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Tritium-Labeled N^1-[3-(1*H*-imidazol-4-yl)propyl]-N^2-propionylguanidine ([^{3}H]UR-PI294), a High-Affinity Histamine H_3 and H_4 Receptor Radioligand

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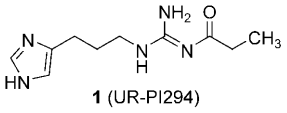
This study reports the synthesis and pharmacological characterization of tritium-labeled N^1-[3-(1*H*-imidazol-4-yl)propyl]-N^2-propionylguanidine ([^{3}H]UR-PI294), a novel and readily accessible radioligand for the human histamine H_3 receptor (hH_3R) and H_4 receptor (hH_4R). The radioligand displays high affinity for both histamine receptor subtypes (K_D (hH_3R) = 1.1 nM, K_D (hH_4R) = 5.1 nM) and is shown to be a valuable pharmacological tool for the determination of hH_3R and hH_4R affinities.

Introduction

The biogenic amine histamine mediates its various (patho)physiological functions via four histamine receptor subtypes, all of which belong to the superfamily of G-protein-coupled receptors (GPCRs).[1,2] The histamine H_1 receptor (H_1R) is involved, for instance, in allergic reactions, whereas the histamine H_2 receptor (H_2R) plays a crucial role in gastric acid secretion.[1] Antagonists for H_1R and H_2R are widely used drugs for the treatment of allergic conditions and gastroesophageal reflux disease, respectively.[1] The histamine H_3 receptor (H_3R), pharmacologically characterized in 1983[3] and cloned in 1999,[4] is mainly found in the central nervous system where it plays an important role as a presynaptic receptor modulating the release of histamine and other neurotransmitters.[1] Antagonists of H_3R are not yet used clinically but are under evaluation for the therapy of narcolepsy, attention-deficit hyperactivity disorder, Alzheimer's disease, and obesity.[5] The histamine H_4 receptor (H_4R) was identified and cloned in 2000 and 2001 by several independent researchers revealing high sequence homology with the H_3R.[2] It is mainly expressed on immune cells such as mast cells, eosinophils, dendritic cells, monocytes, and T lymphocytes.[6] This expression pattern and current in vitro and in vivo studies suggest that the H_4R plays a crucial role in inflammatory and immunological processes.[6] H_4R antagonists are promising drug candidates for the treatment of bronchial asthma, allergic rhinitis, rheumatoid arthritis, and pruritus.[7,8]

To further investigate the biological role of the human (h) H_3R, and in particular that of hH_4R, selective agonists and antagonists as well as special ligands including radioligands are required as pharmacological tools. Very recently, we found N^G-alkanoylimidazolylpropylguanidines to be high-affinity hH_3R antagonists/partial agonists and highly potent full hH_4R agonists,[9] which are among the most potent hH_4R agonists described to date. For example, UR-PI294 (**1**), bearing an N^G-propionyl group (Figure 1), possesses more than 1000- and 100-fold selectivity for hH_3R and hH_4R relative to hH_1R and hH_2R, respectively.

The propionyl group in UR-PI294 is of special interest, as it offers the possibility to introduce a commercially available tritiated propionyl residue. The resulting radioligand, [^{3}H]UR-PI294 (**10**), may be a valuable pharmacological tool for the labeling of hH_3R and in particular of hH_4R. With respect to hH_3R, the low efficacy of UR-PI294 at this histamine receptor subtype may be of interest because partial agonistic radioligands with low intrinsic activities have been shown to behave as antagonists.[10–12] Therefore, [^{3}H]UR-PI294 may complement the commercially available and preferably used full hH_3R agonists [^{3}H](*R*)-α-methylhistamine[13] and [^{3}H]N^α-methylhistamine[14,15] in pharmacological studies at hH_3R. Concerning hH_4R, only three radioligands have been reported: [^{3}H]histamine,[16–19] [^{3}H]JNJ 7777120,[16,20] and [^{125}I]iodophenpropit (shown).[16] The often low specific activity (10–25 Ci mmol^{-1})[16,18,20–23] of the commercially available H_4R radioligand [^{3}H]histamine brings about low sensitivity and requires high concentrations of either the radioligand or the receptor protein. The use of iodinated compounds is compromised by potential chemical instability,[24] the half-life of ^{125}I-labeled ligands (59.4 days) being shorter than that of ^{3}H-labeled ligands (12.4 years), and the



1 (UR-PI294)

	EC_{50} [nM]	E_{max}
hH_1R	3 500	0.30
hH_2R	370	0.83
hH_3R	1.6	0.39
hH_4R	3.0	0.90

Figure 1. The acylguanidine UR-PI294: structure and pharmacological profile at the four histamine receptors determined in steady-state GTPase assays using membrane preparations of Sf9 cells expressing the respective human histamine receptor.[9] Agonistic activity expressed as EC_{50}; E_{max} = efficacy relative to histamine = 1.00.

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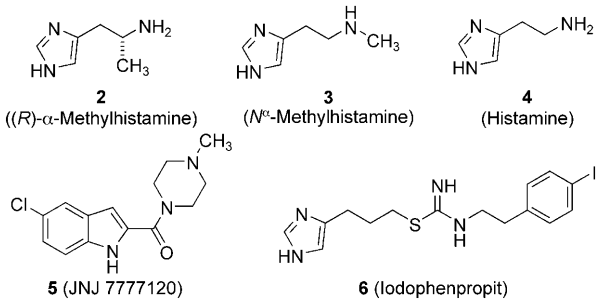
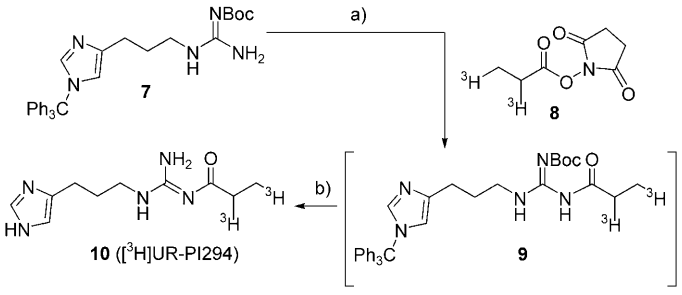


need for special safety precautions (shielding) during preparation and handling. Therefore, tritium-labeled UR-PI294 is considered a valuable radioligand, in particular for hH_4R. Herein we present the synthesis and pharmacological characterization of [^{3}H]UR-PI294, a new high-affinity hH_3R and hH_4R radioligand.

Results and Discussion

Chemistry

The radioligand **10** ([^{3}H]UR-PI294) was prepared as depicted in Scheme 1. Acylation of Boc-guanidine **7**[9] with tritiated propionic acid was achieved by employing the commercially avail-



Scheme 1. Synthesis of the radioligand [^{3}H]UR-PI294 (**10**). Reagents and conditions: a) NEt_3, $CHCl_3$, 16 h, RT; b) TFA 23%, $CHCl_3$, 5 h, RT.

able reactive succinimidyl ester (**8**). Intermediate **9** was not isolated and directly deprotected under acidic conditions. After purification by HPLC, the designated radioligand **10** ([^{3}H]UR-PI294) was obtained in high radiochemical purity (97.8%) with a specific activity of 41.8 Ci mmol^{-1}. The identity of the radioligand was confirmed by HPLC analysis of labeled (**10**) and unlabeled (**1**) UR-PI294, which have identical retention times (Figure 2).

Saturation binding analysis of [^{3}H]UR-PI294 at hH_3R and hH_4R

The specific binding of [^{3}H]UR-PI294 to membranes of Sf9 insect cells co-expressing hH_3R plus $G_{i\alpha2}$ plus $G_{\beta1\gamma2}$ plus RGS4 (regulator of G-protein signaling 4) was saturable (Figure 3A). Up to a radioligand concentration of 5 nM, nonspecific binding determined in the presence of thioperamide (10 μM) was low, amounting to 5–10% of total binding. Specific binding versus

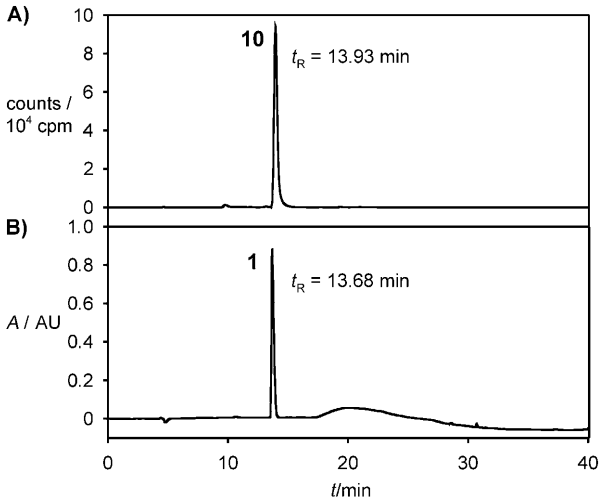


Figure 2. Identity and purity control of [^{3}H]UR-PI294 (**10**). A) Radiochromatogram of the prepared radioligand [^{3}H]UR-PI294 (**10**), conc.: 0.8 μM. B) UV (λ = 210 nm) chromatogram of unlabeled UR-PI294 (**1**), conc.: 100 μM. Conditions: injection volume: 100 μL, gradient: 0.05% TFA in MeCN (*v/v*)/0.05% TFA in H_2O (*v/v*): 0 min: 5:95, 13 min: 11.5:88.5, 25 min: 95:5, 40 min: 95:5), flow: 0.8 mL min^{-1}. The minor difference in t_R (13.68 versus 13.93 min) results from the setup of the UV and radiodetector in series.

[^{3}H]UR-PI294 concentrations was best fitted by nonlinear regression to a one-site binding model (Figure 3A). The determined K_D value of 1.1 nM (Table 1) is consistent with the EC_{50} value determined in functional GTPase assays (EC_{50} = 1.6 nM, Figure 1) confirming that [^{3}H]UR-PI294 acts as a high-affinity radioligand at hH_3R. The estimated B_{max} value was 1.4 pmol per mg membrane protein. Within the investigated concentration range, the Scatchard plot was linear which is in agreement with the binding of [^{3}H]UR-PI294 to a single binding site (Figure 3B).

Binding of [^{3}H]UR-PI294 to hH_4R was determined in Sf9 insect cell membranes co-expressing hH_4R–RGS19 fusion protein plus $G_{i\alpha2}$ plus $G_{\beta1\gamma2}$. Nonspecific binding was determined in the presence of an excess of thioperamide (10 μM). Similar to its binding to hH_3R, [^{3}H]UR-PI294 bound specifically to hH_4R (Figure 3C) in a saturable manner. Specific binding versus the concentration of [^{3}H]UR-PI294 was best described by a one-site model and afforded a K_D value of 5.1 nM (Table 1). This fits very well to the potency determined in the functional GTPase assay (EC_{50} = 3.0 nM, Figure 1). The calculated B_{max} value of 2.0 pmol mg^{-1} membrane protein reveals similar expression levels of hH_3R and hH_4R in the Sf9 insect cell membranes. The corresponding Scatchard plot of the ratio of bound/free versus bound [^{3}H]UR-PI294 revealed a straight line indicating that the radioligand binds to a single binding site (Figure 3D).

Analysis of the association and dissociation kinetics of [^{3}H]UR-PI294 at hH_3R and hH_4R

Association and dissociation experiments were carried out with Sf9 insect cell membranes expressing hH_3R at a [^{3}H]UR-PI294 concentration of 2 nM at 22 °C. As already observed for other hH_3R radioligands,[25,26] the association and dissociation kinetics

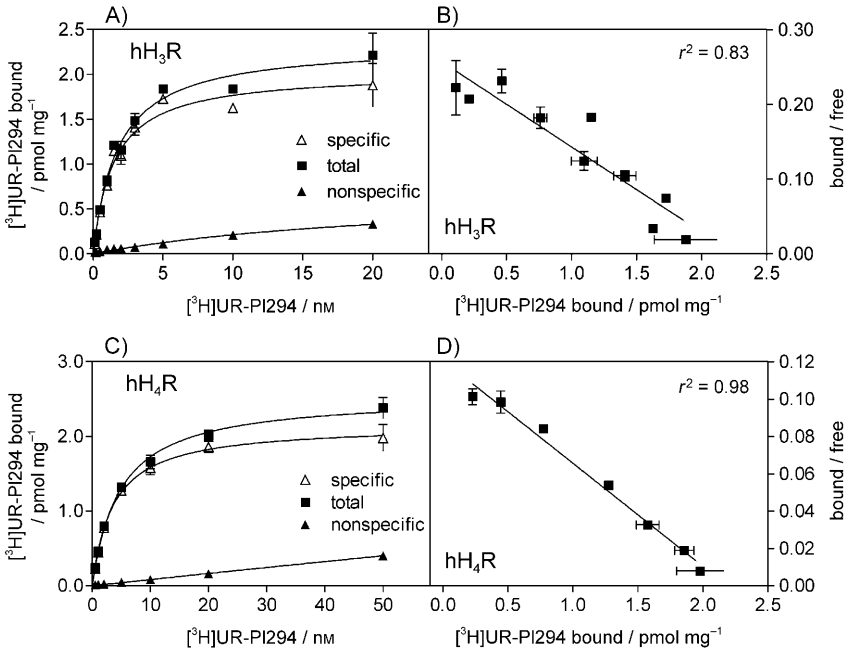


Figure 3. Representative [^{3}H]UR-PI294 saturation binding experiments in Sf9 insect cell membranes expressing A) and B) hH_3R plus $G_{i\alpha2}$ plus $G_{\beta1\gamma2}$ plus RGS4, or C) and D) hH_4R–RGS19 fusion protein plus $G_{i\alpha2}$ plus $G_{\beta1\gamma2}$ performed in duplicate. Membranes were incubated with increasing concentrations of [^{3}H]UR-PI294 as described in the pharmacological methods section. Nonspecific binding was determined in the presence of 10 μmol L^{-1} thioperamide. Specific binding is the difference between the total and nonspecific binding of [^{3}H]UR-PI294 at a given concentration. In panels A) and C) data were best fitted by nonlinear regression to a one-site model. Panels B) and D) show corresponding Scatchard plots of saturation binding data, best fitted by linear regression.

Table 1. Saturation binding parameters of [^{3}H]UR-PI294 at hH_3R and hH_4R.[a]

	K_D [nM]	B_{max} [pmol (mg protein)$^{-1}$]
hH_3R	1.1 ± 0.2	1.4 ± 0.3
hH_4R	5.1 ± 1.9	2.0 ± 0.1

[a] Data are the means of five independent experiments, each performed in duplicate.

of [^{3}H]UR-PI294 at hH_3R were very rapid (Figure 4 A). The specific binding of [^{3}H]UR-PI294 achieved equilibrium after approximately 10 min corresponding to an association half-life ($t_{1/2}$) of 1.4 min. Displacement of [^{3}H]UR-PI294 by thioperamide (10 μM) was complete within 20 min, indicating that [^{3}H]UR-PI294 binds reversibly to the receptor ($t_{1/2}$ = 3.2 min). The resulting association rate constant (k_{on}) was 0.14 min^{-1} nM^{-1}, and the dissociation rate constant (k_{off}) was 0.21 min^{-1}. The K_D value calculated from the ratio of the k_{off} and k_{on} values (1.5 nM) is in very good agreement with the K_D value determined from the saturation binding isotherms (Table 2).

Owing to the lower affinity of [^{3}H]UR-PI294 for hH_4R, a concentration of 10 nM was employed for recording the association and dissociation rates of the radioligand at Sf9 insect cell membranes expressing hH_4R–RGS19 fusion protein (22 °C). Relative to hH_3R, [^{3}H]UR-PI294 showed remarkably slower association and dissociation kinetics at hH_4R (Figure 4 C, D). The specific binding of [^{3}H]UR-PI294 reached equilibrium within 60 min. Association kinetics of [^{3}H]UR-PI294 was almost tenfold slower than hH_3R ($t_{1/2}$ = 11.4 min). Likewise, the dissociation of [^{3}H]UR-PI294 from hH_4R, initiated by thioperamide (10 μM), was slow and had not terminated after 1 h. The resulting k_{on} for [^{3}H]UR-PI294 was 0.0027 min^{-1} nM^{-1} and the k_{off} 0.033 min^{-1} yielding a K_D value of 12 nM, which is consistent with the K_D value from the saturation binding studies (Table 2). The rather slow association rate of the radioligand at hH_4R required an incubation period of at least 60 min for saturation and competition experiments to ensure measurements at equilibrium.

Competition binding experiments of [^{3}H]UR-PI294 with reference ligands at hH_3R and hH_4R

A number of standard ligands for hH_3R and hH_4R were evaluated for their ability to inhibit specific binding of [^{3}H]UR-PI294 to Sf9 insect cell membranes expressing hH_3R or hH_4R–RGS19 fusion protein. The resulting K_I values were determined and compared with affinities reported from competition binding experiments using other radioligands for hH_3R and hH_4R. All obtained competition curves were monophasic and best described by a one-site competition model.

[^{3}H]UR-PI294 was displaced from hH_3R by the H_3R agonists histamine and (*R*)-α-methylhistamine as well as by the H_3R antagonists (inverse agonists) thioperamide and clobenpropit (Figure 5 A). All resulting K_I values were in good agreement with reported K_I values determined with established H_3R radioligands (Table 3).

The H_4R ligands histamine, thioperamide, clobenpropit, and JNJ 7777120 inhibited specific binding of [^{3}H]UR-PI294 to hH_4R in a concentration-dependent manner (Figure 5 B). As for hH_3R, the determined K_I values for hH_4R binding were in good agreement with K_I values from literature (Table 3).

Summary and Conclusions

Binding of [^{3}H]UR-PI294 to Sf9 insect cell membranes expressing hH_3R or hH_4R–RGS19 fusion protein was saturable and highly specific. [^{3}H]UR-PI294 showed high affinities for both receptors. However, affinity for hH_3R was five times higher relative to hH_4R (K_D = 1.1 nM for hH_3R, K_D = 5.1 nM for hH_4R). The saturation binding curves were best described by a one-site model and the Scatchard plots appeared to be linear, suggesting that [^{3}H]UR-PI294 binds to a single binding site at mem-

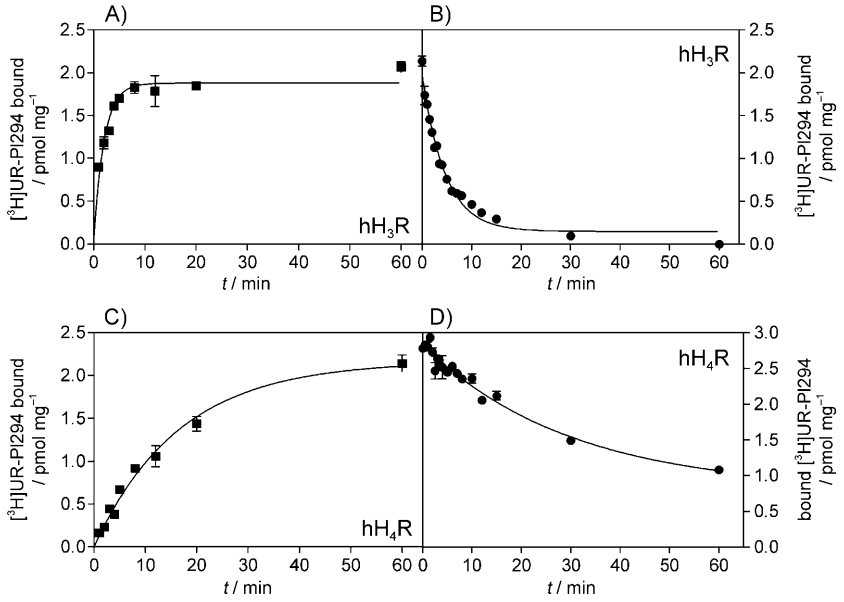


Figure 4. Specific binding kinetics of [^{3}H]UR-PI294 to Sf9 insect cell membranes expressing A) and B) hH_3R plus $G_{i\alpha 2}$ plus $G_{\beta 1\gamma 2}$ plus RGS4, or C) and D) hH_4R–RGS19 fusion protein plus $G_{i\alpha 2}$ plus $G_{\beta 1\gamma 2}$ were measured as described in the pharmacological methods section. Panels A) and C) show association kinetics for 2 and 10 nM [^{3}H]UR-PI294, respectively. Nonspecific binding was determined for each time point in the presence of thioperamide (10 μM). Specific binding is the difference between the total [^{3}H]UR-PI294 and nonspecific [^{3}H]UR-PI294 binding for a given time point. Data were best fitted by nonlinear regression to a one-phase exponential association model. Panels B) and D) show dissociation kinetics for 2 and 10 nM [^{3}H]UR-PI294, respectively. Displacement of [^{3}H]UR-PI294 was induced by thioperamide (10 μM). Nonspecific binding was determined by incubation of [^{3}H]UR-PI294 for 60 min with the respective membrane in the presence of thioperamide (10 μM). Specific binding is the difference between the total and nonspecific [^{3}H]UR-PI294 binding. Data were best fitted by nonlinear regression to a one-phase exponential decay model. All experiments were performed in duplicate at 22 °C.

Table 2. Parameters of the kinetics analysis of [^{3}H]UR-PI294 binding in Sf9 insect cell membranes.[a]

	k_{obs} [min^{-1}][b]	$t_{1/2}$ [min] (association)	k_{on} [min^{-1} nM^{-1}]	k_{off} [min^{-1}]	$t_{1/2}$ [min] (dissociation)	K_D [nM] (kinetic)	K_D [nM] (saturation)[c]
hH_3R	0.50	1.4	0.14	0.21	3.2	1.5	1.1
hH_4R	0.06	11.5	0.0027	0.033	20.7	12	5.1

[a] Expressing hH_3R plus $G_{i\alpha 2}$ plus $G_{\beta 1\gamma 2}$ plus RGS4 or hH_4R–RGS19 fusion protein plus $G_{i\alpha 2}$ plus $G_{\beta 1\gamma 2}$. [b] Observed rate constant. [c] The dissociation constants taken from Table 1 determined in saturation binding experiments are listed for comparison.

branes expressing hH_3R and hH_4R. Kinetics experiments showed a rapid association and dissociation of the radioligand from hH_3R, whereas these processes were about ten times slower for hH_4R. The resulting dissociation constants were in good agreement with the K_D values obtained from the saturation binding studies for both histamine receptor subtypes. Binding constants determined for H_3R and H_4R reference ligands were consistent with data reported in the literature, confirming [^{3}H]UR-PI294 to be a suitable radioligand for the determination of the affinities of unlabeled H_3R and H_4R ligands.

As there is a lack of selectivity between hH_3R and hH_4R, the radioligand is primarily valuable for application in recombinant systems expressing only one histamine receptor subtype. However, in most immune cells such as mast cells or eosinophils where H_4R is mainly located, H_3Rs are not expressed.[6,37,38] Therefore, because of the more than 100-fold selectivity over the hH_1R and hH_2R subtypes, [^{3}H]UR-PI294 may also be applicable for labeling hH_4R in these native cells devoid of hH_3Rs.

Taken together, [^{3}H]UR-PI294 is a new, highly potent acylguanidine-type radioligand for both hH_3R and hH_4R. The facile preparation and rather low costs arising from the employment of commercially available tritiated succinimidyl propionate makes [^{3}H]UR-PI294 readily accessible under common laboratory conditions. The low efficacy of [^{3}H]UR-PI294 at hH_3R may result in similar affinities for both the high-affinity and low-affinity binding site of the receptor. Such radioligands are required for studying constitutive activity of GPCRs.[39] Therefore, [^{3}H]UR-PI294 may be a promising tool for investigating constitutive activity of hH_3R. The high specific activity of [^{3}H]UR-PI294 (41.8 Ci mmol^{-1}) relative to commonly available [^{3}H]histamine combined with high affinity and low nonspecific binding at hH_4R shows that this radioligand in particular is a promising pharmacological probe for hH_4R, a promising new target for the development of, for instance, anti-inflammatory drugs.[6]

Experimental Section

Synthesis

General: Commercial reagents and chemicals were purchased from Acros Organics (Geel, Belgium), Merck KGaA (Darmstadt, Germany), and Sigma–Aldrich Chemie GmbH (Munich, Germany), and used without further purification. Succinimidyl [2,3-$^3H_2$]propionate solution in ethyl acetate (60 Ci mmol^{-1}, 5 mCi mL^{-1}) was from Biotrend, American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). All solvents were of analytical grade or distilled prior to use. Scintillation cocktail was from Carl Roth GmbH (Rotiszint eco plus, Karlsruhe, Germany). Analytical HPLC was performed on a system from

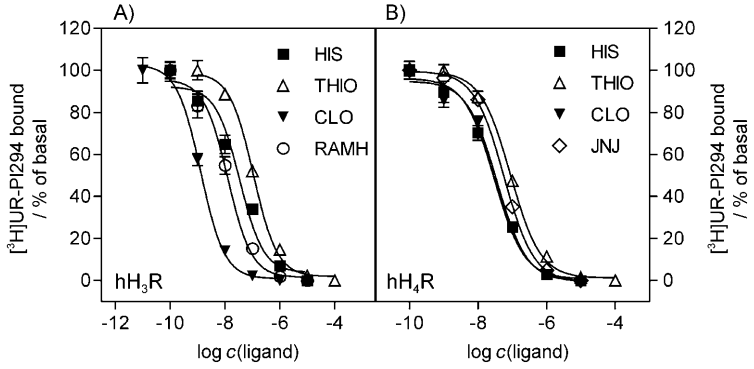


Figure 5. Competition of [^{3}H]UR-PI294 binding by reference H_3R and H_4R ligands in Sf9 insect cell membranes expressing A) hH_3R plus $G_{i\alpha2}$ plus $G_{\beta1\gamma2}$ plus RGS4, or B) hH_4R–RGS19 fusion protein plus $G_{i\alpha2}$ plus $G_{\beta1\gamma2}$. [^{3}H]UR-PI294 concentrations were 2 nM for membranes expressing hH_3R and 5 nM for membranes expressing hH_4R. Data were analyzed by nonlinear regression and best fitted to one-site (monophasic) competition curves. Data points shown are the mean of three independent experiments, each performed in duplicate. (HIS: histamine; THIO: thioperamide; CLO: clobenpropit; RAMH: (*R*)-α-methylhistamine; JNJ: JNJ 7777120).

Table 3. K_i values of reference H_3R and H_4R ligands from competition binding experiments using [^{3}H]UR-PI294 in Sf9 insect cell membranes.[a]

Compd	hH_3R		hH_4R	
	K_i [nM]			
	[^{3}H]UR-PI294[b]	Reported[c]	[^{3}H]UR-PI294[b]	Reported[d]
Histamine	11 ± 3.0	2.8–15	15 ± 2.0	8.1–17
(*R*)-α-Methylhistamine	4.5 ± 1.1	0.56–6.3	–	–
Thioperamide	38 ± 2.0	25–76	45 ± 1.9	27–210
Clobenpropit	0.46 ± 0.02	0.34–3.8	18 ± 2.2	7.2–13
JNJ 7777120	–	–	30 ± 3.8	4.0–32

[a] Expressing hH_3R plus $G_{i\alpha2}$ plus $G_{\beta1\gamma2}$ plus RGS4 or hH_4R–RGS19 fusion protein plus $G_{i\alpha2}$ plus $G_{\beta1\gamma2}$ relative to K_i values reported in literature. [b] Mean K_i values determined in three independent experiments, each performed in duplicate. [c] Reference [16, 27–32]. [d] Reference [16, 21, 23, 30, 33–36].

Waters (Waters GmbH, Eschborn, Germany) equipped with a Waters pump control module, Waters 510 HPLC pump, a Waters 486 UV/VIS detector, and a Packard radiomatic Flo-one beta series A-500 radiodetector. Stationary phase was an Agilent Scalar C_{18} (250×4.6 mm, 5 μm) column. The flow rate was 0.8 mL min^{-1}. Gradients of MeCN/TFA 0.05% (*v*/*v*) and H_2O/TFA 0.05% (*v*/*v*) were used as mobile phase. The absorbance was detected at 210 nm or a radiodetector was used to measure the radioactivity by liquid scintillation counting. Radiochemical purity of [^{3}H]UR-PI294 was calculated as the percentage peak area from the radiochromatogram.

Preparation of N^1-[3-(1*H*-imidazol-4-yl)propyl]-N^2-[2,3-$^3H_2$]propionylguanidine ([^{3}H]UR-PI294, 10): A solution of succinimidyl [2,3-$^3H_2$]propionate in ethyl acetate (600 μL, 50 nmol, 1 eq, 8.76 μg, 3 mCi) was added to a solution of **7**[9] (2.0 μmol, 1.02 mg, 40 eq) and NEt_3 (4.0 μmol, 0.405 mg, 80 eq) in $CHCl_3$ (300 μL). The solvent was removed by a rotary evaporator and the residue taken up in $CHCl_3$ (100 μL). After the mixture was stirred for 16 h at RT, TFA (30 μL) was added and stirring was continued for an additional 5 h. The solvent was removed under reduced pressure and the residue taken up in 500 μL of a mixture of MeCN/H_2O/TFA 5:94.9:0.1 (*v*/*v*/*v*). Insoluble material was removed by centrifugation for 10 min at 13000 rpm (15100 *g*) and aliquots (5×100 μL) of the supernatant were purified by HPLC. The fractions containing the radioligand were collected at approximately 14 min (gradient: 0.05% TFA in MeCN/0.05% TFA in H_2O: 0 min: 5:95 (*v*/*v*), 13 min: 11.5:88.5 (*v*/*v*), 25 min: 95:5 (*v*/*v*), 40 min: 95:5 (*v*/*v*)). The solvent of the combined fractions was evaporated and the residue was taken up in 450 μL of a mixture of EtOH/H_2O 1:1 (*v*/*v*). The concentration of the radioligand in this stock solution was determined after recording a calibration curve with the unlabeled ligand UR-PI294 (HPLC, $\lambda = 210$ nm) from the peak area (11.89 μM, 5.35 nmol, yield: 10.7%). The total activity of [^{3}H]UR-PI294 was determined by dilution of 1.5 μL of the stock solution with 448.5 μL MeCN/TFA (aq) 0.1% 5:95 (*v*/*v*) and counting 9 μL of this dilution (three times in duplicate) in 3 mL scintillation cocktail (total activity in 450 μL stock solution: 223.6 μCi). This results in a specific activity for [^{3}H]UR-PI294 of 41.8 Ci mmol^{-1}. The activity concentration of the stock solution was adjusted to 250 μCi mL^{-1} with a mixture of EtOH/H_2O 1:1 (*v*/*v*) (5.98 μM). HPLC analysis showed a radiochemical purity of 97.8%. The identity of the radioligand was confirmed by HPLC analysis of the labeled and unlabeled UR-PI294 under the same conditions resulting in nearly identical retention times (Figure 2).

Pharmacological methods

General: Histamine dihydrochloride was purchased from Alfa Aesar GmbH & Co. KG (Karlsruhe, Germany). Thioperamide maleate, (*R*)-α-methylhistamine dihydrobromide, and clobenpropit dihydrobromide were from Tocris Bioscience (Ellisville, MO, USA). The H_4R antagonist JNJ 7777120 was a gift from Dr. R. Thurmond (Department of Immunology, Johnson & Johnson Pharmaceutical R&D, San Diego, CA, USA). GF/C filters were from Whatman (Maidstone, UK). Radioactivity was determined by liquid scintillation counting in a Beckman LS-6500 device.

Data analysis and pharmacological parameters: All data were presented as mean ± SEM. IC_{50} values were converted into K_i values using the Cheng–Prussoff equation.[40] K_D values from saturation binding experiments were analyzed by nonlinear regression and best fit to one-site binding (hyperbola) isotherms. K_i values were analyzed by nonlinear regression and best fit to one-site (monophasic) competition curves. Observed rate constants (k_{obs}) were analyzed by nonlinear regression and best fit to one-phase exponential association curves. Dissociation constants (k_{off}) were analyzed by nonlinear regression and best fit to one-phase exponential decay curves. (GraphPad Prism 4.02 software, San Diego, CA, USA). k_{obs} is the observed association rate constant. Association rate constants (k_{on}) were calculated according to following equation: $k_{on} = (k_{obs} - k_{off})/[\text{radioligand}]$.[41] Dissociation constants (K_D) from the kinetics experiments were calculated according to following equation: $K_D = k_{off}/k_{on}$.[41]

[^{3}H]UR-PI294 binding assay: Radioligand binding experiments were performed with a similar procedure to that described for the H_1R and H_2R by Seifert et al.[42] and Kelley et al.[43] For [^{3}H]UR-PI294

binding experiments Sf9 insect cell membranes co-expressing hH_3R, mammalian $G_{i\alpha 2}$, $G_{\beta 1\gamma 2}$, and RGS4 or hH_4R–GAIP fusion protein, mammalian $G_{i\alpha 2}$, and $G_{\beta 1\gamma 2}$ were employed. The respective membranes were thawed and sedimented by 10 min centrifugation at 4 °C and 13000 g. Membranes were resuspended in binding buffer (12.5 mM $MgCl_2$, 1 mM EDTA, and 75 mM Tris-HCl, pH 7.4). Each tube (total volume 250 μL) contained 25–50 μg (hH_3R) or 50 μg (hH_4R) of membrane protein. Saturation binding experiments were performed in a concentration range of 0.125–20 nM (hH_3R) and 0.5–50 nM (hH_4R) of [^{3}H]UR-PI294. Nonspecific binding was determined in the presence of 10 μM thioperamide. Competition binding experiments were performed with 2 nM [^{3}H]UR-PI294 (hH_3R) or 5 nM [^{3}H]UR-PI294 (hH_4R) and increasing concentrations of unlabeled ligands. Incubations were conducted for 60 min (hH_3R) or 60–90 min (hH_4R) at 22 °C and shaking at 250 rpm. Bound radioligand was separated from free radioligand by filtration through 0.3% polyethyleneimine-pretreated GF/C filters followed by three washes with 2 mL of cold binding buffer (4 °C) using a Brandel Harvester. Pretreating the filter with polyethyleneimine reduced filter binding of [^{3}H]UR-PI294 to 0.14%. Filter-bound radioactivity was determined, after an equilibration phase of at least 12 h, by liquid scintillation counting.

[^{3}H]UR-PI294 kinetics studies: For the kinetics experiments the membranes were prepared as described for the [^{3}H]UR-PI294 binding assays. Tubes containing [^{3}H]UR-PI294 at a concentration of 2 nM (hH_3R) or 10 nM (hH_4R) and the respective hH_3R or hH_4R membrane at a concentration of 200 μg mL^{-1} (hH_3R and hH_4R) in binding buffer (12.5 mM $MgCl_2$, 1 mM EDTA, and 75 mM Tris-HCl, pH 7.4) were employed. The reaction mixtures were agitated with a magnetic stir bar and incubated at ambient temperature (22 °C). Association kinetics experiments were started by the addition of membrane suspension to the tube. At each time point 250 μL aliquots (50 μg membrane protein) were withdrawn from the tube. Nonspecific binding was determined for each time point in the presence of thioperamide (10 μM). Bound [^{3}H]UR-PI294 was separated from free [^{3}H]UR-PI294 by filtration through GF/C filters (pretreated with 0.3% polyethyleneimine solution) using a Millipore 1225 vacuum sampling manifold followed by three washes with 2 mL binding buffer (4 °C). Filter-bound radioactivity was determined after an equilibration phase of at least 12 h by liquid scintillation counting. For the dissociation kinetics, the tubes containing [^{3}H]UR-PI294 and the respective membrane were incubated for 60 min (hH_3R) or 90 min (hH_4R), respectively before starting dissociation by addition of thioperamide (10 μM final concentration). Similar to the procedure for determining the association kinetics, a 250 μL aliquot was removed for each time point. Nonspecific binding was determined by incubation [^{3}H]UR-PI294 for 60 min in the presence of thioperamide (10 μM). Bound [^{3}H]UR-PI294 was measured as described for the association kinetics experiment.

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[1] S. J. Hill, C. R. Ganellin, H. Timmerman, J. C. Schwartz, N. P. Shankley, J. M. Young, W. Schunack, R. Levi, H. L. Haas, *Pharmacol. Rev.* **1997**, *49*, 253–278.
[2] I. J. P. de Esch, R. L. Thurmond, A. Jongejan, R. Leurs, *Trends Pharmacol. Sci.* **2005**, *26*, 462–469.
[3] J. M. Arrang, M. Garbarg, J. C. Schwartz, *Nature* **1983**, *302*, 832–837.
[4] T. W. Lovenberg, B. L. Roland, S. J. Wilson, X. Jiang, J. Pyati, A. Huvar, M. R. Jackson, M. G. Erlander, *Mol. Pharmacol.* **1999**, *55*, 1101–1107.
[5] R. Leurs, R. A. Bakker, H. Timmerman, I. J. P. de Esch, *Nat. Rev. Drug Discovery* **2005**, *4*, 107–120.
[6] R. L. Thurmond, E. W. Gelfand, P. J. Dunford, *Nat. Rev. Drug Discovery* **2008**, *7*, 41–53.
[7] J. A. Jablonowski, N. I. Carruthers, R. L. Thurmond, *Mini-Rev. Med. Chem.* **2004**, *4*, 993–1000.
[8] P. J. Dunford, K. N. Williams, P. J. Desai, L. Karlsson, D. McQueen, R. L. Thurmond, *J. Allergy Clin. Immunol.* **2007**, *119*, 176–183.
[9] a) P. Igel, E. Schneider, D. Schnell, S. Elz, R. Seifert, A. Buschauer, (submitted); b) P. Igel, PhD Thesis, 2008, University of Regensburg, Regensburg (Germany).
[10] R. Seifert, *J. Pharmacol. Exp. Ther.* **2001**, *298*, 840–847.
[11] R. Seifert, U. Gether, K. Wenzel-Seifert, B. K. Kobilka, *Mol. Pharmacol.* **1999**, *56*, 348–358.
[12] R. Seifert, T. W. Lee, V. T. Lam, B. K. Kobilka, *Eur. J. Biochem.* **1998**, *255*, 369–382.
[13] J. M. Arrang, M. Garbarg, J. C. Lancelo, J. M. Lecomte, H. Pollard, M. Robba, W. Schunack, J. C. Schwartz, *Nature* **1987**, *327*, 117–123.
[14] R. E. West, Jr., A. Zweig, N. Y. Shih, M. I. Siegel, R. W. Egan, M. A. Clark, *Mol. Pharmacol.* **1990**, *38*, 610–613.
[15] A. Korte, J. Myers, N.-Y. Shih, R. W. Egan, M. A. Clark, *Biochem. Biophys. Res. Commun.* **1990**, *168*, 979–986.
[16] H. D. Lim, R. M. van Rijn, P. Ling, R. A. Bakker, R. L. Thurmond, R. Leurs, *J. Pharmacol. Exp. Ther.* **2005**, *314*, 1310–1321.
[17] F. Gbahou, L. Vincent, M. Humbert-Claude, J. Tardivel-Lacombe, C. Chabret, J.-M. Arrang, *Br. J. Pharmacol.* **2006**, *147*, 744–754.
[18] T. Hashimoto, S. Harusawa, L. Araki, O. P. Zuiderveld, M. J. Smit, T. Imazu, S. Takashima, Y. Yamamoto, Y. Sakamoto, T. Kurihara, R. Leurs, R. A. Bakker, A. Yamatodani, *J. Med. Chem.* **2003**, *46*, 3162–3165.
[19] T. Oda, N. Morikawa, Y. Saito, Y. Masuho, S.-I. Matsumoto, *J. Biol. Chem.* **2000**, *275*, 36781–36786.
[20] R. L. Thurmond, P. J. Desai, P. J. Dunford, W.-P. Fung-Leung, C. L. Hofstra, W. Jiang, S. Nguyen, J. P. Riley, S. Sun, K. N. Williams, J. P. Edwards, L. Karlsson, *J. Pharmacol. Exp. Ther.* **2004**, *309*, 404–413.
[21] K. L. Morse, J. Behan, T. M. Laz, R. E. West, Jr., S. A. Greenfeder, J. C. Anthes, S. Umland, Y. Wan, R. W. Hipkin, W. Gonsiorek, N. Shin, E. L. Gustafson, X. Qiao, S. Wang, J. A. Hedrick, J. Greene, M. Bayne, F. J. Monsma, Jr., *J. Pharmacol. Exp. Ther.* **2001**, *296*, 1058–1066.
[22] J. D. Venable, H. Cai, W. Chai, C. A. Dvorak, C. A. Grice, J. A. Jablonowski, C. R. Shah, A. K. Kwok, K. S. Ly, B. Pio, J. Wei, P. J. Desai, W. Jiang, S. Nguyen, P. Ling, S. J. Wilson, P. J. Dunford, R. L. Thurmond, T. W. Lovenberg, L. Karlsson, N. I. Carruthers, J. P. Edwards, *J. Med. Chem.* **2005**, *48*, 8289–8298.
[23] R. M. van Rijn, P. L. Chazot, F. C. Shenton, K. Sansuk, R. A. Bakker, R. Leurs, *Mol. Pharmacol.* **2006**, *70*, 604–615.
[24] P. W. Mantyh in *Receptor Localization: Ligand Autoradiography, Vol. 13* (Eds.: F. M. Leslie, C. A. Altar), Liss, New York, **1988**, pp. 9–36.
[25] D. Witte, B. B. Yao, T. R. Miller, T. L. Carr, S. Cassar, R. Sharma, R. Faghih, B. W. Surber, T. A. Esbenshade, A. A. Hancock, K. M. Krueger, *Br. J. Pharmacol.* **2006**, *148*, 657–670.
[26] E. A. Harper, N. P. Shankley, J. W. Black, *Br. J. Pharmacol.* **1999**, *128*, 881–890.
[27] X. Ligneau, S. Morisset, J. Tardivel-Lacombe, F. Gbahou, C. R. Ganellin, H. Stark, W. Schunack, J. C. Schwartz, J. M. Arrang, *Br. J. Pharmacol.* **2000**, *131*, 1247–1250.
[28] T. W. Lovenberg, J. Pyati, H. Chang, S. J. Wilson, M. G. Erlander, *J. Pharmacol. Exp. Ther.* **2000**, *293*, 771–778.
[29] K. Wieland, G. Bongers, Y. Yamamoto, T. Hashimoto, A. Yamatodani, W. M. B. P. Menge, H. Timmerman, T. W. Lovenberg, R. Leurs, *J. Pharmacol. Exp. Ther.* **2001**, *299*, 908–914.
[30] C. Liu, X.-J. Ma, X. Jiang, S. J. Wilson, C. L. Hofstra, J. Blevitt, J. Pyati, X. Li, W. Chai, N. Carruthers, T. W. Lovenberg, *Mol. Pharmacol.* **2001**, *59*, 420–426.

[31] B. B. Yao, C. W. Hutchins, T. L. Carr, S. Cassar, J. N. Masters, Y. L. Bennani, T. A. Esbenshade, A. A. Hancock, *Neuropharmacology* **2003**, *44*, 773–786.
[32] A. D. Medhurst, A. R. Atkins, I. J. Beresford, K. Brackenborough, M. A. Briggs, A. R. Calver, J. Cilia, J. E. Cluderay, B. Crook, J. B. Davis, R. K. Davis, R. P. Davis, L. A. Dawson, A. G. Foley, J. Gartlon, M. I. Gonzalez, T. Heslop, W. D. Hirst, C. Jennings, D. N. C. Jones, L. P. Lacroix, A. Martyn, S. Ociepka, A. Ray, C. M. Regan, J. C. Roberts, J. Schogger, E. Southam, T. O. Stean, B. K. Trail, N. Upton, G. Wadsworth, J. A. Wald, T. White, J. Witherington, M. L. Woolley, A. Worby, D. M. Wilson, *J. Pharmacol. Exp. Ther.* **2007**, *321*, 1032–1045.
[33] Y. Zhu, D. Michalovich, H.-L. Wu, K. B. Tan, G. M. Dytko, I. J. Mannan, R. Boyce, J. Alston, L. A. Tierney, X. Li, N. C. Herrity, L. Vawter, H. M. Sarau, R. S. Ames, C. M. Davenport, J. P. Hieble, S. Wilson, D. J. Bergsma, L. R. Fitzgerald, *Mol. Pharmacol.* **2001**, *59*, 434–441.
[34] J. A. Jablonowski, C. A. Grice, W. Chai, C. A. Dvorak, J. D. Venable, A. K. Kwok, K. S. Ly, J. Wei, S. M. Baker, P. J. Desai, W. Jiang, S. J. Wilson, R. L. Thurmond, L. Karlsson, J. P. Edwards, T. W. Lovenberg, N. I. Carruthers, *J. Med. Chem.* **2003**, *46*, 3957–3960.
[35] N. Terzioglu, R. M. van Rijn, R. A. Bakker, I. J. P. De Esch, R. Leurs, *Bioorg. Med. Chem. Lett.* **2004**, *14*, 5251–5256.
[36] E. Schneider, unpublished results, Department of Pharmacology and Toxicology, University of Regensburg, Germany, **2008**.
[37] U. Lippert, M. Artuc, A. Grutzkau, M. Babina, S. Guhl, I. Haase, V. Blaschke, K. Zachmann, M. Knosalla, P. Middel, S. Kruger-Krasagakis, B. M. Henz, *J. Invest. Dermatol.* **2004**, *123*, 116–123.
[38] P. Ling, K. Ngo, S. Nguyen, R. L. Thurmond, J. P. Edwards, L. Karlsson, W.-P. Fung-Leung, *Br. J. Pharmacol.* **2004**, *142*, 161–171.
[39] R. Seifert, T. Wieland, *Methodological Approaches*, Wiley-VCH, Weinheim, **2005**.
[40] Y.-C. Cheng, W. H. Prusoff, *Biochem. Pharmacol.* **1973**, *22*, 3099–3108.
[41] S. Lazareno, *J. Recept. Signal Transduction Res.* **2001**, *21*, 139–165.
[42] R. Seifert, K. Wenzel-Seifert, T. Bürckstümmer, H. H. Pertz, W. Schunack, S. Dove, A. Buschauer, S. Elz, *J. Pharmacol. Exp. Ther.* **2003**, *305*, 1104–1115.
[43] M. T. Kelley, T. Bürckstümmer, K. Wenzel-Seifert, S. Dove, A. Buschauer, R. Seifert, *Mol. Pharmacol.* **2001**, *60*, 1210–1225.

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