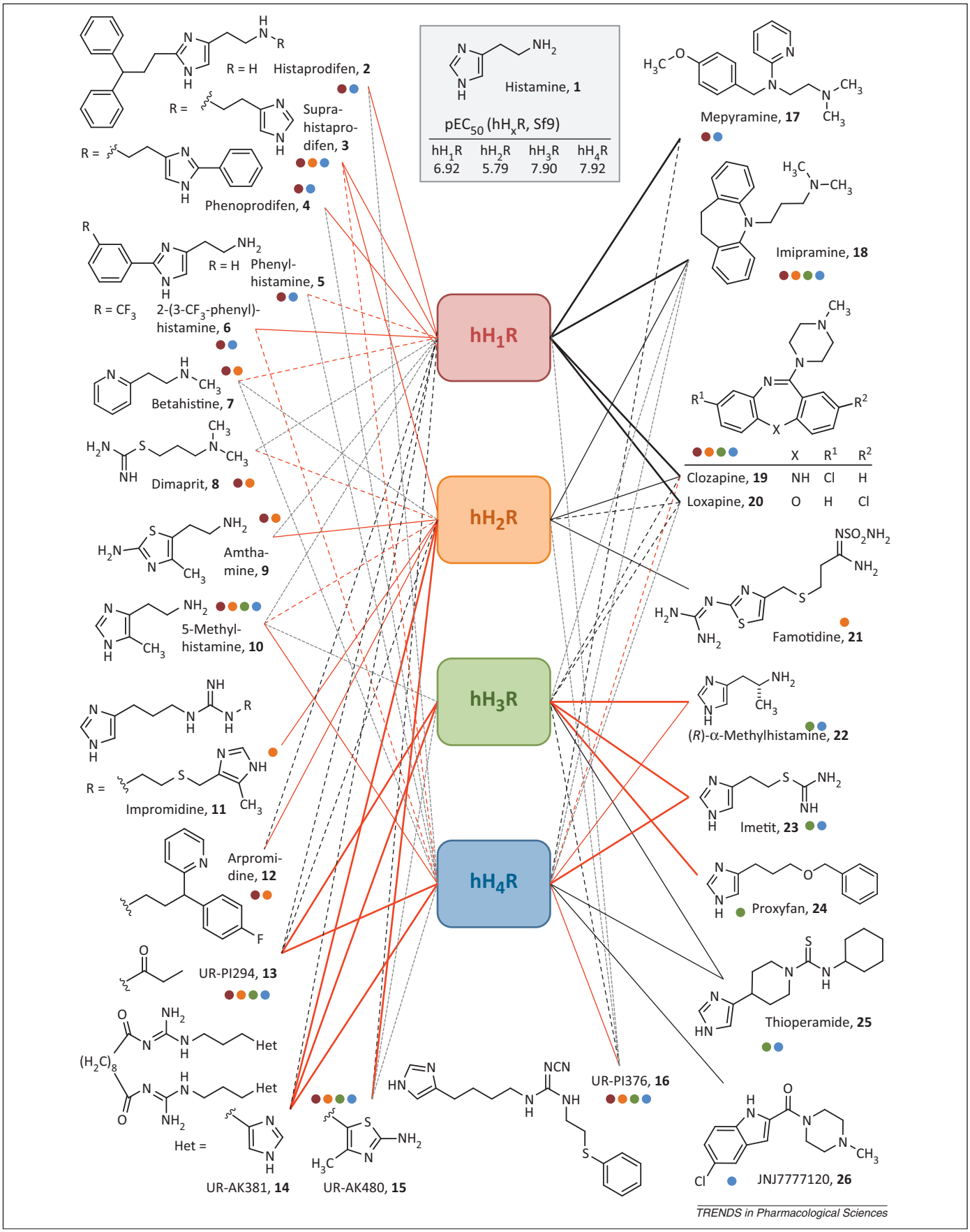


Figure 1. Structures and agonistic/antagonistic activities of selected compounds determined in the GTPase assay on human histamine receptors (hH_xRs) expressed in Sf9 insect cells. Investigations performed in Sf9 cells on the respective H_xR subtype are indicated by the color-coded filled circles. Range of agonistic (red lines) and antagonistic activities (black lines) are indicated as follows: pEC_{50} or pK_B value >8, bold line; 6.5–8.0, thin line; 5.0–6.5, broken line; <5.0, gray broken line. Thickness of lines is based on the data shown in Table 4. Note that within the Sf9 cell system, the pharmacological profiles of several important ligands including dimaprit, amthamine, impromidine, arpromidine,

studies, problems and possible solutions regarding the application of GPCR antibodies are discussed in Box 1.

H_1R is expressed in lymphocytes and monocytes and mediates modulatory effects in the immune system [24]. In immune cells, H_1R is often expressed along with H_2R, complicating detailed pharmacological analysis [24], specifically in light of the fact that there is substantial overlap in interaction of certain ligands with hH_1R and hH_2R (Figure 1). In HL-60 leukemia cells differentiated towards monocytes with 1α,25-dihydroxycholecalciferol and in HL-60 cells differentiated towards neutrophils with dibutyryl-cyclic adenosine 3′,5′-monophosphate (dibutyryl-cAMP), H_1R mediates increases in intracellular calcium concentration ($[Ca^{2+}]_i$) via pertussis toxin-sensitive G_i proteins and pertussis toxin-insensitive mechanisms [25,26]. The biological function of H_1R in HL-60 cells is not yet known. H_1R in HL-60 cells shows some unusual properties such as pronounced noncompetitive antagonism of first-generation H_1R antagonists, poor partial agonism of betahistine and lack of agonism of 2-phenylhistamines [25–27]. In marked contrast, at the recombinant hH_1R expressed in Sf9 insect cells, betahistine and 2-phenylhistamines are potent partial agonists [28]. It is not known whether this is due to low receptor expression in HL-60 cells [29] or due to ligand-specific receptor conformations (also referred to as functional selectivity) [30,31] that differentially direct hH_1R to pertussis toxin-sensitive and -insensitive G proteins.

Analysis of the H_2 receptor in human cells

Among all hH_xRs, hH_2R is the GPCR that can be studied most readily in human cells. Functionally, human neutrophils express H_2R but not H_4R, although at the mRNA level, H_4R is present [11,12,17,32]. The closely related eosinophils express both functional H_2R and H_4R [33]. Neutrophils can be easily obtained in large numbers from peripheral blood and from buffy coat preparations. Hence, in these cells, H_2R has been analyzed in considerable detail. In neutrophils and eosinophils, H_2R mediates increases in cAMP formation and an inhibition of chemotaxis, superoxide anion (O_2^-) formation, and release of cytotoxic enzymes [11,12,32]. The potencies and efficacies of various H_2R agonists with respect to cAMP formation do not match the profile for inhibition of O_2^- formation, suggesting that, in contrast to the general assumption, NADPH oxidase inhibition is not cAMP-mediated [11,12,32,34]. An implication of these data is that various H_2R ligands stabilize distinct active conformations that differentially activate various signaling pathways [30–32]. The existence of ligand-specific receptor conformations is further supported by data from HL-60 leukemia cells that also express H_2R. In these cells, H_2R mediates both an increase in cAMP and in $[Ca^{2+}]_i$, but the potencies and efficacies of various H_2R agonists for both responses are relatively different [35,36]. U937 promonocytes are a model system for AML forms M4/M5 and have been used to study the hH_2R in terms of structure/activity relationships of ligands [37,38], models of receptor activation [39], desensitization [40], gene expression [41], and myeloid differentiation [42].

Analysis of the H_3 receptor in human cells: an elusive goal

H_3R is a receptor exclusively expressed in neurons [43,44], and to the best of our knowledge there is currently no human cell culture model that expresses hH_3R endogenously at sufficient levels to allow for detailed pharmacological studies. To circumvent these methodological problems, hH_3R has to be expressed in mammalian or insect cell expression systems. Studies on hH_3R in mammalian expression systems have been reviewed elsewhere [8,20]. The pharmacological properties of representative hH_xR ligands at hH_3R expressed in mammalian cells are listed in Table 2. Studies on hH_3R in insect cell expression systems are summarized in Tables 3 and 4.

The human H_4 receptor can be studied in eosinophils, but it is a difficult task

The best-studied human cell type expressing H_4R is the eosinophil. Several independent groups have consistently reported on the functional expression of H_4R in human eosinophils [33,45–48]. H_4R mediates increases in $[Ca^{2+}]_i$ and a very moderate activation of chemotaxis but no activation of O_2^- formation or release of cytotoxic enzymes. Thus, in contrast to other typical eosinophil GPCRs such as the eotaxin receptor and formyl peptide receptor, hH_4R mediates only incomplete eosinophil activation. Even blockade of inhibitory hH_2R [32] in eosinophils by famotidine does not largely enhance the stimulatory effect of hH_4R on chemotaxis [33]. These data raise questions

JNJ7777120, thioperamide, proxyfan, famotidine, and mepyramine are still incomplete. However, completion is urgently needed because there is precedence for surprising pharmacological effects of hH_xR ligands [4,28,81]. At hH_1R and hH_2R, the endogenous ligand, histamine (**1**), is approximately 10 and 100 times less potent, respectively, than at hH_3R and hH_4R. Substitution in position 2 of the imidazole ring was described as a key modification to shift the selectivity of histamine towards H_1R compared with H_2R [82,83,86]. As for other known H_xR ligands, the selectivity issue was revisited after the discovery of hH_3R and hH_4R. For instance, studying recombinant hH_xRs expressed in Sf9 cells the 2-phenylhistamines (**5, 6**) and 2-(diphenylpropyl) histamines (**2–4**) turned out to be less selective than previously assumed [4]. Compounds **2–6** also possess remarkable activities at H_4R and/or H_2R. The same holds for other amine-type agonists such as betahistine (**7**), although to a smaller extent. (*R*)-α-Methylhistamine (**22**) and imetit (**23**) are agonists at both hH_3R and hH_4R [55,65]. 5-Methylhistamine (**10**), a molecular tool used by Black *et al.* to define H_2R [86], turned out to be more potent as an agonist at hH_4R [50,55]. The H_2R-agonistic potency of **10** may be of relevance in other species [23], in particular, when **10** is used at high concentrations. Guanidine derivatives such as impromidine (**11**) [167] and arpromidine (**12**) [77] were used as standard H_2R agonists in numerous studies. Meanwhile, imidazolylpropylguanidine moiety was identified as a structural motif also conferring high affinity to hH_xR subtypes other than hH_2R [78,168]. The selectivity profile can be shifted towards H_3R and H_4R by attaching small acyl residues at guanidine-N (**13**). Linkage of two imidazolylpropylguanidine molecules by diacyl spacer groups yielded bivalent ligands with high agonistic potency at hH_2R, hH_3R, and hH_4R. H_2R selectivity was achieved according to the same bioisosteric approach as in the case of amthamine (**9**) [169] compared with histamine: replacement of 4-imidazolyl by 2-amino-5-thiazolyl residue resulted in highly potent and subtype selective H_2R agonists (e.g., **15**) [170,171]. Starting from acylguanidines, H_4R selective agonists such as UR-PI376 [50] were obtained through replacement of guanidine by a cyanoguanidine group, which is completely uncharged at physiological pH. As also demonstrated by proxyfan (**24**) [172], a basic side chain appears to be dispensable in H_3R agonists. Proxyfan has been described to exhibit protean agonism, that is, dependent on the constitutive activity of a given system, the ligand may exhibit agonistic or inverse agonistic activity [173]. At hH_3R expressed in Sf9 cells, there is no evidence for protean agonism or functional selectivity of proxyfan when coupling to G_i/G_o proteins is considered [66]. Owing to the high degree of homology of H_3R and H_4R, numerous H_3R ligands were identified to interact with H_4R as well. Among these ligands are, for example, (*R*)-α-methylhistamine (**22**), imetit (**23**), and thioperamide (**25**) [55,65]. Moreover, H_1R antagonist mepyramine (**17**) was reported to exhibit weak H_4R antagonistic activity [4], and antidepressant or antipsychotic drugs such as imipramine (**18**) [89], clozapine (**19**) [70], and loxapine (**20**) [89] were identified as antagonists or agonists (**19**: H_4R agonist) at hH_xRs with highest activity at hH_1R.

Table 2. Pharmacological properties of ligands at hH_xRs expressed in mammalian expression systems[a]

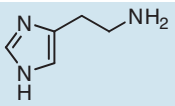
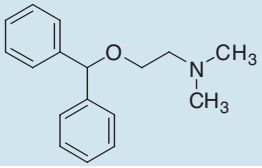
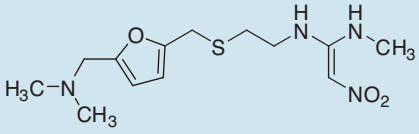
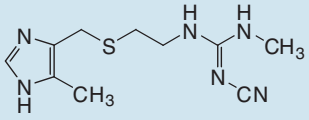
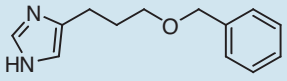
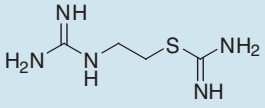
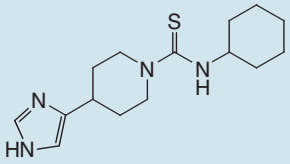
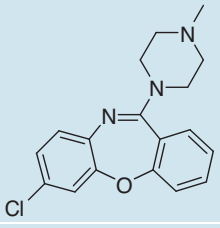
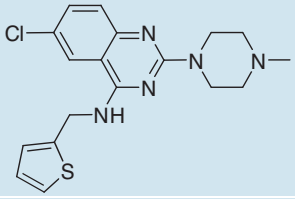
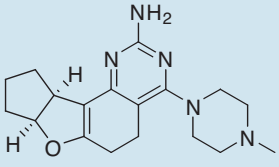
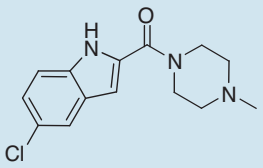
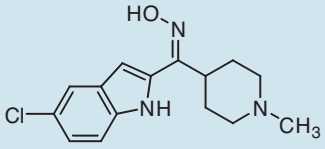
Compound	Structure		pK_i	Expression system	Radioligand for competition binding assay	Refs
1, histamine		**hH_1R**	4.2		[^{3}H]Mepyramine	[55]
		hH_2R	4.3		[^{3}H]Iodoaminopotentidine	
		hH_3R	8.0	Transfected SK-N-MC cells		
		hH_4R	7.8			
27, diphenhydramine		**hH_1R**	7.9			[18]
		hH_2R	<5			
		hH_3R	<5			
		hH_4R	<5			
28, ranitidine		**hH_1R**	<4			[18]
		hH_2R	7.1			
		hH_3R	<5			
		hH_4R	<5			
29, cimetidine		**hH_1R**	<5			[18]
		hH_2R	6.2			
		hH_3R	<5			
		hH_4R	<5			
24, proxyfan		**hH_1R**	n.d.			[55]
		hH_2R	n.d.			
		hH_3R	7.9	Transfected SK-N-MC cells	[^{3}H]N^{α}-Methylhistamine	
		hH_4R	7.3		[^{3}H]Histamine	
30, VUF8430		**hH_1R**	<4	Transfected COS-7 cells	[^{3}H]Mepyramine	[174]
		hH_2R	<4	Transfected CHO cells	[^{3}H]Iodoaminopotentidine	
		hH_3R	6.0	Stably transfected SK-N-MC cells	[^{3}H]N^{α}-Methylhistamine	
		hH_4R	7.5		[^{3}H]Histamine	
25, thioperamide		**hH_1R**	<5			[18]
		hH_2R	<4			
		hH_3R	7.3			
		hH_4R	7.2			
31		**hH_1R**	8.11	Transiently transfected COS-7 cells	[^{3}H]Mepyramine	[175]
		hH_2R	5.05		[^{3}H]Iodoaminopotentidine	
		hH_3R	5.04	Transfected SK-N-MC cells	[^{3}H]N^{α}-Methylhistamine	
		hH_4R	7.55		[^{3}H]Histamine	
32		**hH_1R**	7.70		[^{3}H]Mepyramine	[176]
		hH_2R	n.d.	–	–	
		hH_3R	n.d.	–	–	
		hH_4R	8.12	Transiently transfected HEK293T cells	[^{3}H]Histamine	
33		**hH_1R**	5.45			[177]
		hH_2R	<5.04			
		hH_3R	6.03			
		hH_4R	8.24	Transiently transfected HEK293T cells	[^{3}H]Histamine	

Table 2 (*Continued*)

Compound	Structure		pK_i	Expression system	Radioligand for competition binding assay	Refs
26		**hH$_1$R**	6.01 ($n = 1$), >5[b]	[b]Transfected SK-N-MC cells	[b][^{3}H]Mepyramine	
		hH$_2$R	5.07 ($n = 1$)	[b]Transfected CHO cells		[b][18]
		hH$_3$R	5.65, 5.29[b]	Transiently transfected HEK293T cells, [b]Transfected SK-N-MC cells	[b][^{3}H]N^{α}-Methylhistamine	[177]
		hH$_4$R	7.92, 8.39[b]		[b][^{3}H]Histamine	
34		**hH$_1$R**	<5	Transfected SK-N-MC cells	[^{3}H]Mepyramine	[139]
		hH$_2$R	<6	Transfected CHO cells	[^{3}H]Iodoaminopotentidine	
		hH$_3$R	<5	Transfected SK-N-MC cells	[^{3}H]N^{α}-Methylhistamine	
		hH$_4$R	7.51		[^{3}H]Histamine (10 nM)	

[a]Table 2 summarizes the pharmacological profiles of selected ligands at hH$_x$Rs expressed in mammalian cells. The basis of the comparisons includes radioligand competition binding studies. In this assay, no assessment of ligand efficacies and potencies can be made. It is not trivial to compare potencies and efficacies of ligands in functional assays when mammalian expression systems are considered because the parameters determined for the various receptors are often very different, for example, gene reporter assays versus second messenger accumulation or coupling to a cognate G protein versus coupling to a chimeric G protein [55,58,60,63,69–71]. Also, stoichiometry of signaling components constitutes an issue in determining potencies and efficacies in functional assays [68,89,178]. These problems are addressed in the Sf9 insect cell system, using an identical parameter for all hH$_x$Rs (Tables 3 and 4), although even in this system caution has to be exerted in comparisons [89]. Both in the mammalian expression systems and the Sf9 expression system, pharmacological characterization of ligands at hH$_x$Rs is still incomplete.

[b]All data labeled with b were reported in Ref. [18].

regarding the (patho)physiological relevance of hH$_4$R in eosinophils. Perhaps hH$_4$R possesses greater functional relevance in eosinophils localized in inflamed tissue than in eosinophils isolated from peripheral blood. hH$_4$R is also expressed in mast cells. Thus, it is possible that in human mast cells, H$_4$R is of greater functional importance than in eosinophils, but detailed studies on H$_4$R in human mast cells have yet to come forward [49]. As for hH$_2$R ligands, there is evidence for functional selectivity of hH$_4$R ligands in eosinophils. This has been particularly well elaborated for the potent and selective hH$_4$R agonist UR-PI376 [33,50]. However, a serious drawback of studies with human eosinophils is the fact that it is difficult to obtain these cells in sufficient purity, quantity, and viability [33].

Peripheral human blood monocytes have also been suggested to express H$_4$R [51]. However, it is not certain how specific the H$_4$R antibody used for analysis of H$_4$R expression in these cells was (Box 1) [52–54]. Also, classic proximal H$_4$R-stimulated signaling pathways, specifically increases in $[Ca^{2+}]_i$, have only been incompletely studied so far in monocytes [51]. The signaling pathways leading to inhibition of chemokine CCL2 formation in monocytes also remain to be clarified. Moreover, the exceptionally high potency of clobenpropit ($EC_{50} \sim 3$ pM) with respect to inhibition of CCL2 formation should be noted because this value is approximately four orders of magnitude lower than the corresponding EC_{50} at recombinant hH$_4$R [51,55]. Even if one takes the concept of functional selectivity [33,56] into account, resulting in parameter-dependent differences of ligand potencies, this is an exceptionally large difference. Taken together, the expression of functionally active hH$_4$R in monocytes has to be corroborated in future studies.

Analysis of hH$_x$R subtypes in mammalian expression systems

Based on the limitations of hH$_x$R analysis in native human cells, recombinant systems are essential for comprehensive hH$_x$R characterization. hH$_x$Rs have been expressed in classic mammalian expression systems, including HEK293 cells, CHO cells, NIH-3T3 cells, and COS-7 cells (Table 2) [57–61]. hH$_3$R and hH$_4$R have also been expressed in SK-N-MC cells (Table 2) [55]. In most studies, only one particular hH$_x$R was studied, and there are only a limited number of studies in which several or all hH$_x$Rs were studied side-by-side in one laboratory [44,62]. Table 2 summarizes data on the pharmacological analysis of hH$_x$Rs in mammalian expression systems with a set of selected ligands. There are still important gaps in the systematic characterization of ligands at hH$_x$Rs expressed in mammalian cells. Another problem in the analysis of hH$_x$Rs in mammalian expression systems is the fact that the functional parameters determined for any given hH$_x$R are relatively different and rather distal in the signal transduction cascade. For hH$_1$R, gene reporter assays are widely used [60,63]. In these assays, hH$_1$R exhibits high constitutive activity with most H$_1$R antagonists being inverse agonists [60,63]. However, when a more proximal parameter of the signal transduction cascade was determined in Sf9 insect cell membranes, namely high-affinity GTPase activity, most H$_1$R antagonists still displayed inverse agonistic activity, but the apparent constitutive activity of H$_1$R was very low [28,64], particularly in comparison to hH$_3$R and hH$_4$R [65,66]. This is an excellent illustration of the fact that constitutive activity of a receptor is highly parameter-dependent [67]. Specifically, when

Table 3. Summary of the most important properties of hH_xRs expressed in Sf9 cells[a]

Parameter	hH_1R	hH_2R	hH_3R	hH_4R
Most sensitive system(s)	hH_1R + RGS4/GAIP + insect cell $G\alpha_q$ [28]	hH_2R–$Gs\alpha_s$ fusion protein [74]	hH_3R + $G\alpha_{i2}$ + $G\beta_1\gamma_2$ [66,98]	**S1**[b],: hH_4R + $G\alpha_{i2}$ + $G\beta_1\gamma_2$ [65] **S2**: [b]hH_4R – GAIP + $G\alpha_{i2}$ + $G\beta_1\gamma_2$ [76,168]
High-affinity agonist binding	**No**	**Yes**	**Yes**	**Yes**
GTPγS binding	**No**	**Yes**	**Yes**	**Yes**
Steady-state GTPase	**Yes**	**Yes**	**Yes**	**Yes**
Effector regulation	**No**	**AC**[b] ↑	**No**	**No**
Constitutive activity	**Low** (steady-state GTPase assay)	**Low** (steady-state GTPase assay and [^{35}S]GTPγS binding) **High** in AC assay	**High** (steady-state GTPase assay)	**High** (steady-state GTPase assay and [^{35}S]GTPγS binding)
Regulation by Na^+	Not determined in Sf9 cells	Not determined in Sf9 cells	**Yes**, dependent on the coexpressed Gα subunit [98]	**No**: **S1** [65], **S2** [76]
Radioligands	*Antagonist:* [^{3}H]mepyramine K_d = 4.49 ± 0.35 nM [28] B_{max} = 5.85 ±1.67 pmol/mg [28] nonspecific binding: 10–15% (5 nM) [89]	*Antagonist:* [^{3}H]tiotidine K_d = 32.0 ± 4.6 nM [74] B_{max} = 0.43 ± 0.02 pmol/mg [74] nonspecific binding: 50–60% (20 nM) [89]	*Agonist:* [^{3}H]N^α-methylhistamine K_d = 0.60 ± 0.07 nM [98] B_{max} = 0.62 ± 0.02 pmol/mg [98] nonspecific binding: 20–30% (3 nM) [89]	*Agonist:* [^{3}H]histamine [65] (**S1**) K_d = 9.7 ± 1.7 [65] (**S1**) B_{max} = 1.6 ± 0.3 pmol/mg [65] (**S1**) nonspecific binding: 30% (10 nM) [89]
		Antagonist: [^{3}H]trimipramine K_d = 45.2 nM [89] B_{max} = 0.91 pmol/mg [89] nonspecific binding: 80–90% [89]	*Agonist:* [^{3}H]UR-PI294 [168] K_d = 1.1 ± 0.2 nM [168] B_{max} = 1.4 ± 0.3 pmol/mg [168] nonspecific binding: 5–10% (5 nM) [168]	*Agonist:* [^{3}H]UR-PI294 [168] (**S2**) K_d = 5.1 ± 1.9 nM [168] (**S2**) B_{max} = 2.0 ± 0.1 pmol/mg [168] (**S2**) nonspecific binding: 5–10% (5 nM) [168]
	All binding assays were performed with GF/C filters. In the case of hH_3R and hH_4R binding assays, the filters were pretreated with 0.3% (m/v) polyethyleneimine solution			
Evidence for ligand-specific receptor conformations (examples)	**Yes** N^G-Acylated imidazolylpropylguanidines, but not guanidines, stabilize a partially active state of hH_1R [79]	**Yes** Arpromidine-derived guanidines and N^G-acylated imidazolylpropylguanidines show substantial differences in ternary complex formation and guanine nucleotide sensitivity of ternary complex formation relative to GTPase activation [74,79]	**No** No ligand-specific conformations detected for proxyfan and other imidazole compounds [66]	**Yes** Discrepancies between potency in the GTPase assay and affinity in competition binding observed, e.g., for astemizole and dimeric histaprodifen [4]
Summary of mutagenesis studies	Phe^{153} and Ile^{433}: critical for proper folding and expression of hH_1R [28]	• Ala^{271} (hH_2R) and Asp^{271} (gpH_2R) are responsible for species differences in potency of guanidines [74,179] • H-bond between Tyr^{17} and Asp^{271} (not present in hH_2R) stabilizes active gpH_2R receptor conformation [179]	$Asp80^{2.50}$ is a key residue for hH_3R-mediated $G\alpha_{i3}$ activation. Charge-neutralizing mutation to Asn reduces hH_3R/G protein interaction [98]	• hH_4R-R3.50A mutant represents an inactive state with increased inverse agonist and reduced agonist affinity, showing that R3.50 is crucial for hH_4R–G protein coupling [180] • hH_4R-A6.30E mutant did not show a significant reduction of constitutive activity and G protein coupling efficiency, suggesting limited applicability of the traditional 'ionic lock' model [180]
Summary of chimeric studies	Exchange of hH_1R E2 loop or hH_1R E2 loop plus N terminus against the corresponding gpH_1R sequence ligand specifically influence H_1R pharmacology. Increased similarity of hH_1R mutants with gpH_1R was not reflected by pK_i and pEC_{50} values of phenoprodifens [181] The mutant receptors show an alteration of extracellular receptor surface, which changes ligand binding kinetics and affinity [182]	Chimeras of N-terminal half of hH_2R or gpH_2R and C-terminal half of gpH_2R or hH_2R, fused to $Gs\alpha_S$ confirm the role of position 271 for the pharmacological differences between hH_2R and gpH_2R [74]	No chimeric studies in Sf9 cells performed	The hH_4R N-terminus was replaced with the corresponding canine H_4R (cH_4R) sequence to yield $h_{cNT}H_4R$. A replacement of the hH_4R extracellular loops E1, E2 and E3 by the canine sequences resulted in $h_{cE1}H_4R$, $h_{cE2}H_4R$ and $h_{cE3}H_4R$, respectively. Exchange of E3 reduced the potency of histamine and 5-methylhistamine (**S1**). Alteration of E2 or E3 reduced the efficacy of thioperamide and converted the partial inverse agonist JNJ7777120 to a partial agonist (GTPase assay, System **S1**), indicating a role of E2- and E3 in H_4R activation induced by specific ligands [183].

Structural stability	Not determined in Sf9 cells		Not determined in Sf9 cells		Not determined in Sf9 cells		Structurally unstable: 45 min incubation at 37 °C leads to a loss of ~60% of binding sites. The receptor is stabilized by histamine and to an even larger extent by thioperamide [65]	
Impact of G protein on pharmacology	Interaction of hH_1R with $G\alpha_i$ or $G\alpha_s$ not investigated in Sf9 cells; effective coupling to Sf9 cell $G\alpha_q$; mammalian $G\alpha_q$ expression in Sf9 cells failed [73]		Unlike β_2AR[b] [184], hH_2R does not show differences in constitutive activity, when fused to $Gs\alpha_S$ or $Gs\alpha_L$ [75]		• Coexpression of hH_3R and $G\alpha_{i3}$ shows the highest NaCl sensitivity compared with $G\alpha_{i1}$, $G\alpha_{i2}$, or $G\alpha_o$ (GTPase assays) [98] • No substantial influence of G protein isoform ($G\alpha_{i1-3}$ or $G\alpha_o$) on histamine stimulation or thioperamide inhibition of steady-state GTPase signals [66] • Pharmacological profile of hH_3R for imidazole ligands is similar when coexpressed with $G\alpha_{i1-3}$ or $G\alpha_o$ [66]		• hH_4R activates $G\alpha_{i/o}$ proteins in the steady-state GTPase assay with the following specificity: $G\alpha_{i2} > G\alpha_{i1} \sim G\alpha_{i3} > G\alpha_o$. Fusion of hH_4R to GAIP increases signal-to-noise ratio without changing G protein specificity [76] • A weak interaction with insect cell $G\alpha_q$ is unmasked in hH_4R–GAIP fusion protein (system **S2**), compared with system **S1** [76]	
	Sf9 cells expressing guinea pig H_1R or rat H_2R showed a histamine-induced rise in $[Ca^{2+}]_i$ and phospholipase C activity [185–187] These results could not be reproduced in another study [73]. The reason for these contradictory results is unknown, but it may have been caused by subtle differences between laboratories, e.g., in cell culture or experimental conditions							
K_i values for prototypical ligands	Histamine	2.06 ± 0.18 μM [28]	Histamine	Not determined	Histamine	6.31 nM [188][c]	Histamine	K_d = 9.7 ± 1.7 nM [65] (**S1**)
	Methylhistaprodifen	0.37 ± 0.07 μM [28]	Impromidine	64 nM (low affinity state) [74]	(*R*)-α-Methylhistamine	1.23 nM [188][c]	JNJ 7777120	32 ± 7 nM [65] (**S1**)
	Triprolidine	3.01 ± 0.54 nM [28]	Arpromidine	11 nM (high affinity state), 461 nM (low affinity state) [74]	Imetit	0.63 nM [188][c]	Thioperamide	106 ± 21 nM [65] (**S1**)
					Thioperamide	45.7 nM [188][c]		
K_i values in mammalian systems (for comparison)	CHO-hH_1R cells, [^{3}H]mepyramine [189]		Owing to extremely high nonspecific binding (up to 60%) → [^{3}H]tiotidine was only rarely used in binding studies. [^{3}H]Trimipramine showed even higher nonspecific binding [89] Examples: membranes from left guinea pig atrium [190], H_2R transfected Colo-320DM cells [191], or MKN-45 human gastric carcinoma cells [192]. U937-promonocytes [39] There is no comparison of H_2R ligand affinity with [^{3}H]tiotidine in mammalian systems versus Sf9 cells in the literature		CHO-K1-hH_3R cells, [^{125}I]iodoproxyfan binding [193]:		HEK 293-hH_4R cells, [^{3}H]histamine [194]:	
	Histamine	1.26 μM[c]			Histamine	13 ± 2 nM	Histamine	4.7 ± 0.3 nM
	Triprolidine	1.0 nM[c]			(*R*)-α-Methylhistamine	2.7 ± 0.9 nM	Thioperamide	43 ± 3 nM
					Thioperamide	60 ± 12 nM	SK-N-MC-hH_4R cells, [^{3}H]histamine [55]	
							JNJ7777120	15.8 nM
K_i versus K_B	K_i and K_B are very similar in most cases. Exception: K_i is 5- to 7-fold higher than K_B for BU-E-84 and Bu-E-82 [195]		No Sf9 cell data available		No Sf9 cell data available		Subset of JNJ 777120-related partial inverse agonists: large differences between K_i ([^{3}H]-histamine binding) and EC_{50} values (steady-state GTPase). K_i and K_B values are similar [96]	
Impact of fusion, coexpression on pharmacology	Increased steady-state GTPase signal after coexpression with RGS proteins (unmasking of hH_1R/insect cell $G\alpha_q$ interaction), RGS4 > GAIP [73]		Fusion to $Gs\alpha_S$ increases AC[d] activation [73], compared with coexpression		• Coexpression systems: large differences in $hH_3R/G\alpha$ coupling stoichiometry: $G\alpha_{o1}$ (1:11) > $G\alpha_{i2}$ (1:6) > $G\alpha_{i3}$ (1:3) > $G_{\alpha i1}$ (1:2) [66] • Similar pharmacological profiles of standard ligands at hH_3R–$G\alpha_{i2}$ and hH_3R–$G\alpha_{o1}$ fusion proteins in the GTPase assay → stoichiometry differences in the coexpression system have no impact on the pharmacological profile of hH_3R [66]		• hH_4R–GAIP fusion protein shows increased agonist signal-to-noise ratio in the GTPase assay, compared with hH_4R coexpressed with $G\alpha_{i2}$ and $G\beta_1\gamma_2$ [76] • hH_4R–$G\alpha_{i2}$ fusion protein shows no improvement of agonist signal-to-noise ratio in the GTPase assay, compared with the coexpression system	

Table 3 (*Continued*)

Parameter	hH_1R	hH_2R	hH_3R	hH_4R
Running behavior of receptor protein on western blots	Two glycosylation sites at the N terminus and one in the E2 loop, band appears at ~85 kDa [181] (predicted M_W: ~ 56 kDa)	hH_2R–$Gs\alpha_s$ runs at ~80 kDa. Predicted M_W: 86 kDa (40 kDa for hH_2R and 46 kDa for $Gs\alpha_s$). Diffuse bands indicate differently glycosylated forms [196]	The hH_3R band appears exactly at the predicted size of ~49 kDa. Doublets probably represent differently glycosylated forms [187]	Different glycosylation states or conformations at 43 and 46 kDa. Additional weak bands between 26 and 35 kDa may represent atypically migrating species [65] Predicted M_W: ~44.5 kDa
Additional comments, advantages, and disadvantages of the system	• hH_1R cannot be reconstituted with mammalian $G\alpha_q$ in Sf9 cells. RGS proteins have to be coexpressed that unmask the interaction of hH_1R with insect cell $G\alpha_q$ [73] • Specifically in the case of cannabinoid CB_2R, fusion to $G\alpha_{i2}$ and coexpression with RGS4 can alter the pharmacological properties of ligands [197] • Effector coupling to PLC/Ca^{2+} signaling cannot be reconstituted (this is controversially discussed in the literature [73,185–187]). For more information on $G\alpha_q$ coupling of hH_1R and hH_2R, cf. [73]	• Fusion of hH_2R with $Gs\alpha_s$ increases signal intensity in AC assays, compared with coexpression systems [73] • [^{3}H]Tiotidine binding shows low signal-to-noise ratio, requiring high protein amounts (>200 μg) [74,75] • [^{3}H]Trimipramine showed up to 90% nonspecific binding [89] → No optimal radioligand available • AC coupling of hH_2R shows only poor efficiency, because the number of AC molecules is the limiting factor and hH_2R has not yet been reconstituted with mammalian ACs	• High constitutive activity causes reduced relative intensity of agonist signals in GTPase assays [66] • The ternary complex of hH_3R is GTPγS-insensitive ([^{3}H]N^{α}-methylhistamine binding, [188]) • Membranes expressing hH_3R without mammalian G proteins were not characterized in radioligand binding assays	• High constitutive activity leads to low signal-to-noise ratio (histamine-induced relative signal in GTPase assays ~25% above baseline in S1 [65] and ~50% in S2 [76]) • G protein-independent high-affinity state precludes determination of ternary complex formation in high-affinity agonist binding [65]
			No reconstitution of effector coupling achieved for hH_3R and hH_4R in insect cells	
	Advantage of all systems: under optimized conditions, a very proximal parameter (G protein activation) can be investigated before signal amplification occurs in the signaling cascade This enables the quantification of fine differences in ligand efficacies not visible in other systems			

[a]It is difficult to conduct a comprehensive pharmacological characterization of hH_xRs in native human cells (Table 1). In principle, a comparison of hH_xRs can be conducted in mammalian expression systems, radioligand competition binding being the most feasible parameter (Table 2). However, comparison of hH_xRs in mammalian cells at the functional level already becomes more difficult because the parameters and G proteins are fairly different, introducing bias into the analysis. The Sf9 cell system is suitable for the comparison of hH_xRs both at the level of radioligand competition binding and at the functional level, steady-state GTPase activity being a common parameter determined for all hHxRs. A general discussion on the usefulness and specific advantages and disadvantages of Sf9 cells as an expression system for GPCRs and the transfer of data from this system to mammalian cells has recently been presented [198]. The purpose of Table 3 is to compare the specific properties of hH_xRs expressed in Sf9 cells. Epitope-tagged hH_xRs expressed in Sf9 cells have also been most helpful for unmasking the lack of specificity of commercial H_4R antibodies [52,54] (Box 1). Protocols of pharmacological methods that can be applied for analysis of hH_xRs expressed in Sf9 cells have recently been reviewed [199,200]. The pharmacological data for selected ligands obtained in this expression system are listed in Table 4.

[b]Abbreviations: AC, adenylyl cyclase; β_2AR, β_2-adrenoceptor; **S1**, System 1; **S2**, System 2.

[c]Calculated from the pK_i values given in this publication.

Table 4. Pharmacological properties of ligands at hH_xRs expressed in Sf9 cells in the GTPase assay and radioligand competition binding assays[a]

Compound	Compound name (Refs)	hH_1R + RGS4			hH_2R–$G_{s\alpha S}$			hH_3R + $G_{\alpha i2}$ + $G_{\beta 1\gamma 2}$			hH_4R–GAIP + $G_{\alpha i2}$ + $G_{\beta 1\gamma 2}$		
		pEC_{50} (pK_B)	E_{max}	pK_i	pEC_{50} (pK_B)	E_{max}	pK_i	pEC_{50} (pK_B)	E_{max}	pK_i	pEC_{50} (pK_B)	E_{max}	pK_i
1	**Histamine** [28,64,66,74,81,183,188,196]	6.92 [64] 6.74 [28]	1.00	5.62 [64] 5.69 [28]	6.00 [64] 5.90 [74]	1.00	n.d.	7.60 [81] 7.90 [66]	1.00	8.20	7.92 [81] 7.60 [183]	1.00	7.89
2	**Histaprodifen** [64,84]	6.95	0.62	6.47	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.44	–0.48	4.56
3	**Suprahistaprodifen** [4,28,64,178]	6.62	0.84	6.33	6.24 [28] 6.62 [178]	0.39 [28] 0.54 [178]	n.d.	n.d.	n.d.	n.d.	5.82	0.25	5.77
4	**Phenoprodifen** [28,84]	6.67	0.52	6.60	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.60	–0.63	4.95
5	**Phenylhistamine** [4,28,201]	6.06 [28] 6.14 [201]	0.79 [28] 0.72 [201]	5.36	4.41	0.20	n.d.	n.d.	n.d.	n.d.	4.92	0.32	4.79
6	**2-(3-Trifluoromethylphenyl) histamine** [4,28,201]	6.61 [28] 6.71 [201]	0.74 [28] 0.61 [201]	5.83	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5.83	0.51	5.91
7	**Betahistine** [4,28,74]	5.84	0.86	n.d.	4.47	0.73	n.d.	n.d.	n.d.	n.d.	<4	0.37	4.09
8	**Dimaprit** [28,74,196]	n.d.	0.06	n.d.	5.71 [74] 6.04 [196]	0.85		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
9	**Amthamine** [28,74,196]	n.d.	0.01	n.d.	6.35 [74] 6.72 [196]	0.90 0.91	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
10	**5-Methylhistamine** [50,65,183]	4.80	0.90	n.d.	5.54	1.01	n.d.	n.d.	No agonistic activity up to a concentration of 1 mM	n.d.	7.15 [50] 7.49 [65] 7.11 [183]	0.90 [50] 0.87 [65] 1.03 [183]	7.61
11	**Impromidine** [74,196]	n.d.	n.d.	n.d.	6.70 [74] 6.80 [196]	0.84 [74] 0.82 [196]		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
12	**Arpromidine** [28,74,196]	(6.48)	n.d.	6.45	6.72 [74] 7.14 [196]	0.79 [74] 0.84 [196]		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
13	**UR-PI294** [81]	5.46	0.30	n.d.	6.43	0.83	n.d.	8.80	0.39	n.d.	8.52	0.90	n.d.
14	**UR-AK381** [171]	(6.32)	n.d.	n.d.	8.21	0.81	n.d.	8.75	0.63	n.d.	8.07	0.44	n.d.
15	**UR-AK480** [171]	(6.01)	n.d.	n.d.	8.11	0.53	n.d.	(<5)	n.d.	n.d.	(<6)	n.d.	n.d.
16	**UR-PI376** [50]	(<5)	0.07	4.59	(<5)	0.08	5.39	(6.00)	–0.28	6.28	7.47	0.93	7.24
17	**Mepyramine** [4,28,202]	(8.25)	n.d.	8.35	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5.17	–0.21	<4
18	**Imipramine** [89]	(8.25)	–0.10	8.12	(6.10)	–0.13	6.26	<4 (pIC_{50})	–0.54	<4	<4	–0.78	4.62
19	**Clozapine** [89]	(8.36)	–0.04	8.58	(6.28)	–0.09		<4 (pIC_{50})	–0.49	<4	5.78	0.66	5.93
20	**Loxapine** [89]	(8.14)	–0.07	8.65	(5.91)	–0.10		<4 (pIC_{50})	–0.71	4.26	<4	Ineffective	5.06
21	**Famotidine** [74,178]	n.d.	n.d.	n.d.	7.32 [178] 7.53 [74]	–0.10 [178] –0.07 [74]		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
22	**(*R*)-α-Methylhistamine** [65,188]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	8.91	6.56	0.92	n.d.

Table 4 (*Continued*)

Compound	Compound name (Refs)	hH_1R + RGS4			hH_2R–$G_{s\alpha S}$			hH_3R + $G_{\alpha i2}$ + $G_{\beta 1\gamma 2}$			hH_4R–GAIP + $G_{\alpha i2}$ + $G_{\beta 1\gamma 2}$		
		pEC_{50} (pK_B)	E_{max}	pK_i	pEC_{50} (pK_B)	E_{max}	pK_i	pEC_{50} (pK_B)	E_{max}	pK_i	pEC_{50} (pK_B)	E_{max}	pK_i
23	**Imetit** [65,188]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	9.20	8.17	0.69	n.d.
24	**Proxyfan** [188]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	7.87	n.d.	n.d.	n.d.
25	**Thioperamide** [50,65,81,183,188]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	7.01	–0.71	7.34	6.96 [81] 7.01 [65] 6.87 [183]	–0.95 [81] –1.00 [65] –0.87 [183]	6.94
26	**JNJ7777120** [65,183,203]	n.d.	n.d.	4.33	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	7.42 [65] 8.25 [183]	–0.31 [65] –0.16 [183]	7.49

[a]Table 4 summarizes the pharmacological data for selected hH_4R ligands at hH_xRs expressed in Sf9 cells. The structures of ligands are depicted in Figure 1. It should be noted that even in this system, data sets are far from complete. In some cases, the incompleteness is due to the fact that when a specific study was performed, the other relevant hH_xRs were not yet available. In other cases, data are incomplete because of the tacit assumption that data regarding receptor selectivity of ligands in standard test systems (often animal organ experiments) can be transferred to Sf9 cells. Now, we are aware of the fact that this assumption is incorrect, and we aim at completing the data sets for all ligands at all hH_xRs. In several studies, data for key ligands were reproduced to avoid making reference to 'historic data'. The data obtained from independent analyses are listed separately with the corresponding reference, and no mean values are calculated to allow an estimate of data variability over a period of up to several years. Data reproducibility is very good. Hence, the Sf9 cell system is very robust at serving as a reference system for ligand analysis at hH_xRs. By contrast, in native systems, for example, neutrophils, data variability for hH_2R characterization over a long period of time has been greater, different isolation methods of cells being one possible variable [11,12,33]. Abbreviation: n.d., not determined.

constitutive activity is determined at a level where the availability of a signal transduction component is limiting, constitutive activity appears to be very high [68].

hH_2R activity has been classically determined by the cAMP accumulation assay [58,61]. The cAMP accumulation assay is certainly of physiological relevance. With regard to the G_i/G_o-protein-coupled hH_3R and hH_4R, commonly determined parameters are the inhibition of forskolin-stimulated cAMP accumulation and cAMP-regulated gene expression [55,59,69,70], but because forskolin is not an endogenous constituent of the human body, the physiological relevance of these assay formats is unclear. Another parameter determined in mammalian expression systems is GPCR-mediated β-arrestin activation [71]. Because ligands may differ in their efficacy at activating G protein- and β-arrestin-dependent pathways [72], this assay is particularly important for studying the concept of functional selectivity. So far, the β-arrestin pathway has only been assessed for hH_4R [71] but not other hH_xRs. These studies revealed that the prototypical hH_4R antagonist JNJ7777120 behaves as a partial agonist with respect to β-arrestin activation [71]. The relevance of these data for the situation in native cells is still elusive [33,56]. One also has to keep in mind that the current focus on β-arrestin-dependent pathways in context with functional selectivity is driven, at least in part, by the availability of experimental techniques. Other pathways relevant for functional selectivity may be overlooked, introducing bias into data analysis.

Analysis of human histamine receptor subtypes in *Spodoptera frugiperda* Sf9 insect cells

In Sf9 cells, all hH_xRs were functionally reconstituted with G proteins, and unlike in mammalian expression systems, all hH_xRs could be studied using one and the same parameter, namely high-affinity GTPase activity. Table 3 summarizes the most important properties of hH_xR expression systems in Sf9 insect cells, and Table 4 summarizes the results of the pharmacological analysis of representative hH_xR ligands in this system. This analysis reveals that, despite intense research in this field for more than a decade, complete data sets for all hH_xRs are available only for a minority of ligands. Moreover, there is considerable overlap in the interactions of ligand classes with hH_xRs (Figure 1).

Although GTPase activity can be determined for all hH_xRs, nonetheless, the systems are not identical. Specifically, for hH_1R, coupling to insect cell G_q proteins is determined because mammalian G_q proteins cannot be expressed in functionally active form in Sf9 cells [73]. In addition, because the concentration of insect cell G_q proteins is low, GTP hydrolysis must be enhanced with regulator of G protein signaling (RGS) proteins RGS4 or RGS19, indicating that under these circumstances, GTP hydrolysis becomes the rate-limiting step of the G protein cycle [73]. For analysis of hH_2R at the GTPase level, fusion of the GPCR to the short splice variant of $G_s\alpha$, $G_s\alpha_S$, or the long splice variant of $G_s\alpha$, $G_s\alpha_L$, is necessary [74,75]. For hH_3R, coexpression of the GPCR with $G_i\alpha_1$, $G_i\alpha_2$, $G_i\alpha_3$, or $G_o\alpha_1$ in conjunction with $G\beta_1\gamma_2$ was used. There is no evidence for functional selectivity of hH_3R ligands in these

systems [66]. However, these 'negative' findings do not preclude functional selectivity of hH_3R ligands because several variables such as distinct $\beta_x\gamma_y$ complexes or different RGS proteins have yet to be examined. Lastly, for hH_4R, the most effective analysis system is represented by a fusion protein of hH_4R with RGS19, also referred to as GAIP, in coexpression with $G_i\alpha_2$ and $G\beta_1\gamma_2$ [76].

Overlap in interaction of ligands with human histamine receptor subtypes

As was already observed for native H_1R and H_2R in guinea pig organs [77], arpromidine and related imidazolylpropylguanidines bearing an H_1R antagonist-derived moiety at the guanidine group instead of the cimetidine-like portion of impromidine are dual hH_1R antagonists/hH_2R agonists [28,74] (Figure 1 and Table 4). Such a pharmacological profile may actually be clinically useful because H_2R activation results in anti-inflammatory effects (Table 1). Hence, dual H_1R antagonists/H_2R agonists could be superior to H_1R antagonists in type I allergies.

Compared to arpromidine-derived guanidines, corresponding acylated imidazolylpropylguanidines (AIPGs) are considerably less basic, show better absorption following oral administration, and penetration of the BBB [78]. Although AIPGs are potent hH_2R agonists, some AIPG derivatives are also rather potent hH_1R agonists [79,80]. Moreover, AIPGs were the starting point for the development of a novel class of potent hH_4R agonists [81]. However, these compounds also exhibit agonistic activity at hH_3R (Figure 1 and Table 4) [81]. The selectivity of AIPG-derived compounds for H_2R was dramatically increased by bioisosteric replacement of the imidazole ring by a 2-aminothiazole moiety as in amthamine (cf. bivalent ligand **15** vs **14**, Figure 1, Table 4).

The 2-phenylhistamines and histaprodifens were originally designated as potent and selective H_1R agonists [82,83]. However, a more detailed analysis revealed that certain histaprodifens are also fairly potent partial hH_2R agonists [28]. Moreover, compounds of these two classes turned out to be either hH_4R agonists or hH_4R inverse agonists [3,84]. Along the same line, overlap in pharmacology between hH_1R and hH_4R is also an issue with first- and second-generation H_1R antagonists, although there is still unresolved controversy on this point [3,85]. Several of these compounds are low-potency hH_4R antagonists/inverse agonists (Figure 1 and Table 4).

The case of 5-methylhistamine is an informative example of the ambiguity of the term 'receptor subtype selectivity'. Originally, 5-methylhistamine (also designated as 4-methylhistamine) was one of the molecular tools used to pharmacologically define H_2R using the electrically stimulated rat uterus, rat gastric acid secretion, and the spontaneously beating isolated guinea pig atrium as biological systems [86]. At recombinant hH_4R expressed in Sf9 cells, 5-methylhistamine is ~15-fold more potent than at hH_2R (Table 4) [50,65]. In experiments with native cells, 5-methylhistamine is typically used at a concentration of 10 μM, assuming that hH_4R selectivity is preserved [51,87]. However, at this high concentration, considerable activation of hH_2R can take place (Table 4) so that rigorous discrimination between hH_2R and hH_4R is ambiguous unless inhibition studies of 5-methylhistamine effects with both H_2R antagonists and H_4R antagonists are performed. So far, in most studies, these important control studies combining supposedly selective agonists with antagonists have not been routinely performed. A suitable alternative for the use of 5-methylhistamine is the cyanoguanidine UR-PI376 that exhibits potent H_4R-agonistic effects at native hH_4R in eosinophils and at recombinantly expressed hH_4R [33,50]. However, because of the issue of functional selectivity, potencies of UR-PI376 can vary by up to 40-fold, depending on which specific parameter in which cell type is determined [33]. Hence, to exclude unexpected off-target interactions of UR-PI376 with hH_xRs other than hH_4R, other (biogenic amine) GPCRs than hH_xRs or even non-GPCR targets, specificity of the agonist was corroborated by blocking its effects with the H_4R antagonist JNJ7777120 [33]. To exclude off-target effects of ligands and data misinterpretation it is also highly advisable to examine antagonists from structurally distinct chemical classes. Taken together, these results show that characterization of hH_4R in native human cells with ligands is more challenging and requires much more experimental effort than it appears at first glance. Coexpression of multiple hH_xRs, lack of high receptor subtype-selectivity, and functional selectivity constitute potential confounding issues of ligands used.

Clozapine is an atypical antipsychotic with considerable affinity for numerous GPCRs [88]. Owing to its pleiotropic effects, the precise molecular mechanism of action in psychiatric diseases is still not known [88]. Clozapine is an antagonist at hH_1R and hH_2R and a partial hH_4R agonist (Figure 1) [55,89]. A comparison of the pK_b values of clozapine for hH_1R and hH_2R and pEC_{50} values for hH_4R with therapeutic plasma concentrations indicates that interaction of clozapine with all of the three mentioned hH_xRs can occur under clinical conditions [89]. Obesity in clozapine-treated patients could be related to H_1R antagonism [90]. It remains to be determined if hH_2R antagonism of clozapine and partial hH_4R agonism is of relevance for agranulocytosis in clozapine-treated patients, constituting the most serious adverse effect of the drug [89,91].

Comparison of hH_xR ligand binding sites

Figure 2 shows an alignment of transmembrane (TM) regions and extracellular loops (ECLs) of hH_xRs and hH_xR models docked with selected ligands from Figure 1. The alignment and phylogenetic trees indicate that hH_xRs are rather distantly related, with only H_3R and H_4R showing a high degree of sequence similarity. Nevertheless, the binding sites of all hH_xRs are structurally and spatially similar, leading to overlaps in ligand interactions and complicating the design of selective agonists and antagonists.

A common hH_xR binding site may be defined, consisting of an identical negatively charged anchor (D3.32), a hydrophobic pocket (TM3, 6), subtype-specific residues in TM5 responsible for agonistic activity by selective interactions with the imidazole moiety of histamine and with other bioisosteric groups, and an affinity-conferring pocket mainly formed by amino acids of TM2, 3, and 7.

Nearly all known ligands interact with D3.32. However, a basic moiety is not required. The docking mode of

thioperamide on hH_3R indicates that a salt bridge may be replaced by charge-assisted hydrogen bond(s). Hydrophobic interactions with Y3.33 (hH_2R: V) and Y6.51 occur in all subtypes. Position 6.55 probably contributes to H_3R/H_4R selectivity (M vs T). For some ligands, the hydrophobic pocket extends to residues 6.44, 6.48, 6.52, and 6.54. C3.36 (hH_1R: S) does also belong to the binding site, suggested to discriminate via *cis* and *trans* configuration between active and inactive states [92].

Supported by results from *in vitro* mutagenesis studies, it is assumed that imidazolyl and similar groups form bidentate hydrogen bonds with specific residues in partially different positions of TM5 – hH_1R: K5.39, N5.46; hH_2R: D5.42, T5.46 (or Y5.38, D5.42 [93]); hH_3R: S5.43 (not supported by binding to the S5.43A mutant), E5.46; hH_4R: T5.42 (and/or S5.43), E5.46. However, in the case of hH_3R and hH_4R, a 'reverse' binding mode of histamine may be proposed with a salt bridge between the protonated

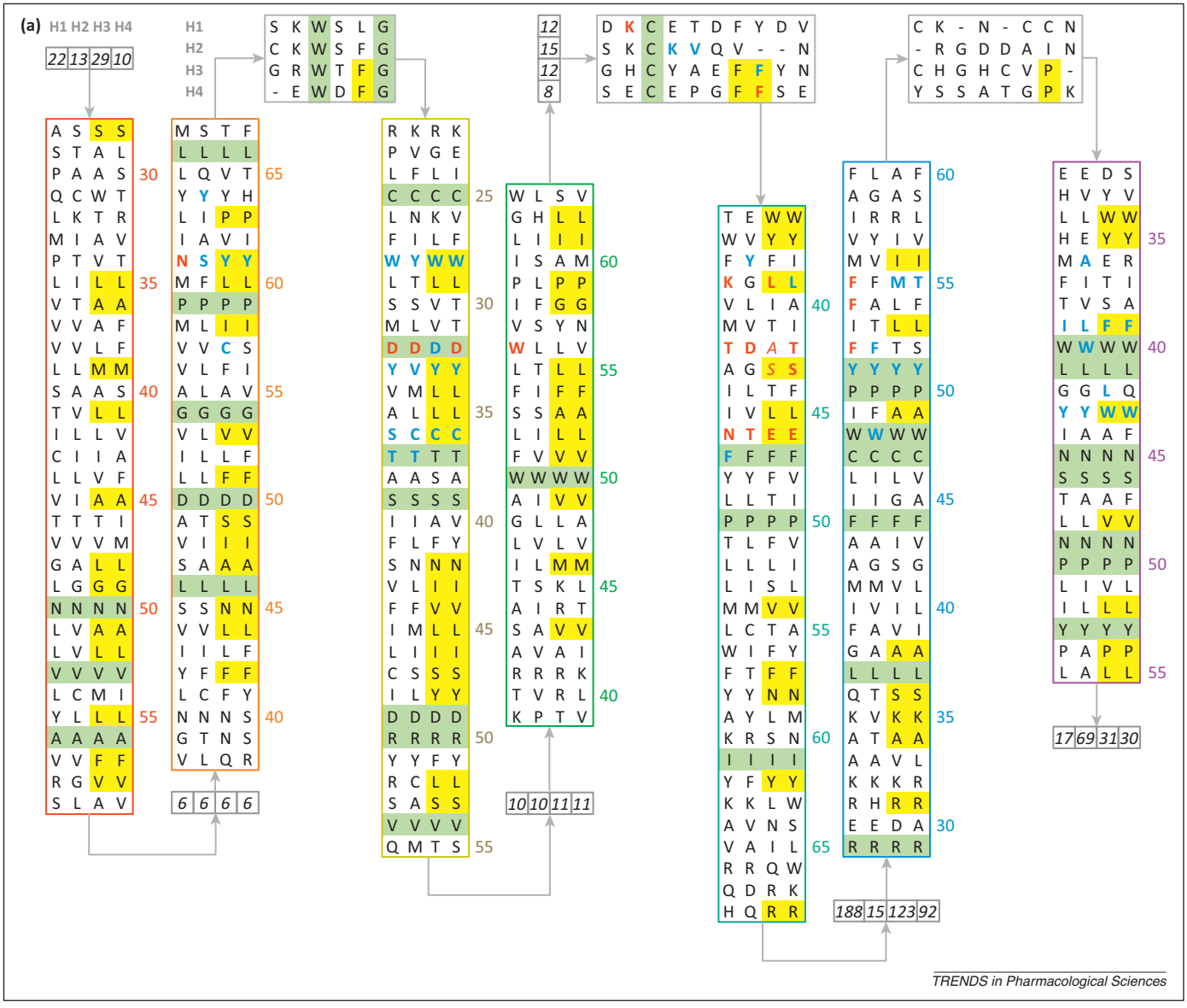


Figure 2. Alignment of human histamine receptors, hH_xRs, and models of binding sites with docked ligands. **(a)** Alignment of transmembrane (TM) regions and extracellular loops (ECLs) of hH_xRs. N- and C-termini, intracellular loops, and parts of ECL2 are only represented by their lengths (count of amino acids). TM residues are numbered according to Ballesteros and Weinstein [204] with no. 50 for the most conserved residue in each TM. The order is always hH_1R, hH_2R, hH_3R, hH_4R from left to right (TM) and from top to bottom (ECL), respectively. Green bars represent identical residues in all hH_xRs, yellow bars additional identities of hH_3R and hH_4R. Amino acids belonging to the ligand binding sites are in bold red if verified by *in vitro* mutagenesis (hH_1R [205–208], hH_2R [209], hH_3R [210], hH_4R [211,212]) or bold blue if proven by X-ray structure (hH_1R [213]) or suggested by homology models (other receptor subtypes). Residues where mutants did not affect ligand binding are in italics. The vertical arrangement of the TMs is based on a horizontal line through D3.32 parallel to the membrane, resulting from comparison of the 3D structures. The identities of TM residues amount to 35.7% for hH_1R versus hH_2R, 32.4% for hH_1R versus hH_3R, 29.5% for hH_1R versus hH_4R, 31.0% for hH_2R versus hH_3R, 26.7% for hH_2R versus hH_4R, and 53.3% for hH_3R versus hH_4R. **(b–e)** Models of hH_xR binding sites with docked ligands, presented in stereo view (parallel mode). Source of the models: hH_1R – X-ray structure [213], hH_2R, hH_3R, hH_4R – X-ray structures of the inactive [214] or active [215] β_2-adrenergic receptor in the case of antagonists and agonists, respectively. The ligands were manually docked and the models in turn minimized with Amber-FF99 [216] (receptor with fixed ligand) and Tripos force field [217] (ligand and a 'hot' receptor region of amino acids up to 3 Å around) using the modeling suite Sybyl 8.0 (Tripos, L.P., St Louis, MO, USA). Shown are the side chains and Cα atoms of all amino acids of the binding sites as sticks and the Cα trace of the TM regions as lines. The backbone and the carbon atoms of the amino acids are individually drawn in spectral colors (cf. panel a): TM2, orange; TM3, yellow; ECL2, cyan; TM4, green; TM5, green–blue; TM6, blue; TM7, magenta. Labels correspond to the Ballesteros and Weinstein nomenclature (except ECL2 residues). The ligands are presented as ball and stick models with gray C atoms. Other ligand and receptor atom colors: nitrogen, blue; oxygen, red; sulfur, yellow; chlorine, green. (b) hH_1R in complex with imipramine. (c) hH_2R in complex with arpromidine. (d) hH_3R in complex with thioperamide. (e) hH_4R in complex with UR-PI294.

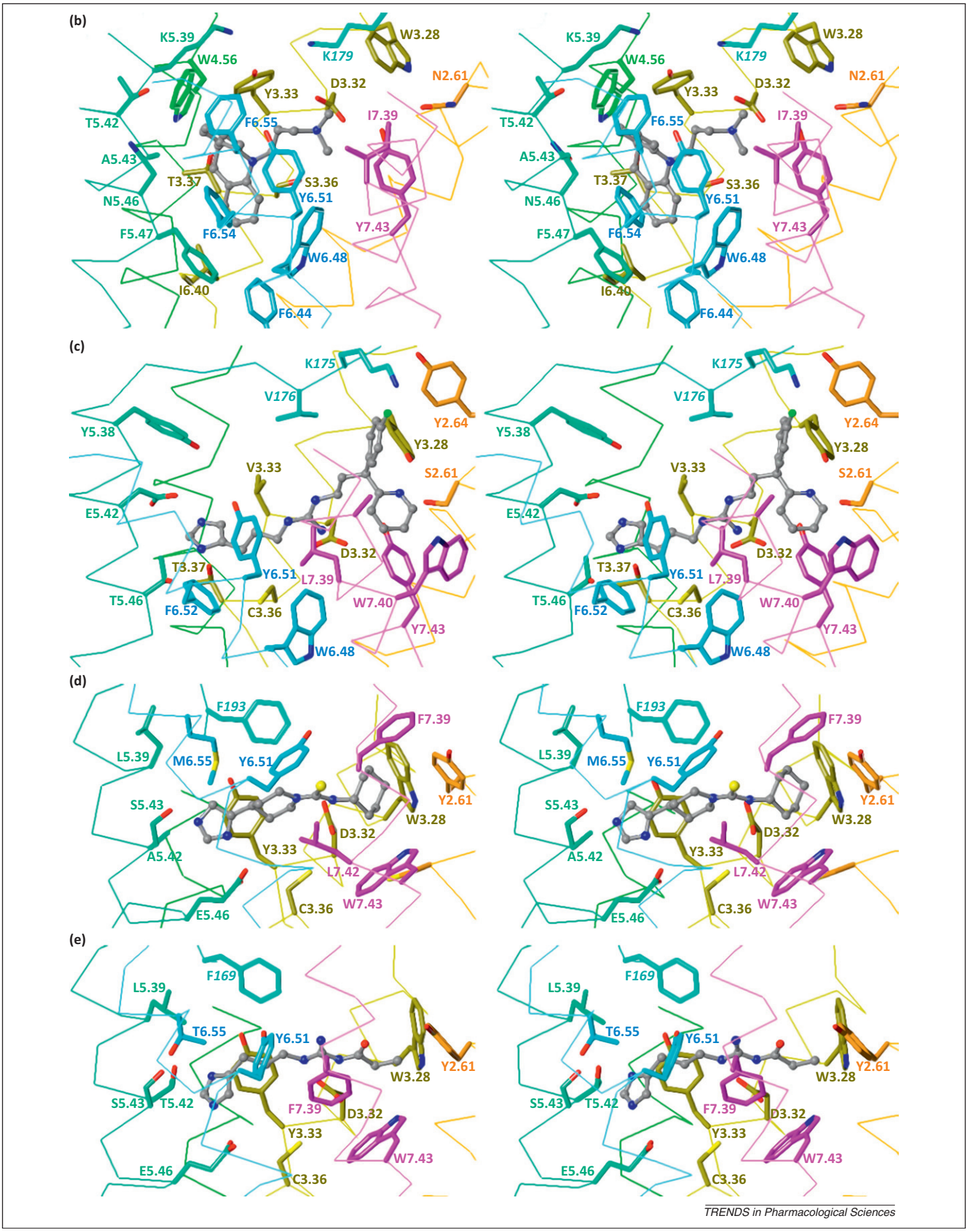


Figure 2. (*Continued*).

amino group and E5.46 [94]. Tricyclic hH_xR antagonists such as doxepin and imipramine (Figure 1) [89] do not form hydrogen bonds with hH_1R but penetrate into a lower hydrophobic pocket (I3.40, F5.47, F6.44, W6.48) and make additional unique interactions with W4.56. Owing to the high similarity of inactive and active state binding sites, further conclusions about different interactions of agonists, antagonists, and inverse agonists cannot be drawn.

'Eastern' ligand moieties (Figures 1 and 2, right-hand moieties) may interact with an affinity-conferring pocket mainly consisting of hydrophobic amino acids in positions 2.61, 3.28, 7.39, and 7.43. Unfortunately, the identity of hH_3R and hH_4R residues in these positions does not provide patterns of hH_3R- and hH_4R-selective interactions. Some ligands additionally interact with amino acids in different positions of the C-terminal part of ECL2, forming a cap of the common binding site (Figure 2).

Functional selectivity of agonists and antagonists

The pharmacological profile of hH_2R in terms of agonist potencies and efficacies is almost identical when the receptor is coupled to the long and short splice variants of $G_s\alpha$ [75]. However, when hH_2R coupling to insect cell G_s is studied measuring adenylyl cyclase activity as parameter, the pharmacological profile is different from the profile of hH_2R coupled to mammalian G_s proteins, indicating that the various hH_2R conformations differ in their ability to couple to mammalian and insect cell $G_s\alpha$ [79]. Although, of course, the insect cell $G_s\alpha$ is not of physiological relevance, these data constitute an important case study showing that the impact of a given G protein isoform even within the same G protein family on functional selectivity of ligands cannot be predicted with certainty but needs to be evaluated experimentally. Analysis of the effects of agonists at recombinant hH_2R expressed in Sf9 cells (parameter: GTPase activity) and hH_2R in human neutrophils (parameters: stimulation of cAMP accumulation and inhibition of O_2^- formation) revealed several differences, which also supports the concept of functional selectivity [32]. It remains to be determined whether other G proteins than the two $G_s\alpha$ splice variants account for the parameter-dependent pharmacological profiles of ligands in native cells.

It is generally assumed that, in contrast to agonists, the effects of antagonists are system-independent [95]. However, at hH_1R and hH_4R expressed in Sf9 cells, there are dissociations between pK_i values (antagonist and agonist radioligand competition binding, respectively) and pK_b values (inhibition of histamine-stimulated GTP hydrolysis) [89,96]. Moreover, differences in the potencies of H_2R antagonists were observed at recombinant hH_2R relative to hH_2R in human neutrophils [32]. Along the same line, comparison of pK_b values of antagonists (inhibition of histamine-stimulated GTP hydrolysis) and pIC_{50} values of these compounds (inverse agonist activity in the GTPase assay) revealed differences, suggesting that the histamine-bound active hH_4R conformation and the constitutively active agonist-free hH_4R conformation are functionally distinct from each other [96]. When comparing the affinities of agonists at hH_2R in the [^{3}H]tiotidine (antagonist) competition binding assay with the potencies of agonists in the GTPase assay, additional discrepancies became apparent [74,79]. The B_{max} of [^{3}H]tiotidine binding is much lower than the actual hH_2R expression level, indicating that only a small fraction of the available receptors binds [^{3}H]tiotidine [74]. The majority of hH_2R molecules may either be inaccessible to tiotidine or exist in a low-affinity state for tiotidine. Hence, it will be important to document incomplete and/or biphasic antagonist inhibition curves in functional experiments.

At recombinant hH_4R expressed in Sf9 cells, JNJ7777120 exhibits the properties of a partial inverse agonist in the GTPase assay [65,96]. However, in U2OS osteosarcoma cells stably expressing hH_4R, JNJ7777120 exhibits the properties of a partial agonist with regard to β-arrestin activation [71]. It has been suggested that this β-arrestin activation is independent of G_i proteins [71], but the evidence for such an assumption is not fully convincing [56]. Specifically, the exclusion of the involvement of G_i proteins in a given signal transduction process by using the ADP-ribosylating pertussis toxin is much more difficult than is generally appreciated [56]. Also, a stimulatory effect of JNJ7777120 on extracellular ligand-regulated protein kinase (ERK) activation, assumed to be a downstream β-arrestin effect, was observed only with the exceedingly high concentration of 100 μM [71], at least four orders of magnitude above the K_i value of JNJ7777120 for hH_4R [62,96]. Thus, hH_4R-independent off-target effects of JNJ7777120 on ERK activation cannot be excluded. Currently, it is unclear whether the effects of JNJ7777120 on ERK activation in recombinant systems are of relevance in native cells, also in light of the fact that osteosarcoma cells as an expression system are only very distantly related to the physiological myeloid cell context of hH_4R. There is no evidence for inverse agonist activity of JNJ7777120 or paradoxical stimulatory effects of JNJ7777120 in human eosinophils, the maximum ligand concentration being 10 μM [33]. A recent study revealed that β-arrestin activation in U2OS cells is not restricted to JNJ7777120 but is also found for numerous other hH_4R ligands. Some ligands are biased towards G_i protein activation, and some ligands are biased towards β-arrestin activation, but there is no clear relationship between ligand structure and pharmacological profile [97]. Similarly, it has been difficult to relate ligand structure and hH_4R inverse agonistic activity [96].

Unresolved questions and future studies

Both hH_3R and hH_4R exhibit high constitutive activity when expressed in Sf9 cells [65,66]. Na^+ acts as an allosteric inverse agonist at hH_3R and reduces constitutive hH_3R activity [98]. The structural basis of allosteric modulation of GPCRs by Na^+ has been resolved recently, (i.e., interaction of Na^+ with a highly conserved aspartate residue in the second transmembrane domain plays a critical role [99]). In marked contrast to hH_3R [98], the constitutive activity of hH_4R is resistant to allosteric modulation by Na^+ [65]. The molecular basis for this discrepancy between hH_3R and hH_4R is unknown; both GPCRs possess the highly conserved asparate residue in the second TM domain (Figure 2). The reason for the discrepancy between high constitutive activity of hH_4R in terms of guanine

nucleotide exchange in membranes and lack of constitutive activity of hH_4R in intact human eosinophils also needs to be clarified [33,65]. In contrast to H_4R, there is evidence for constitutive activity of H_3R in native systems [43].

As is evident from Figure 1 and Tables 2 and 4, the pharmacological profiles of ligands at hH_xRs in native cells and mammalian and insect cell expression systems are far from complete. In many cases, it has been taken for granted that compounds previously classified as being selective for a given H_xR subtype in animal organ or intact animal experiments [78,82,83,86] actually show the same behavior at hH_xRs. However, case studies for several compound classes including 2-phenylhistamines, histaprodifens, and imidazolylpropylguanidines revealed that this is clearly not the case (Figure 1) [3,28,79,80,84]. Hence, it will be very important to generate complete pharmacological profiles of all available compounds at all hH_xRs, even though such studies may appear to be 'boring' at first glance. Compounds with a mixed pharmacological profile may be of therapeutic interest, too. For example, dual H_1R antagonists/H_2R agonists may exhibit superior anti-inflammatory properties compared with H_1R antagonists in type I allergies. Similarly, dual H_1R/H_4R antagonists may be more effective in this condition than H_1R antagonists, specifically with regard to the symptoms of pruritus and asthma [3,4]. Lastly, ligands with antagonistic activity at H_1R and H_4R plus agonistic activity at H_2R may be highly efficacious in various inflammatory conditions (Table 1). Owing to the overlap in pharmacophores binding to these GPCRs (Figure 1), the achievement of this goal is not elusive. Elucidation of the crystal structures of hH_2R and hH_4R, complementing the already available hH_1R structure [99], will constitute an important step towards this goal. In the field of psychiatric drugs, broad interaction profiles at GPCRs appear to be clinically important [100–102], and this concept may be extended to inflammatory conditions entailing hH_xRs.

H_2R agonists may be useful drugs for the treatment of the M4/M5 forms of AML [13,14,35,36,42]. These compounds can act by inhibiting O_2^- formation [13], and they exhibit some degree of cell type-specificity in their effects [32]. Thus, the introduction of highly potent and selective H_2R agonists activating physiological signal transduction pathways in defined cell types could constitute an important conceptual advance in current tumor therapy paradigms in terms of a targeted therapy based on GPCR-mediated signaling. The greatest concern in the implementation of H_2R agonists in AML therapy is the induction of gastroduodenal ulcers due to enhanced parietal cell H^+ secretion. This problem could effectively be controlled by proton pump inhibitors [10], but the development of highly potent and effective H_2R agonists with selectivity for AML cells is the preferred long-term goal.

Concluding remarks

All four hH_xRs constitute established or promising drug targets, particularly for antagonists, and to a much more limited extent, for agonists. Human cell systems endogenously expressing hH_1R, hH_2R, and hH_4R but not hH_3R are available. Mammalian and insect cell expression systems exist for all hH_4Rs. In Sf9 cells, hH_xR ligands can be assessed using the same parameter for all hH_xRs, namely high-affinity GTPase activity. There is substantial overlap in the interactions of various chemical ligand groups with hH_xRs. This overlap in activity does not necessarily constitute a disadvantage but can also be exploited for future drug development. Studies on hH_xRs have revealed that functional selectivity does not only apply for agonists but also for antagonists/inverse agonists. Lastly, it has become clear that there is no single parameter that fully assesses the pharmacological properties of a ligand. Thus, any given ligand must be assessed in multiple dimensions, both with respect to the test cell systems and the particular parameters in any given system, yielding complex pharmacological profiles. The lessons learned from the comprehensive and systematic analysis of hH_xRs expressed in Sf9 cells can be applied to other GPCRs and can guide future GPCR ligand development in general.

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