Evaluation of Histamine H₁-, H₂-, and H₃-Receptor Ligands at the Human Histamine H₄ Receptor: Identification of 4-Methylhistamine as the First Potent and Selective H₄ Receptor Agonist

Herman D. Lim,¹ Richard M. van Rijn,¹ Ping Ling, Remko A. Bakker, Robin L. Thurmond, and Rob Leurs


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ABSTRACT

The histamine H₄ receptor (H₄R) is involved in the chemotaxis of leukocytes and mast cells to sites of inflammation and is suggested to be a potential drug target for asthma and allergy. So far, selective H₄R agonists have not been identified. In the present study, we therefore evaluated the human H₄R (hH₄R) for its interaction with various known histaminergic ligands. Almost all of the tested H₁R and H₂R antagonists, including several important therapeutics, displaced less than 30% of specific[^3H]histamine binding to the hH₄R at concentrations up to 10 μM. Most of the tested H₁R agonists and imidazole-based H₂R ligands show micromolar-to-nanomolar range hH₄R affinity, and these ligands exert different intrinsic hH₄R activities, ranging from full agonists to inverse agonists. Interestingly, we identified 4-methylhistamine as a high-affinity H₄R ligand (Kᵢ = 50 nM) that has a >100-fold selectivity for the hH₄R over the other histamine receptor subtypes. Moreover, 4-methylhistamine potently activated the hH₄R (pEC₅₀ = 7.4 ± 0.1; α = 1), and this response was competitively antagonized by the selective H₄R antagonist JNJ 7777120 [1-[(5-chloro-1H-indol-2-yl)-carbonyl]-4-methylpiperazine] (pA₂ = 7.8). The identification of 4-methylhistamine as a potent H₄R agonist is of major importance for future studies to unravel the physiological roles of the H₄R.

Histamine exerts many (patho-)physiological effects through its interaction with four histamine receptor subtypes that all belong to the family of G protein-coupled receptors (Hough, 2001). The histamine H₁ receptor (H₁R) and H₂ receptor (H₂R) were pharmacologically identified long before the cDNAs were cloned (Gantz et al., 1991; Yamashita et al., 1991), and they have been successful blockbuster targets for more than two decades. The cDNA encoding the histamine H₃ receptor (H₃R) was cloned more recently (Lovenberg et al., 1999), and bioinformatic analysis of human genome databases resulted in identification of the gene encoding the human H₄R (hH₄R) based on its sequence homology to the H₃R (37%) (Oda et al., 2000; Liu et al., 2001a; Morse et al., 2001; Nguyen et al., 2001; Zhu et al., 2001). Although the hH₄R is mainly present in the nervous system, the hH₄R is distributed mainly in hematopoietic cells (Oda et al., 2000; Liu et al., 2001a; Morse et al., 2001; Zhu et al., 2001). The H₄R shows a different pharmacological profile compared with the closely related H₂R, although many H₂R ligands also interact with the hH₄R.

ABBREVIATIONS: H₁R, histamine H₁ receptor; H₂R, human histamine H₂ receptor; IL, interleukin; JNJ 7777120, 1-[(5-chloro-1H-indol-2-yl)carbonyl]-4-methylpiperazine; VUF 6002, 1-[(5-chloro-1H-benimidazol-2-yl)carbonyl]-4-methylpiperazine; VUF 4742, N-(4-chlorobenzyl)-N-[(4-(5)-imidazolyl)pentyl]thiourea; OUP-16, 2-cyano-1-methyl-1–3-{(2R,5R)-5-[1H-imidazol-4(5)-yl]tetrahydrofuran-2-yl}methylguanidine; PEA, 2-pyridylethylamine; TEA, 2-(2-thiazolyl)ethylamine; VUF 8328, S-[3-(4-(5)-imidazolyl)propyl]isothiourea; ORG37770, 1,2,3,4,10,14b-hexahydro-2-methylpyrazino[2,1-a]pyrido[2,3-c][2]benzazepine; CRE, cAMP response element; BMMC, bone marrow mast cell; JNJ 637940, 7-methyl-2-[4-(3-piperidin-1-yl-propoxy)-phenyl]-imidazo[1,2-a]pyridine; A-349821, 4′-3-[[R(R)2,5-dimethyl-pyrolidin-1-yl]-propoxy]-biphenyl-4-yl)-morpholin-4-yl-methanone; VUF 4683, 1-[4-[(imidazol-4-yl)-butyl]-3-isopropyl-thiourea; VUF 4616, 1-[5-(imidazol-4-yl)-penty]-3-isopropyl-thiourea; VUF 4617, 1-cyclohexyl-3-[5-((imidazol-4-yl)-pentyl]-thiourea.
with the H₄R. Like the H₂R, the H₄R couples to pertussis toxin-sensitive Gi/o proteins and thereby inhibits forskolin-induced cAMP production (Oda et al., 2000; Liu et al., 2001a; Zhu et al., 2001). In addition, the H₄R also activates mitogen-activated protein kinase (Morse et al., 2001) and mobilizes calcium in eosinophils and mast cells (Buckland et al., 2003; Hofstra et al., 2003).

The presence of the hH₄R on leukocytes and mast cells suggests that this new histamine receptor plays an important role in the modulation of the immune system. This hypothesis is supported by the fact that IL-10 and IL-13 modulate hH₄R expression (Morse et al., 2001) and that binding sites for cytokine-regulated transcription factors, such as interferon-stimulated response element, interferon regulatory factor-1, nuclear factor-κB, and nuclear factor-IL-6, are present upstream of the hH₄R gene (Cogé et al., 2002).

Physiological roles of the hH₄R include the control of IL-16 release by human CD₈⁺ T cells (Gantner et al., 2002), chemotactic responses and cytoskeletal changes of human eosinophils (O’Reilly et al., 2002; Buckland et al., 2003; Ling et al., 2004), chemotaxis and intracellular calcium mobilization in mast cells (Hofstra et al., 2003), and control of leukotriene B₄ production by mast cells that subsequently leads to neutrophil recruitment into peritoneum (Takeshita et al., 2003; Thurmond et al., 2004). These studies suggest that the hH₄R is a potential drug target for immune system-related diseases.

Until recently, potent and selective H₄R ligands were not available. In the early studies, the H₄R antagonist thipiperazine (Jablonski et al., 2003; Thurmond et al., 2004) and the related benzimidazole analog VUF 6002 (Terzgliu et al., 2004) as selective and potent hH₄R antagonists. Studies directed toward selective H₄R agonists have so far been less successful. Burimamide, clozapine, and clobenpropit are all known to act as H₂R agonists, and clozapine and clobenpropit have been proven useful for initial pharmacological studies (Gantner et al., 2002; Buckland et al., 2003; Bell et al., 2004; Ling et al., 2004). Currently, the most selective H₄R agonist is the imidazoline JNJ 7777120, which displays a 15-fold selectivity for the hH₄R compared with its binding affinity for the hH₂R (Hashimoto et al., 2003). However, the lack of selectivity of currently known agonists for the H₄R limits their use as H₂R agonists.

In our search for selective H₄R agonists, many known histaminergic ligands of different structural classes, including several important therapeutics, were evaluated for their interaction with the hH₄R. Our studies resulted in the identification of 4-methylhistamine, a presumed moderately active and selective H₄R agonist (Durant et al., 1975), as a high-affinity H₄R agonist with a more than 100-fold selectivity over the H₃R, H₂R, and H₁R.

**Materials and Methods**

**Materials.** Aminopotentidetine, amthamine dihydrobromide, amselamine dihydrobromide, burimamide oxalate, and burimamide analogs (Vollinga et al., 1985); clobenpropit dihydrochloride, dimaprit dihydrobromide, histaprodifen dimaleate, homohistamine dihydrobromide, imbutamine dihydrobromide, imetit dihydrobromide, imipenep dihydrobromide, immethidine dihydrobromide, iodopropenil dihydrobromide, JNJ 7777120, 2'-3-bromophenylhistamine dihydrobromide, methimiprop dihydrobromide, 2'-pyridylmethylethylamine (TEA) dihydrochloride, thioipramer fumarate, and VUF 8326 were synthesized at the Department of Medicinal Chemistry (Vrije Universiteit Amsterdam, Amsterdam, The Netherlands). Fatmotide, ketotifen fumarate, and S(+)-lansoprazole were purchased from MP Biomedicals (Irvine, CA). Amoxapine, d-chloropromazine maleate, clozapine, cimetidine, N-desmethyl clozapine, diphenhydramine hydrochloride, doxepin hydrochloride, fexofenadine, histamine dihydrochloride, imipramine hydrochloride, loxapine, mepramine (pyrilamine maleate), (R)α-methylhistamine dihydrochloride, (S)α-methylhistamine dihydrochloride, N,N-methylhistamine dihydrochloride, N oxide clozapine, octoclotheptin, pertussis toxin, polyethyleneamine, ranitidine hydrochloride, triphenylamine dihydrochloride, and triprolidine hydrochloride were purchased from Sigma/RBI (Natick, MA). 2-Nitrophenol-β-pyranoside and G418 (geneticin) were from Duchefa (The Netherlands); promethazine was from UVM-Chemistry Amsterdam (Amsterdam, The Netherlands); fexofenadine was from Ultrafine Chemicals (Manchester, UK); tiotidine was from Imperial Chemical Industries PLC (London, UK); and [3H]N⁺N⁺-methylhistamine (85 Ci/mmol), [3H]histamine (12.4 Ci/mmol), and [3H]mepipramine (23 Ci/mmol) were from PerkinElmer Life and Analytical Sciences (Boston, MA). [125I]Iodoaminopotentidetine and [125I]iodopropenil were labeled at the Department Nuclear Medicine and PET Research (Vrije Universiteit Medical Centre, Amsterdam, The Netherlands) as described previously (Jansen et al., 1992), whereas [3H]JNJ 7777120 (84 Ci/mmol) was synthesized at Johnson & Johnson Pharmaceutical Research and Development, L.L.C. (La Jolla, CA) (Thurmond et al., 2004). Gifts of astemizole (Janssen Pharmaceuticaals, Antwerp, Belgium); ciproheptadine hydrochloride (MSD, Haarlem, The Netherlands); cetirizine hydrochloride and hydroxyzine dihydrochloride (UCB Pharma, Brussels, Belgium); ebastine (Almirall Prodesfarma, Barcelona, Spain); loratidine (Schering Plough, Kenilworth, N.J.); mianserin hydrochloride and ORG37770 (Organon NV, Oss, The Netherlands); mifentidine (Instituto De Angel, Milan, Italy) and mizolastine (Synthélabo Recherche, Bagneux, France); propyfan dihydrochloride and propanoxyfan dihydrochloride (Sepracor, Marlborough, MA); 2-methylhistamine dihydrochloride, 4-methylhistamine dihydrochloride, and imipramine hydrochloride (GlaxoSmithKline, Welwyn Garden City, Hertfordshire, UK) are greatly acknowledged.

**Cell Culture.** SK-N-MC cell lines, which stably express either the human H₃R (SK-N-MC/hH₃), or H₄R (SK-N-MC/hH₄) as well as a cosmammalian reporter cell line (NAG-responsive element (CRE)-driven β-galactosidase reporter gene vector SK-N-MCH or SK-N-MC/hH₄ cells (Lovénberg et al., 1999; Jansen et al., 1992), were cultured in Eagle’s minimum essential medium supplemented with 5% fetal calf serum, 0 mg/ml streptomycin, 100 U/ml penicillin, and 600 μg/ml G418 at 37°C in 5% CO₂ and 95% humidity.

**Radioligand Binding Assays.** The SK-N-MC/hH₃ cells homogenates were incubated for 40 min at 25°C with approximately 1 nM [3H]N⁺N⁺-methylhistamine in 25 mM KPO₄ buffer and 140 mM NaCl (pH 7.4 at 25°C), with or without competing ligands, whereas the SK-N-MC/hH₄ cell homogenates were incubated 1 h at 37°C in 10 mM [3H]histamine and 50 mM Tris-HCl (pH 7.4 at 37°C), with or without competing ligands. Bound radioligands were collected on 0.3% polyethleneimine-pretreated Whatman GF/C (and washed three times with 3 ml of ice-cold washing buffer (4°C) containing 5 mM KPO₄ buffer and 140 mM NaCl (pH 7.4 at 4°C)) for the hH₃R and 50 mM Tris-HCl (pH 7.4 at 4°C) for the hH₄R. Binding analysis of 10 nM [3H]JNJ 7777120 and 0.1 nM [125I]iodopropenil to the hH₄R was performed with the same conditions as described for [3H]histamine. Inatura...
tion binding analysis, the nonspecific binding of [3H]histamine or [3H]JNJ 7777120 was determined with 1 \( \mu M \) clobenpropit. The binding analysis of [3H]mepyramine and [125I]iodoaminopotentidine binding to human H1R and human H2R, respectively, was performed according to Bakker et al. (2004). The binding data were analyzed with Prism 4.0 (GraphPad Software Inc., San Diego, CA), and data are presented as mean ± S.E.M. Mouse and rat H4R radioligand binding assays were performed according to Liu et al. (2001b).

Colometric Cyclic AMP Assay. A reporter CRE-\( \beta \)-galactosidase reporter gene assay was used to determine (inverse) agonistic or antagonistic activity of either the hH1R or hH4R. Approximately 4 million cells/96-well plate of SK-N-MC/hH4 and SK-N-MC/hH4 cells were exposed for 6 h to histaminergic ligands in serum-free Eagle's minimum essential medium containing 1 \( \mu M \) forskolin. Thereafter, the medium was discarded, the cells were lysed in 100 \( \mu l \) of assay buffer (100 mM sodium phosphate buffer at pH 8.0, 4 mM 2-nitrophenol-\( \beta \)-D-pyranoside, 0.5% Triton X-100, 2 mM MgSO4, 0.1 mM MnCl4, and 40 mM \( \beta \)-mercaptoethanol), incubated overnight at room temperature, and the \( \beta \)-galactosidase activity was determined at 420 nm with a PowerwaveX340 plate reader (Bio-Tek Instruments Inc., Winooski, VT). The OD420 might differ between experiments due to intra-assay variability; therefore, intrinsic activity of agonists was determined relatively to activity of histamine.

Primary Cell Experiments. Cell culture of BALB/c mice-derived bone marrow mast cells (BMMCs) and in vitro BMMC chemotaxis assay was performed as described previously (Hofstra et al., 2003). Purification of human polymorphonuclear leukocytes and the human eosinophil shape change assay were performed as described previously (Ling et al., 2004). The mouse-derived BMMCs were obtained following approved protocols that follow National Institutes of Health/International Animal Care and Use Guidelines.

Results

Pharmacological Characterization of the hH4R Expressed in SK-N-MC Cells. Stable transfection of the hH4R cDNA in SK-N-MC cells resulted in the expression of functional hH4R proteins. The hH4R could be labeled with both agonist and antagonist radioligands. The H4R agonist [3H]histamine shows saturable binding to the expressed H4R with a minimal amount of nonspecific binding (Fig. 1A).

![Fig. 1](https://example.com/figure1.png)

Fig. 1. The cell line SK-N-MC/hH4 stably expresses functional hH4R and CRE-control \( \beta \)-galactosidase. The homogenate of SK-N-MC/hH4 cells shows a saturable binding for the H4R agonist [3H]histamine (A) and also for the H4R antagonist [3H]JNJ 7777120 (B). The binding of [3H]histamine to the hH4R is inhibited by H3/4R ligands (C). The full agonist histamine and partial agonist clobenpropit hH4R inhibit the 1 \( \mu M \) forskolin-induced CRE activation, as measured by a \( \beta \)-galactosidase reporter gene, whereas the inverse agonist thioperamide dose-dependently blocks the hH4R constitutive activity (D). Data shown are from representative experiments, each performed in triplicate.
hands, the nonimidazole JNJ 7777120 shows a 300-fold selectivity for the hH4R (pKᵢ = 7.8 ± 0.1 against [³H]histamine) over the hH3R (pKᵢ = 5.3 ± 0.1 against [³H]N'-methylhistamine), allowing the use of [³H]JNJ 7777120 to label the hH3R (Thurmond et al., 2004). The hH3R antagonist [³H]JNJ 7777120 exhibits a somewhat higher level of nonspecific binding to hH3R expressing SK-N-MC cells, but it also binds saturaively and shows an equipotent affinity (Kᵢ = 11 ± 3.6 nM; n = 3) and results in a Bₐ₀,max value of 1.7 ± 0.4 pmol/mg protein (Fig. 1B). The binding of either 10 nM [³H]histamine (Fig. 1C) or 10 nM [³H]JNJ 7777120 (data not shown) to the hH3R is fully displaced by histamine (pKᵢ = 7.8 ± 0.1), the H3/H4R antagonist thioperamide (pKᵢ = 6.9 ± 0.1) and the H4R agonist/H3R antagonist clobenpropit (pKᵢ = 8.1 ± 0.1), in a good agreement with the results reported previously (Oda et al., 2000; Liu et al., 2001a; Morse et al., 2001).

The SK-N-MC/hH4 cells used in this study coexpress a CRE-controlled β-galactosidase reporter gene and can therefore also be used for a functional analysis of H4R ligands. Stimulation of the hH3R with histamine resulted in the inhibition (58 ± 3%; n = 16) of the forskolin-stimulated (1 μM) cAMP-mediated reporter gene transcription with a pEC₅₀ value of 7.7 ± 0.1 (n = 16) (Fig. 1D). Treatment of SK-N-MC/hH4 cells with the Gαᵪ₂ protein inhibitor pertussis toxin (100 ng/ml for 16 h) completely inhibited histamine induced responses, confirming the coupling of the H3R to Gαᵪ₂ proteins (Oda et al., 2000; Liu et al., 2001a; Zhu et al., 2001). In our hands, histamine exerted the maximally observed level of inhibition in this assay and is therefore referred to as a full agonist (intrinsic activity α = 1). As reported previously (Oda et al., 2000; Liu et al., 2001a; Morse et al., 2001), clobenpropit acts as a potent partial hH3R agonist with a pEC₅₀ value of 7.7 ± 0.1 (n = 3) and an intrinsic activity of 0.8 (Fig. 1D).

Treatment of SK-N-MC/hH4 cells with pertussis toxin (100 ng/ml for 16 h) resulted in an increase of 1 μM forskolin-stimulated CRE activity by 130 ± 3%, suggesting that the hH3R shows a detectable level of constitutive activity in the SK-N-MC/hH4 cells. In line with previous observations on inverse agonism by thioperamide (Liu et al., 2001a; Morse et al., 2001), 1 μM forskolin-stimulated CRE activity was increased by thioperamide with a pEC₅₀ value of 7.0 ± 0.1 (Fig. 1D). The inhibition of the constitutive activity of the hH3R by thioperamide was of the same magnitude as observed after treatment with pertussis toxin and thioperamide is referred to as a full inverse hH3R agonist (intrinsic activity α = −1).

In SK-N-MC/hH4 cells, the cAMP-driven β-galactosidase reporter-gene transcription also can be activated by endogenously expressed Gαᵪ₂ protein–coupled β adrenergic receptors (Bahouth et al., 2001). The β₂ adrenergic receptor agonist fenoterol induced β-galactosidase activity to a similar extent to that of forskolin, with a pEC₅₀ value of 6.9 ± 0.1 (n = 6). H3R activation by histamine inhibited the 100 nM fenoterol-induced β-galactosidase activity for 39 ± 3% with a pEC₅₀ value of 7.4 ± 0.1 (n = 7). However, inverse agonistic activity of thioperamide, at the hH3R, could not be easily demonstrated with a fenoterol-based assay system (data not shown). The evaluation of the functional activity of all the various histaminergic ligands was therefore performed using forskolin (1 μM) stimulated SK-N-MC/hH4 cells.

All compounds were preliminarily tested as displacers of [³H]histamine binding to the hH3R expressed in SK-N-MC/hH4 cells at a concentration of 10 μM. Compounds inhibiting the specific binding of 10 nM [³H]histamine to the hH3R by ≥30% are expected to have a Kᵢ > 10 μM based on the Cheng and Prusoff (Cheng and Prusoff, 1973) equation: Kᵢ = IC₅₀/(1 + [radioligand]/Kᵢ) and were excluded for further testing. Active compounds (displacement ≥30%) were tested more extensively in both [³H]histamine displacement studies and the CRE-β-galactosidase-based functional hH3R assay.

**Most H4R Ligands Are Devoid of H3R Activity.** Histamine potently displaces [³H]histamine from the hH3R with a pKᵢ value of 7.8 ± 0.1 (Table 1), whereas H3R agonists with substituents at the 2-position of the imidazole ring show significantly lower affinities. Substitution of the imidazole ring with either a small methyl or large 3-bromophenyl substituent is not tolerated and causes an almost 100-fold drop of affinity. Bulkier substituents at the 2-position (1,1-diphenylpropyl in histaprodifen) even result in a total loss of affinity for the hH3R (Table 1). Agonists, lacking the imidazole ring, such as TEA, PEA, or 8R-lisuride (Bakker et al., 2004), are also not active at the hH3R (Table 1).

After an initial report that the H4R can be labeled with [³H]mepyramine (Nguyen et al., 2001), a large number of H4R antagonists (Table 1), including many clinically relevant drugs, were evaluated for their hH3R affinity as well. Almost all tested H4R antagonists, including mepyramine, showed pKᵢ values <5 (Table 1) and did not show functional activity at 1 and 10 μM at the hH3R (data not shown). Although structurally similar to some tricyclic H3R antagonists devoid of H3R affinity, clozapine binds with moderate potency to the hH3R (pKᵢ = 6.7 ± 0.1) and exerts full agonistic activity at the hH3R with a pEC₅₀ value of 6.8 ± 0.1 (n = 5). N-Desmethyl clozapine, a clozapine metabolite, showed a slightly decreased affinity (pKᵢ = 6.5 ± 0.1), whereas N-oxide clozapine, another clozapine metabolite, is totally devoid of H4R affinity. Furthermore, we evaluated clozapine analogs of therapeutic importance as well. Loxapine and amoxapine showed >10-fold lower affinity (pKᵢ 5.4 ± 0.1 and 5.3 ± 0.1, respectively), whereas octoclozepine did not show binding for the hH3R at all.

**Some H4R Ligands Act as hH3R Agonists.** Within the series of known H3R agonists that we have tested, only some ligands retain H3R activity. Replacement of the imidazole ring of histamine in the selective H4R agonists amthamine and amselamine (Leurs et al., 1994) results in a total loss of hH3R activity at concentrations up to 10 μM. Dimaprit, a H4R agonist/H3R antagonist lacking an imidazole group, binds the H3R with moderate affinity, showing a pKᵢ value of 6.5 ± 0.1, and exerts partial H3R agonistic activity (Table 1). Impromidine, which was reported to bind to both H3R and H4R, also binds potently to the hH3R with a pKᵢ value of 7.6 ± 0.1 and acts as a partial H3R agonist (α = 0.5). Both the R- and S-enantiomers of the related ropivamidine bind, respectively, >10 and >100 times less potently. In fact, the first reported H3R selective agonist 4-methylhistamine (Durant et al., 1975) is the only known H3R agonist that also acts as full agonist at the H4R (Table 1). 4-Methylhistamine binds two times less potently than histamine to the hH3R, exhibiting a pKᵢ value of 7.3 ± 0.1 (n = 3).

Most tested H4R antagonists, including cimetidine, mifen- tidine, aminopotentidine, ranitidine, famotidine, and tiotidine, only displaced <30% of 10 nM [³H]histamine binding to the hH3R. Only the H3 antagonist bisuramide shows a high affinity for the hH3R pKᵢ = 7.4 ± 0.1 (Table 1). Moreover,
various burimamide analogs as H3R antagonists (Vollinga et al., 1994). Reduced H4R agonistic activity. A total loss of agonistic H4R activity is observed for VUF 4614. As can be seen in Fig. 2B, VUF 4614 was able to competitively block the hH4R agonistic responses of histamine, resulting in a pA2 value of 6.8. Finally, within this series we identified VUF 4742 as an hH4R inverse agonist (Fig. 2A). This burimamide analog bound with moderate affinity to the H4R (pK_i = 6.9 ± 0.1; n = 4) and acted as a full inverse agonist with a pEC50 value of 7.2 ± 0.1 (n = 5), in accordance with its binding affinity.

**Evaluation of H3R Ligands at the hH4R**. The H3R shares its highest sequence homology with the H4R, and it is therefore not surprising that in the initial studies some H3R ligands were identified as H4R ligands as well. We therefore characterized in this study a large set of known H3R ligands for their interaction with the H4R. The histamine analogs N4-methylhistamine, (R)-α-methylhistamine, and (S)-α-methylhistamine show an almost 2 order of magnitude lower affinity for the hH3R than for the hH4R. However, the hH4R retains some level of stereoselectivity for (R)-α-methylhistamine (pK_i = 5.6 ± 0.1) and (S)-α-methylhistamine (pK_i = 5.4 ± 0.1) (Table 3).

**Table 1** Activity of H3R and H4R ligands at the hH4R

<table>
<thead>
<tr>
<th>H3R ligand</th>
<th>pEC50</th>
<th>H4R Activity</th>
<th>pEC50</th>
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<td>Mepyramine</td>
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<td>N.D.</td>
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<td>Antagonist</td>
<td>&lt;5</td>
<td>N.D.</td>
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<th>H4R ligand</th>
<th>pK_i at hH4R</th>
<th>H4R Activity</th>
<th>pK_i</th>
<th>pEC50</th>
<th>α</th>
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<td>Mefentidine</td>
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<td>Agonist</td>
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<td>N.D.</td>
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<tr>
<td>Triptidine</td>
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<td>Agonist</td>
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α intrinsic activity (1 designated for full agonistic, 0 for neutral antagonist, and – for full inverse agonistic activity); N.D., not determined; —, due to non-H4R-mediated effects of SR-141716, the pEC50 value was not determined.
et al., 2001), also potently bind the hH4R with p

immepip, imetit, and VUF 8328 (an imetit analog) (Wieland

hH4R( affinity, impentamine also looses intrinsic activity for the

H3R agonist impentamine shows only moderate affinity at

1000 nM. The latter effect is not mediated by the hH4R, because it cannot be blocked by H4R antagonist JNJ 7777120.

Increasing the spacer length between imidazole and amine
group from two carbon atoms (histamine, p

K

M forskolin-stimulated β-galactosidase activity at concentrations up to 1 μM and an increase at concentrations ≥10 μM. The latter effect is not mediated by the hH4R, because it cannot be blocked by H4R antagonist JNJ 7777120.

Table 2
Activity of burimamide analogs at the hH4R
The ligands were tested as described under Materials and Methods. Data shown are mean ± standard error of mean of at least three independent experiments, each performed in triplicate. pKi values were determined with [3H]histamine displacement assay; pEC50 values show the inhibition of 1 μM forskolin-stimulated β-galactosidase activity in SK-N-MC/hH4 cells.

<table>
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<tr>
<th>Ligand</th>
<th>n</th>
<th>R</th>
<th>pK_i at hH4R</th>
<th>pKi</th>
<th>pEC50</th>
<th>α</th>
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<td>7.0</td>
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<tr>
<td>VUF 4616</td>
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<tr>
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<td>VUF 4742</td>
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α, intrinsic activity (1 designated for full agonistic, 0 for neutral antagonist, and −1 for full inverse agonistic activity); N.D., not determined.

b pKi values determined with [125I]iodoaminopotentidine displacement assay (Leurs et al., 1994).

pKi values determined with [125I]iodoaminopotentidine displacement assay (Vollinga et al., 1995).

Fig. 2. Burimamide analogs exert different intrinsic activities at the hH4R. A, burimamide acts as a H4R partial agonist, which dose-dependently inhibits 1 μM forskolin-induced CRE activation in SK-N-MC/hH4 cells. Various burimamide analogs act as H4R agonists (VUF 4683, burimamide, and VUF 4686), a neutral H4R antagonist (VUF 4614), or an inverse H4R agonist (VUF 4742). B, neutral H4R antagonist VUF 4614 competitively antagonizes the histamine response at the hH4R, resulting in a rightward shift of histamine dose-response curve. Data shown are from representative experiments, each performed in triplicate.

Increasing the spacer length between imidazole and amine group from two carbon atoms (histamine, pKi = 7.8 ± 0.1) to three carbon atoms (homohistamine, pKi = 7.5 ± 0.1) slightly decreases the affinity for the hH4R, whereas four carbon atoms (imbutamine, pKi = 8.0 ± 0.1) results in a slightly higher hH4R affinity. A further increase of the spacer length proved to be detrimental for hH4R affinity. The highly potent H4R agonist impentamine shows only moderate affinity at the H4R (pKi = 6.6 ± 0.1) (Table 3). Interestingly, besides the affinity, impentamine also looses intrinsic activity for the hH4R (α = 0). Previously identified H4R agonists, including immeep, imetit, and VUF 8328 (an imetit analog) (Wieland et al., 2001), also potently bind the hH4R with pKi values of 7.7 ± 0.1, 8.2 ± 0.1, and 8.0 ± 0.1, respectively. At the hH4R these ligands also act as agonists, but exert somewhat lower intrinsic activity (α values of 0.9, 0.9, and 0.6, respectively) (Table 3). As reported previously (Kitbunnadaj et al., 2004), the recently identified H4R agonist immethridine (pKi = 9.1 ± 0.1) binds much less potently to the hH4R (pKi = 6.6 ± 0.1) and is also not able to fully activate the H4R (Table 3). In agreement with our findings with N-methylhistamine, the methylated immeep analog methimeep shows a large selectivity for the hH4R (pKi = 9.0 ± 0.1) over the hH4R (pKi = 5.7 ± 0.1), as reported previously (Kitbunnadaj et al., 2005).

Also various H4R antagonists bind to the hH4R (Oda et al., 2000; Liu et al., 2001a; Morse et al., 2001; Zhu et al., 2001). In our hands the isotheiourea-based H4R antagonists clobenpropit and iodophenpropit both potently bind to the hH4R (Table 3). Yet, both ligands have very distinguishable intrinsic activities at the H4R. Clobenpropit, which acts as inverse...
agonist at the hH4R (Wieland et al., 2001) behaves as a
potential partial agonist at the hH4R (Table 3; Fig. 3, A and B).
In contrast, iodophenpropit, which also acts as an inverse
agonist at the hH3R (Wieland et al., 2001), behaves as a
neutral antagonist at the hH4R with a pKᵢ value of 7.9 ± 0.1
(n = 6) (Table 3; Fig. 3, A and B). As expected, iodophenpropit
competitively antagonized the action of histamine at the
hH4R, yielding a linear Schild-plot and a pA₂ value of 8.0
(Fig. 3, C and D), in accordance with its binding affinity.
Besides clophenpropit, also the known H3R ligands propit
and the related idopropit show reasonable hH4R affinity
with pKᵢ values of 7.3 ± 0.1 and 7.9 ± 0.1, respectively.
As observed for their action at the hH3R, both compounds act
as partial agonist at the hH4R (Table 3).

**Evaluation of the Potential Use of [125I]Iodophen-
propit as hH4R Radioligand.** As reported in the previous
section and in other studies, the hH4R can be labeled with
either [3H]histamine or the H3R antagonist [3H]JNJ 7777120
(Oda et al., 2000; Liu et al., 2001a; Morse et al., 2001; Nguyen
et al., 2001; Zhu et al., 2001; Thurmond et al., 2004). Previ-
ously, we described [125I]iodophenpropit as a suitable high
affinity H3R radioligand (Jansen et al., 1992). Considering,
the relatively high affinity of iodophenpropit at the hH4R and
its high sensitivity, we investigated the potential of this
radioligand to label the hH4R. Due to the hH4R affinity of
[125I]iodophenpropit, saturation binding experiments were
not feasible. We therefore used homologous [125I]iodophen-
propit displacement analysis to determine a Kᵢ value of
34.4 ± 4.1 nM for [125I]iodophenpropit. The B_max value ob-
tained using [125I]iodophenpropit-binding displacement ex-
periments (3.8 ± 0.4 pmol/mg protein) is approximately 2
times higher than those obtained with either [3H]histamine
(1.8 ± 0.4 pmol/mg protein) and [3H]JNJ 7777120 (1.7 ± 0.4
pmol/mg protein). [125I]iodophenpropit binding to mem-
branes of SK-N-MC/hH4 cells was competitively displaced by
a variety of H3/R ligands (Fig. 4), despite a high level of
nonspecific binding of approximately 60% as determined
with 10 μM imetit. However, iodophenpropit and the related
clophenpropit also displaced the nonspecific binding, resulting
in a multiple site binding profile. The pKᵢ values of com-
ounds for the hH4R obtained using [125I]iodophenpropit dis-
placement studies are consistent with their corresponding
values obtained using displacement of either [3H]histamine or
[3H]JNJ 7777120 binding to the hH4R; only the pKᵢ value of
thiopropitamine obtained using [125I]iodophenpropit dis-
placement studies seems to deviate somewhat form the pKᵢ
values obtained using either [3H]histamine or [3H]JNJ 7777120
displacement studies (Fig. 4; Table 4).

**4-Methylhistamine as a Selective H4R Agonist.** After
our initial observation of the relative high affinity of 4-methyl-
histamine for the hH4R, this histamine analog was evaluated
in more detail. 4-Methylhistamine not only has high
affinity for the hH4R (pKᵢ = 7.3 ± 0.1; n = 3) but also exhibits
considerable selectivity for the hH4R over the other three
human histamine receptors (Fig. 5A). The human histamine
H1, H2, and H3 receptors were tested for their interaction
with 4-methylhistamine, using respectively 1 nM [3H]me-
pyramine (Kᵢ = 1.6 nM), 0.5 nM [125I]iodoaminopotentidine
(Kᵢ = 0.5 nM) and 1 nM [3H]N⁴-methylhistamine (Kᵢ = 2.9
nM) binding to homogenates of transfected cells. As can be
seen in Fig. 5A, 4-methylhistamine shows highest affinity for
the hH4R and binds considerably less potently to the other
histamine receptors, resulting in a >100-fold and >100,000-
fold selectivity over the H3R and H2R, and H3R, respectively.
4-Methylhistamine not only binds to the hH4R but also has a
high affinity, albeit reduced compared with the H3R, for the
mouse and rat H4R with Kᵢ values of 73 and 55 nM,
respectively (Fig. 5B). Moreover, 4-methylhistamine exerts full
agonistic activity at the hH4R (Fig. 5C), resulting in a pEC₅₀
value of 7.4 ± 0.1 (α = 1; n = 5). In contrast, 4-methylhista-
mine exhibits only moderate affinity for the hH4R (pKᵢ =
5.1 ± 0.1; n = 3) and hH3R (pKᵢ = 5.2 ± 0.1; n = 4), and
partial agonistic hH4R activity (Fig. 5D). The hH4R agonistic
effects of 4-methylhistamine can be antagonized by the se-

### Table 3

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<tr>
<th>Ligand</th>
<th>pKᵢ</th>
<th>pEC₅₀</th>
<th>α</th>
<th>pKᵢ</th>
<th>pEC₅₀</th>
<th>α</th>
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<td>Histamine</td>
<td>8.0 ± 0.1</td>
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α, intrinsic activity (1 designated for full agonistic, 0 for neutral antagonist, and –1 for full inverse agonistic activity.)
lective H4R antagonist JNJ 7777120 (Fig. 5E). Schild-plot analysis of the JNJ 7777120 antagonism of the 4-methylhistamine-induced hH4R-mediated inhibition of forskolin-induced β-galactosidase activity yields a $pA_2$ value of 7.8 (data not shown), which is in agreement with the hH4R affinity of JNJ 7777120 (Table 3) (Jablonski et al., 2003; Thurmond et al., 2004). At the mouse and rat H4R, 4-methylhistamine also acts as a full H4 agonist, although with reduced pEC50 values of 5.8 and 5.6, respectively.

Previously, the H4R has been shown to be involved in the regulation of eosinophil and mast cell function (O’Reilly et al., 2002; Buckland et al., 2003; Hofstra et al., 2003; Takeshita et al., 2003; Ling et al., 2004; Thurmond et al., 2004). Indeed, 4-methylhistamine also acts as a full H4 agonist, although with reduced pEC50 values of 5.8 ± 0.1 and 5.6 ± 0.1, respectively.

Previously, the H4R has been shown to be involved in the regulation of eosinophil and mast cell function (O’Reilly et al., 2002; Buckland et al., 2003; Hofstra et al., 2003; Takeshita et al., 2003; Ling et al., 2004; Thurmond et al., 2004). Indeed, 4-methylhistamine also acts as a full H4 agonist, although with reduced pEC50 values of 5.8 ± 0.1 and 5.6 ± 0.1, respectively.
Ling et al. (2004) have previously demonstrated that the H3R antagonist JNJ 637940 did not affect histamine-induced eosinophil shape change and that the H3R was not expressed in eosinophils. Finally, 4-methylhistamine was tested as an H4R agonist at mouse BMMCs as described previously (Hofstra et al., 2003). Like histamine, 4-methylhistamine dose-dependently induced migration of murine BMMCs with an EC50 value of 12 $\mu$M (Fig. 6C), again a somewhat lower potency compared with histamine (Hofstra et al., 2003). Furthermore, we found that the effect of 4-methylhistamine on murine BMMCs was completely inhibited by the selective H4R antagonist JNJ 7777120 in a dose-dependent manner (Fig. 6D).

**Discussion**

With the addition of the H4R to the histamine receptor family (Oda et al., 2000; Liu et al., 2001a; Morse et al., 2001; Nguyen et al., 2001; Zhu et al., 2001), this potential new drug target has created a lot of excitement in the field. The predominant expression of the histamine H4R on hematopoietic cells (Oda et al., 2000; Liu et al., 2001a; Morse et al., 2001; Zhu et al., 2001) and the H4R effects on, e.g., eosinophil and mast cell functions (Gantner et al., 2002; O'Reilly et al., 2002; Buckland et al., 2003; Hofstra et al., 2003; Takeshita et al., 2003; Ling et al., 2004; Thurmond et al., 2004) imply that this new histamine receptor subtype may play a role in various...

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**TABLE 4**

hH4R affinity for selected H4R ligands as determined with displacement of the binding of [3H]histamine, [3H]JNJ 7777120, or [125I]iodophenpropit.

Data shown are mean ± standard error of mean of at least three independent experiments, each performed in triplicate.

<table>
<thead>
<tr>
<th>Compound</th>
<th>[3H]Histamine</th>
<th>[3H]JNJ 7777120</th>
<th>[125I]Iodophenpropit</th>
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</thead>
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<td>7.8 ± 0.1</td>
<td>7.7 ± 0.1</td>
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<td>4-Methylhistamine</td>
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</tr>
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<td>Immepip</td>
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<td>Clozapine</td>
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<td>Clobenpropit</td>
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<td>6.9 ± 0.1</td>
<td>7.1 ± 0.1</td>
<td>7.4 ± 0.1</td>
</tr>
<tr>
<td>JNJ 7777120</td>
<td>7.8 ± 0.1</td>
<td>7.8 ± 0.1</td>
<td>7.8 ± 0.1</td>
</tr>
<tr>
<td>$K_d$ (nM)</td>
<td>20.9 ± 1.6</td>
<td>11.1 ± 3.6</td>
<td>34.4 ± 4.1*</td>
</tr>
<tr>
<td>$R_{max}$ (pmol/mg protein)</td>
<td>1.8 ± 0.4</td>
<td>1.7 ± 0.4</td>
<td>3.8 ± 0.4</td>
</tr>
</tbody>
</table>

*a* Determined by homologous displacement analysis, using the equation $K_d = IC_{50} - \text{[radioilgand]}$.  

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Fig. 5. 4-Methylhistamine is a potent and selective H4R full agonist. A, 4-methylhistamine displacement of the binding of 1 nM [3H]mepyramine, 0.5 nM [125I]iodoaminopotentidinedine, 1 nM [3H]N-methylhistamine, and 10 nM [3H]histamine to the human H1, H2, H3, and H4 receptor, respectively. The chemical structure of 4-methylhistamine is presented as an insert. B, 4-methylhistamine displacement of [3H]histamine binding to mouse, rat, and human H4Rs. C and D, functional effects of 4-methylhistamine at the human H3R (D) and the human H4R (C) compared with the effects of histamine. E, 4-methylhistamine exerts a full hH4R agonistic activity that is competitively antagonized by JNJ 7777120. Data shown are from representative experiments, each performed in triplicate.
allergic and inflammatory conditions. So far, the search for selective H4R ligands has resulted in the discovery of potent neutral hH4R antagonists as JNJ 7777120 (Jablonski et al., 2003; Thurmond et al., 2004) and VUF 6002 (Terzioglu et al., 2004), whereas potent and selective H4R agonists or inverse agonists have so far not been described. In search for new hH4R ligands, we therefore screened a library of known histaminergic ligands, using SK-N-MC cells stably expressing the hH4R. In this cell line the hH4R binds [3H]histamine and [3H]JNJ 7777120 with high affinity (Fig. 1, A and B) and functionally inhibits forskolin-induced CRE-mediated responses through pertussis toxin-sensitive Gi/o proteins (Fig. 1D). In these cells the hH4R also exhibits constitutive activity, which is blocked by pertussis toxin or the nonselective inverse agonist thioperamide (Fig. 1D).

Considering the H4R shares its highest sequence similarity with the H3R, it is not surprising that the H4R is targeted by various imidazole containing H3R ligands (Oda et al., 2000; Liu et al., 2001a; Morse et al., 2001; Zhu et al., 2001). The standard H3R inverse agonist thioperamide (Arrang et al., 1983) also acts as an inverse agonist at the hH4R. Moreover, in the present study we confirm that the presumed H3R agonists immeip, imetit, (R)-α-methylhistamine, and imbutoamine also act as potent hH4R agonists. Furthermore, the H4R is activated by the H3R/H4R antagonist burimamide, the H3R antagonists clobenpropit, and the H3R agonist iodo-\(\text{proxyfan}\), indicating that for hH4R agonism considerable structural diversity (piperidine, isothiourea, thiourea, and ether) in the side chain of imidazole ring is allowed, including aromatic substitutions as indicated by the hH4R agonism displayed by clobenpropit. However, our detailed analysis of various H3R ligands indicates that hH4R efficacy can be modulated by differential hydrophobic substitution on the side chain. In the burimamide series we observed that differential substitution on the thiourea group gives rise to H4R (partial) agonists, a neutral antagonist (VUF 4614; \(pK_i = 7.6\)) and a full inverse agonist (VUF 4742; \(pK_i = 6.9\)). In addition, in the clobenpropit series, we observe that a slight change on the isothiourea substituent results in a modulation of H4R efficacy. The clobenpropit analog iodophenpropit (a phenyl-ethyl substituent instead of a benzyl group) retains high H4R affinity (\(pK_i = 7.9\)), but it has lost agonistic activity completely. In this study, we identified iodophenpropit as a high-affinity, neutral antagonist for the H4R. In view of the \(\sim 15\)
nM affinity of iodophenopropit for the hH4R, we evaluated \(^{125}\)Iiodophenopropit as a potential new H4R radioligand. The hH4R can be labeled to the same extent with both the agonist \(^{3}H\)histamine and the neutral antagonist \(^{3}H\)JNJ 7777120. Surprisingly, the \(B_{\text{max}}\) value determined with \(^{125}\)Iiodophenopropit was twice as much as that determined with either \(^{3}H\)histamine or \(^{3}H\)JNJ 7777120, suggesting that the radioligands might bind to different hH4R subpopulations, similarly to recent findings on the binding of two H4R radioligands to the hH4R (Booth et al., 2002). Yet, the potential existence of different H4R subpopulations needs further investigation. The binding of the three radioligands to membranes of SK-N-MC/hH4 cells was displaced by a variety of H4R ligands, and the \(pK_{d}\) values obtained from these displacement studies show a high correlation. Despite being shown as a potential hH4R radioligand, \(^{125}\)Iiodophenopropit has to be used with caution, as in our hands a high level of nonspecific binding limits its use.

From our screening of many H4R ligands, only the tricyclic clozapine shows reasonable H4R affinity, as reported previously (Oda et al., 2000; Liu et al., 2001a; Zhu et al., 2001). Despite their structural similarity to clozapine, other tested receptor subtypes. The identification of 4-methylhistamine as the first high-affinity H4R agonist (\(K_{i} = 50\) nM) that has a >100-fold selectivity for the hH4R over the other histamine receptor subtypes. The identification of 4-methylhistamine as a potent H4R agonist will be of major importance for future studies to unravel the physiological roles of the H4R.

Acknowledgments

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References


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in conclusion, from a large screening of many known histamine receptor ligands we have identified a variety of compounds with interesting H4R activities. The major signifi-

ance of these findings is the reevaluation of numerous histaminergic ligands at the new histamine receptor subtypes. Based upon our data, many imidazol-containing H4R ligands, including various H4R reference compounds, show potent H4R activities and should be treated with caution. More recently developed H4R agonists, such as immethrinide (Kitbunpadaj et al., 2004) or methimemepip (Kitbunpadaj et al., 2005), or nonimidazole H4R antagonists, such as JNJ 6379490 (Ling et al., 2004) or A-349821 (Esbenshade et al., 2004), hardly act at the H4R and will therefore provide good tools to selectively target the H4R. In the series of tested H4R ligands, we have identified iodophenopropit as potent neutral H4R antagonist and the burimamide analog VUF 4742 as the second identified H4R inverse agonist. From the screening of H4R ligands, we have identified 4-methylhistamine as the first high-affinity H4R agonist (\(K_{i} = 50\) nM) that has a >100-fold selectivity for the hH4R over the other histamine receptor subtypes. The identification of 4-methylhistamine as a potent H4R agonist will be of major importance for future studies to unravel the physiological roles of the H4R.


