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

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4-methylhistamine is a potent and selective histamine H₄R agonist

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d) List of nonstandard abbreviations: A-349821, 4'-(3-[(R,R)2,5-dimethylpyrrolidin-1-yl]-propoxy)-biphenyl-4-yl]-morpholin-4-yl-methanone; BMMC, bone marrow mast cells; CRE, cAMP response element; EMEM, Eagle’s Minimum Essential Medium; hH₄R, human histamine H₄ receptor; JNJ 637940, 7-methyl-2-[4-(3-piperidin-1-yl-propoxy)-phenyl]-imidazo[1,2-a]pyridine; JNJ 7777120, 1-[(5-chloro-1H-indol-2-yl)carbonyl]-4-methylpiperazine; ONPG, 2-nitrophenol-β-D-pyranoside; ORG3770, 1,2,3,4,10,14b-hexahydro-2-methylpyrazino[2,1-a]pyrido[2,3-c][2]benzazepine; OUP-16, 2-cyano-1-methyl-
1-3-{(2R,5R)-5-[1H-imidazol-4(5)-yl]tetrahydrofuran-2-yl}methylguanidine;

VUF 6002, 1-[(5-chloro-1H-benzimidazol-2-yl)carbonyl]-4-methylpiperazine;

VUF 4742, N-(4-chlorobenzyl)-N’-[5-(4(5)-imidazolyl)pentyl]thiourea.

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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 H₂R agonists and imidazole-based H₃R 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 (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.
INTRODUCTION

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) had been pharmacologically identified long before their cDNAs were cloned (Gantz et al., 1991, Yamashita et al., 1991) and have been successful blockbuster targets for more than two decades. The cDNA encoding the histamine 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 gene (37%) (Oda et al., 2000; Liu et al., 2001a; Morse et al., 2001; Nguyen et al., 2001; Zhu et al., 2001). While the hH₃R 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 to the closely related H₃R, although many H₃R ligands also interact with the H₄R. Like the H₃R, the H₄R couples to pertussis toxin-sensitive G₁₆ₒ-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 MAP 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, like ISRE, IRF-1, NF-κB, and NF-IL6, are present upstream of the hH4R gene (Coge 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 H3R antagonist thioperamide was identified as an equally effective H4R antagonist (Oda et al., 2001). High-throughput screening and subsequent medicinal chemistry efforts recently identified the indolylpiperazine JNJ 7777120 (Jablonowski et al., 2003; Thurmond et al., 2004) and the related benzimidazole analog VUF 6002 (Terzioglu et al., 2004) as selective and potent H4R antagonists. Studies directed towards selective H4R agonists have so far been less successful. Burimamide, clozapine, and clobenpropit are all known to act as H4R 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 imifuramine analog OUP-16, which displays a 15-fold selectivity for the H4R compared to its binding
affinity for the H₃R (Hashimoto et al., 2003). However, the lack of selectivity of currently known agonists for the H₄R limits their use as H₄R 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 H₂R agonist (Durant et al., 1975), as a high affinity H₄R agonist with a more than 100-fold selectivity over the H₁R, H₂R, and H₃R.
METHODS

Materials. Aminopotentidine, anthamine dihydrobromide, amselamine dihydrobromide, burimamide oxalate and burimamide analogs (Vollinga, 1995), clobenpropit dihydrochloride, dimaprit dihydrobromide, histaprodifen dimaleate, homohistamine dihydrobromide, imbutamine dihydrobromide, imetit dihydrobromide, impentamine dihydrobromide, immepip dihydrobromide,immethridine dihydrobromide, iodopenpropit dihydrobromide, JNJ 7777120, 2-(3-bromophenyl)histamine dihydrobromide, methimmepip dihydrobromide, 2-pyridylethylamine (PEA) dihydrochloride, 2-(2-thiazolyl)ethylamine (TEA) dihydrochloride, thioperamide fumarate, and VUF 8328 (S-[2-(4-imidazolyl)propyl]isothiourea dihydrobromide) were synthesized at the Department of Medicinal Chemistry, Vrije Universiteit Amsterdam. Famotidine, ketotifen fumarate and 8R-lisuride were purchased from ICN Biomedicals Inc. (USA), amoxapine, d-chlorpheniramine maleate, clozapine, cimetidine, N-desmethyl clozapine, diphenhydramine hydrochloride, doxepin hydrochloride, forskolin, histamine dihydrochloride, imipramine hydrochloride, loxapine, mepyramine (pyrilamine maleate), \((R)\)-\(\alpha\)-methylhistamine dihydrochloride, \((S)\)-\(\alpha\)-methylhistamine dihydrochloride, \(N^\alpha\)-methylhistamine dihydrochloride, \(N\)-oxide clozapine, octoclothepin, pertussis toxin, polyethyleneimine (PEI), ranitidine hydrochloride, \(S^+\)- and \(R^(-)\)-sopromidine (Institute of Pharmacy, Free University Berlin), tripelennamine hydrochloride, and triprolidine hydrochloride were purchased from Sigma RBI (USA). 2-Nitrophenol-\(\beta\)-D-pyranoside (ONPG) and G418 were from Duchefa (The Netherlands), promethazine was from VUMC Pharmacy Amsterdam, fexofenadine from Ultrafine Chemicals (UK), tiotidine
from Imperial Chemical Industries PLC (UK), and $[^3\text{H}]N^\text{\textalpha}-\text{methylhistamine}$ (85 Ci/mmol), $[^3\text{H}]\text{histamine}$ (12.4 Ci/mmol), and $[^3\text{H}]\text{mepyramine}$ (23 Ci/mmol) from Perkin-Elmer Life Science, Inc. (USA). $[^{125}\text{I}]\text{iodoaminopotentidinide}$ and $[^{125}\text{I}]\text{iodophenpropit}$ were labeled at the Department Nuclear Medicine and PET Research, Vrije Universiteit Medical Centre, Amsterdam, as described previously (Jansen et al., 1992), while $[^3\text{H}]\text{JNJ 7777120}$ (84 Ci/mmol) was synthesized at Johnson & Johnson Pharmaceutical Research and Development, L.L.C. La Jolla, CA, USA (Thurmond et al., 2004). Gifts of astemizole (Janssen Pharmaceutica NV, Belgium), cyproheptadine hydrochloride (MSD, The Netherlands), cetirizine hydrochloride and hydroxyzine dihydrochloride (UCB Pharma, Belgium), ebastine (Almirall Prodesfarma, Spain), loratidine (Schering Plough, USA), mianserin hydrochloride and Org 3770 (Organon NV, The Netherlands), mifentidine (Instituto De Angeli, Italy), mizolastine (Synthelabo, France), proxyfan dihydrochloride and iodoproxyfan dihydrochloride (Dr. J.A.M. Christiaans (Kovaleinen et al., 1999)), $R(+)$- and $S(-)$-terfenadine carboxylate (Sepracor, Inc., USA), 2-methylhistamine dihydrochloride, 4-methylhistamine dihydrochloride, and impromidine dihydrochloride (SmithKline, Beecham, UK), are greatly acknowledged.

**Cell culture.** SK-N-MC cell lines, which stably express either the human H$_3$R (SK-N-MC/hH$_3$) or H$_4$R (SK-N-MC/hH$_4$) as well as a cAMP responsive element (CRE)-driven $\beta$-galactosidase reporter gene SK-N-MC/hH$_3$ or SK-N-MC/hH$_4$ cells (Lovenberg et al., 1999; Liu et al., 2001a), were cultured in EMEM medium supplemented with 5% fetal
calf serum, 0.1 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 cell homogenates were incubated for 40 minutes at 25°C with approximately 1 nM [³H]Nα-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 hour at 37°C in 10 nM [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% polyethyleneimine pretreated Whatman GF/C (and washed three times with 3 ml of ice-cold washing buffer (4°C) containing 25 mM Tris-HCl 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]iodophenpropit to the hH₄R was performed with the same conditions as described for [³H]histamine. In saturation binding analysis, the nonspecific binding of [³H]histamine or [³H]JNJ 7777120 was determined with 1 µM 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.) and data are presented as mean ± SEM. Mouse and rat H₄R radioligand binding assays were performed according to Liu et al. (2001b).

**Colometric cyclic AMP Assay.** A reporter CRE-β-galactosidase reporter gene assay was employed to determine (inverse) agonistic or antagonistic activity of either the hH₃R or
hH₄R. Approximately 4 million cells/96-well plate of SK-N-MC/hH₃ and SK-N-MC/hH₄ cells were exposed for six hours to histaminergic ligands in serum-free EMEM medium containing 1 µM forskolin. Thereafter, the medium was discarded, the cells were lysed in 100 µl assay buffer (100 mM sodium phosphate buffer at pH 8.0, 4 mM ONPG, 0.5% Triton X-100, 2 mM MgSO₄, 0.1 mM MnCl₂, 40 mM β-mercaptoethanol), incubated overnight at room temperature, and the β-galactosidase activity was determined at 420 nm with a PowerwaveX340 plate reader (Bio-Tek Instruments, Inc., USA). 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 (BMMC) and in vitro BMMC chemotaxis assay was performed as previously described (Hofstra et al., 2003). Purification of human polymorphonuclear leukocytes (PMNL) and the human eosinophil shape change assay were performed as previously described (Ling et al., 2004). The mouse-derived BMMC were obtained following approved protocols that follow NIH/International Animal Care and Use guidelines.
RESULTS

Pharmacological characterization of the hH4R expressed in SK-N-MC cells

Stable transfection of the human H4R (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 non-specific binding (Figure 1A). Analysis of the [3H]histamine saturation binding yielded a $K_d$ value of $11 \pm 1.0$ nM (n=6) and a $B_{max}$ value of $1.8 \pm 0.4$ pmol/mg protein. Recently, JNJ 7777120 was described as a selective H4R antagonist (Jablonowski et al., 2003). In our hands, the non-imidazole JNJ 7777120 shows a 300-fold selectivity for the hH4R ($pK_i = 7.8 \pm 0.1$ against $[3H]$histamine) over the hH3R ($pK_i = 5.3 \pm 0.1$ against $[3H]$Nα-methylhistamine), allowing the use of $[3H]$JNJ 7777120 to label the H4R (Thurmond et al., 2004). The H4R antagonist $[3H]$JNJ 7777120 exhibits a somewhat higher level of non-specific binding to hH4R expressing SK-N-MC cells, but also binds saturably and shows an equipotent affinity ($K_d = 11 \pm 3.6$ nM, n=3) and results in a $B_{max}$ value of $1.7 \pm 0.4$ pmol/mg protein (Figure 1B). The binding of either 10 nM $[3H]$histamine (Figure 1C) or 10 nM $[3H]$JNJ 7777120 (data not shown) to the hH4R is fully displaced by histamine ($pK_i = 7.8 \pm 0.1$), the H3/4R antagonist thioperamide ($pK_i = 6.9 \pm 0.1$) and the H4R agonist/H3R antagonist clobenpropit ($pK_i = 8.1 \pm 0.1$), 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 co-express a CRE-controlled β-galactosidase reporter gene and can therefore also be used for a functional analysis of H4R ligands. Stimulation of the hH4R 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) (Figure 1D). Treatment of SK-N-MC/hH4 cells with the Gαi/o protein inhibitor pertussis toxin (100ng/ml for 16 hours) completely inhibited histamine induced responses, confirming the coupling of the H4R to Gαi/o 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 before (Oda et al., 2000; Liu et al., 2001a; Morse et al., 2001), clobenpropit acts as a potent partial hH4R agonist with a pEC50 value of 7.7 ± 0.1 (n = 3) and an intrinsic activity of 0.8 (Figure 1D).

Treatment of SK-N-MC/hH4 cells with pertussis toxin (100ng/ml for 16 hours) resulted in an increase of 1 µM forskolin-stimulated CRE activity by 130 ± 3%, suggesting that the hH4R shows a detectable level of constitutive acitivity in the SK-N-MC/hH4 cells. In line with earlier 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 (Figure 1D). The inhibition of the constitutive activity of the hH4R by thioperamide was of the same magnitude as observed after treatment with pertussis toxin and thioperamide is referred to as a full inverse hH4R agonist (intrinsic activity α=-1).
In SK-N-MC/hH4 cells the cAMP-driven β-galactosidase reporter-gene transcription can also be activated by endogenously expressed Gαs protein coupled β adrenergic receptors (Bahouth et al., 2001). The β2 adrenergic receptor agonist feneterol induced β-galactosidase activity to a similar extent to that of forskolin with a pEC50 value of 6.9 ± 0.1 (n = 6). H4R activation by histamine inhibited the 100 nM feneterol-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 hH4R, could not be easily demonstrated with a feneterol-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 hH4R 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 hH4R by ≤30% are expected to have a Ki > 10 µM based on the Cheng and Prusoff equation (Cheng and Prusoff, 1973): K_i = IC50/(1+[radioligand]/K_d)), 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 H4R assay.

**Most H1R ligands are devoid of H4R activity**

Histamine potently displaces [3H]histamine from the hH4R with a pK_i value of 7.8 ± 0.1 (Table 1), while H1R 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 substitutent 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 hH4R (Table 1). Agonists, lacking the imidazole ring, like 2-(2-thiazolyl)ethylamine (TEA), 2-pyridylethylamine (PEA) or 8R-lisuride (Bakker 2004), are also not active at the hH4R (Table 1).

Following an initial report that the H4R can be labeled with [3H]-mepyramine (Nguyen et al., 2001), a large number of H1R antagonists (Table 1), including many clinically relevant drugs, were evaluated for their hH4R affinity as well. Almost all tested H1R antagonists, including mepyramine, showed pKi values <5 (Table 1) and did not show functional activity at 1 and 10 µM at the hH4R (data not shown). Although structurally similar to some tricyclic H1R antagonists devoid of H4R affinity, clozapine binds with moderate potency to the hH4R (pKi 6.7 ± 0.1) and exerts full agonistic activity at the hH4R 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), while N-oxide clozapine, another clozapine metabolite, is totally devoid of hH4R affinity. Further, 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), while octoclothepin did not show binding for the hH4R at all.

**Some H2R ligands act as hH4R agonists**

Within the series of known H2R agonists that we have tested, only some ligands retain H4R activity. Replacement of the imidazole ring of histamine in the selective H2R
agonists amthamine and amselamine (Leurs et al., 1994) results in a total loss of hH₄R activity at concentrations up to 10 µM. Dimaprit, a H₂R agonist/H₃R antagonist lacking an imidazole group, binds the H₄R with moderate affinity, showing a pKᵢ value of 6.5 ± 0.1, and exerts partial H₄R agonistic activity (Table 1). Impromidine, which was reported to bind to both H₂R and H₃R, also binds potently to the hH₄R with a pKᵢ value of 7.6 ± 0.1 and acts as a partial H₄R agonist (α = 0.5). Both the R and S enantiomers of the related sopromidine bind respectively >10 and >100 times less potently. In fact, the first reported H₂R selective agonist 4-methylhistamine (Durant et al., 1975) is the only known H₂R agonist that also acts as full agonist at the H₄R (Table 1). 4-Methylhistamine binds two times less potently than histamine to the hH₄R, exhibiting a pKi value of 7.3 ± 0.1 (n = 3).

Most tested H₂R antagonists, including cimetidine, mifentidine, aminopotentidine, ranitidine, famotidine, and tiotidine, only displaced < 30% of 10 nM [³H]histamine binding to the hH₄R. Only the H₂/₃R ligand burimamide shows a high affinity for the hH₄R pKi = 7.4 ± 0.1 (Table 1). Moreover, burimamide acted as a potent, albeit partial H₄R agonist (pEC₅₀ = 7.7 ± 0.1, α = 0.7). Previously, we reported on various burimamide analogs as H₃R antagonists (Vollinga, 1995). In our search for H₄R selective ligands, various burimamide analogs were therefore investigated for their H₄R activity. In this series of compounds, the presence of an isopropyl (VUF 4683 and VUF 4616) or cyclohexyl (VUF 4617) moiety adjacent to the thiourea group improved the affinity for the hH₄R (Table 2). Interestingly, this series of closely related compounds exerts partial agonistic, neutral antagonistic, and inverse agonistic activities at the hH₄R (Table 2,
Figure 2A). Substitution on the thiourea with aromatic substituents, like a benzyl group in VUF 4686, results in a reduced H₄R agonistic activity. A total loss of agonistic H₄R activity, but not affinity (pKᵢ = 7.6 ± 0.1) is surprisingly observed for VUF 4614. As can be seen in figure 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 (Figure 2A). This burimamide analog bound with moderate affinity to the H₄R (pKᵢ = 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 H₄R shares its highest sequence homology with the H₃R 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 Nβ-methylhistamine, (R)-α-methylhistamine, and (S)-α-methylhistamine show an almost 2 order of magnitude lower affinity for the hH₄R than for the hH₃R. However, the hH₄R retains some level of stereoselectivity for (R)-α- (pKᵢ = 6.6 ± 0.1) and (S)-α-methylhistamine (pKᵢ = 5.4 ± 0.1) (Table 3). Increasing the spacer-length between imidazole and amine group from 2 carbon atoms (histamine, pKᵢ = 7.8 ± 0.1) to 3 carbon atoms (homohistamine, pKᵢ = 7.5 ± 0.1) slightly decreases the affinity for the hH₄R, while 4 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 H₃R agonist
Impentamine shows only moderate affinity at the H₄R (pKᵢ = 6.6 ± 0.1) (Table 3). Interestingly, besides the affinity, impentamine also loses intrinsic activity for the hH₄R (α = 0). Previously identified H₃R agonists, including immepip, 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 H₃R 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 Nα-methylhistamine, the methylated immepip analog methimepip shows a large selectivity for the hH₃R (pKᵢ = 9.0 ± 0.1) over the hH₄R (pKᵢ = 5.7 ± 0.1), as reported previously (Kitbunnadaj et al., 2005). Also various H₃R 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 isothiourea-based H₃R 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 hH₃R (Wieland et al., 2001) behaves as a potent partial agonist at the hH₄R (Table 3 and Figure 3A 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 and Figure 3A 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 (Figure 3C and D), in accordance with its binding affinity. Besides clobenpropit, also the known H₃R ligands proxyfan and the related iodoproxyfan 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]iodophenpropit as H₄R radioligand**

As reported in the previous section and in other studies, the hH₄R can be labeled with either [³H]histamine or the H₄R 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). Previously, 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 H₄R. Due to the hH₄R affinity of [¹²⁵I]iodophenpropit, saturation binding experiments were not feasible. We therefore used homologous [¹²⁵I]iodophenpropit displacement analysis to determine a Kᵥ value of 34.4 ± 4.1 nM for [¹²⁵I]iodophenpropit. The Bₘₐₓ value obtained using [¹²⁵I]iodophenpropit-binding displacement experiments (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 membranes of SK-N-MC/hH₄ cells was competitively displaced by a variety of H₃/₄R ligands (Figure 4), despite a high level of non-specific binding of approximately 60% as determined with 10 µM imetit. However, iodophenpropit and the related clobenpropit also displaced the non-specific binding, resulting in a multiple site binding profile. The pKᵢ values of compounds for the hH₄R obtained using [¹²⁵I]iodophenpropit displacement studies are consistent with their
corresponding values obtained using displacement of either $[^3\text{H}]$histamine or $[^3\text{H}]$JNJ 7777120 binding to the hH$_4$R; only the pK$_i$ value of thioperamide obtained using $[^{125}\text{I}]$iodophenpropit displacement studies appears to deviate somewhat from the pK$_i$ values obtained using either $[^3\text{H}]$histamine or $[^3\text{H}]$JNJ 7777120 displacement studies (Figure 4 and Table 4).

4-methylhistamine as a selective H$_4$R agonist

Following our initial observation of the relative high affinity of 4-methylhistamine for the hH$_4$R, this histamine analog was evaluated in more detail. 4-Methylhistamine does not only have high affinity for the hH$_4$R (pK$_i$ = 7.3 ± 0.1, n = 3), but it also exhibits considerable selectivity for the hH$_4$R over the other three human histamine receptors (Figure 5A). The human histamine H$_1$, H$_2$ and H$_3$ receptors were tested for their interaction with 4-methylhistamine, using respectively 1 nM $[^3\text{H}]$mepyramine (K$_d$ = 1.6 nM), 0.5 nM $[^{125}\text{I}]$iodoaminopotentidine (K$_d$ = 0.5 nM) and 1 nM $[^3\text{H}]N^\alpha$-methylhistamine (K$_d$ = 2.9 nM) binding to homogenates of transfected cells. As can be seen in figure 5A, 4-methylhistamine shows highest affinity for the hH$_4$R and binds considerably less potently to the other histamine receptors, resulting in a >100-fold and >100,000-fold selectivity over the H$_3$R and H$_2$R, and H$_1$R, respectively. 4-Methylhistamine does not only bind to the hH$_4$R, but also has a high affinity, albeit reduced compared to the hH$_4$R, for the mouse and rat H$_4$R with K$_i$ values of 73 and 55 nM, respectively (Figure 5B). Moreover, 4-methylhistamine exerts full agonistic activity at the hH$_4$R (Figure 5C), resulting in a pEC$_{50}$ value of 7.4 ± 0.1 (α = 1, n = 5). In contrast, 4-methylhistamine exhibits only moderate affinity for the hH$_2$R (pK$_i$ = 5.1 ± 0.1, n = 3).
and hH3R (pK_i = 5.2 ± 0.1, n = 4), and partial agonistic hH3R activity (Figure 5D). The hH4R agonistic effects of 4-methylhistamine can be antagonized by the selective H4R antagonist JNJ 7777120 (Figure 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 pA2 value of 7.8 (data not shown), which is in agreement with the hH4R affinity of JNJ 7777120 (Table 3) (Jablonowski 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 respectively 5.8 ± 0.1 and 5.6 ± 0.1.

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; Ling et al., 2004; Takeshita et al., 2004; Thurmond et al., 2004). Indeed, 4-methylhistamine also acted as H4R agonist at human eosinophils and induced a rapid change in eosinophil cell shape, as measured by the gated autofluorescence forward scatter assay (Ling et al., 2004). The H4R agonist 4-methylhistamine acted as a full agonist and dose dependently induced a change in forward scatter (Figure 6A) with an EC50 value of 0.36 ± 0.09 µM, which was 3 fold less active compared to histamine. The effects of 4-methylhistamine on human eosinophils were not inhibited by H1R antagonist mepyramine or H2R antagonist ranitidine, but could be antagonized by the H4R antagonist JNJ 7777120 (Figure 6B). H3R antagonists were not included in this assay as 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 bone marrow derived mast cells.
(BMMC) as described previously (Hofstra et al., 2003). Like histamine, 4-
methylhistamine dose-dependently induced migration of murine BMMCs with an EC$_{50}$
value of 12 µM (Figure 6C), again a somewhat lower potency compared to histamine
(Hofstra et al., 2003). Further, we found that the effect of 4-methylhistamine on murine
BMMC was completely inhibited by the selective H$_4$R antagonist JNJ 7777120 in a dose-
dependent manner (Figure 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; Ling et al., 2004; Takeshita et al., 2003; Thurmond et al., 2004) implies that this new histamine receptor subtype may play a role in various 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 (Jablonowski 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 (Figure 1A and 1B) and functionally inhibits forskolin-induced CRE-mediated responses through pertussis toxin-sensitive G\textsubscript{i/o} proteins (Figure 1D). In these cells the hH4R also exhibits constitutive activity, which is blocked by pertussis toxin or the non-selective inverse agonist thioperamide (Figure 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 immepip, imetit, \((R)-\alpha\)-methylhistamine, and imbutamine also act as potent hH4R agonists. Furthermore, the H3R is activated by the H2R/H3R antagonist burimamide, the H3R antagonists clobenpropit, and the H3R agonist iodoproxyfan, indicating that for hH4R agonism considerable structural diversity (piperidine, isothiourea, thiourea, 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, pKi = 7.6) and a full inverse agonist (VUF 4742, pKi = 6.9). Also, 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 phenylethyl substituent instead of a benzyl group) retains high H4R affinity (pKi = 7.9), but has lost agonistic activity completely. In this study we identified iodophenpropit as a high affinity, neutral antagonist for the H4R. In view of the ~15 nM affinity of iodophenpropit for the hH4R, we evaluated \([^{125}\text{I}]\)iodophenpropit as a potential new H4R radioligand. The hH4R can be labeled to the same extent with both the agonist \([^{3}\text{H}]\text{histamine}\) and the neutral antagonist \([^{3}\text{H}]\text{JNJ 7777120}\). Surprisingly, the Bmax value determined with \([^{125}\text{I}]\)iodophenpropit was twice as much as that determined with either \([^{3}\text{H}]\text{histamine}\) or \([^{3}\text{H}]\text{JNJ 7777120}\), suggesting that the radioligands might bind to different hH4R subpopulations, similarly to recent findings on the binding of two H1R
radioligands to the H₁R (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 H₃/₄R ligands and the pKi values obtained from these displacement studies show a high correlation. Despite being shown as a potential hH₄R radioligand, [¹²⁵I]iodophenpropit has to be used with caution, as in our hands a high level of non-specific binding limits its use.

From our screening of many H₁R ligands, only the tricyclic clozapine shows reasonable H₁R affinity, as reported before (Oda et al., 2000; Liu et al., 2001a; Zhu et al., 2001). Despite their structural similarity to clozapine, other tested H₁R antagonists do not show any appreciable affinity for the hH₄R. We can therefore not confirm that mepyramine binds to the hH₄R (Nguyen et al., 2001), either studied by displacement of [³H]histamine binding to the hH₄R, by saturation [³H]mepyramine binding assays (data not shown), or by functional H₄R assays. Clinically used H₁R antagonists, such as cetirizine, ebastine, fexofenadine, and loratidine, demonstrate significant in vitro anti-inflammatory activity, which are not related to their H₁R activity (Gelfand et al., 2004). The data from our study do not support the involvement of the hH₄R in the anti-inflammatory effects of these H₁R antagonists.

An important finding of this study is the discovery of 4-methylhistamine as a potent and selective hH₄R agonist in both recombinant and endogenously expressing H₄R systems. Whereas this compound is originally described as a relatively selective H₂R agonist (Durant et al., 1975), our present data show that this histamine analog exhibits more than
100-fold selectivity over the recombinant \( H_1 \)R, \( H_2 \)R, and \( H_3 \)Rs. 4-Methylhistamine not only acts as a full agonist at the recombinant \( hH_4 \)R, but also induces migration of mouse bone marrow derived mast cells and a shape change of human eosinophils. Both processes have recently been shown to be induced by histamine via interaction of the \( H_4 \)R (Hofstra et al., 2003; Ling et al., 2004). The relative potencies of histamine and 4-methylhistamine on human eosinophils are similar to those observed in recombinant systems. Similar to the observations with histamine (Hofstra et al., 2003; Ling et al., 2004), the potency of 4-methylhistamine on mouse mast cells is somewhat lower than in recombinant systems. Although the mouse \( H_4 \)R shows a lower affinity for both histamine and 4-methylhistamine compared to the \( hH_4 \)R, the low potency at BMMC seems not merely an issue of species difference, but might also be related to a low \( H_4 \)R expression level. In fact, the \( H_4 \)R in BMMC is present at a very low density as it can not be detected by radioligand binding studies (Thurmond, unpublished observations). Moreover, the cellular environment might dictate the potency of an agonist, such as composition of G proteins and accessories proteins in the cells (Kenakin, 2004).

In conclusion, from a large screening of many known histamine receptor ligands we have identified a variety of compounds with interesting \( H_4 \)R activities. The major significance of these findings is the reevaluation of numerous histaminergic ligands at the new histamine receptor subtypes. Based upon our data, many imidazol-containing \( H_3 \)R ligands, including various \( H_3 \)R reference compounds show potent \( H_4 \)R activities and should be treated with caution. More recently developed \( H_3 \)R agonists, like immethridine (Kitbunnadaj et al., 2004) or methimmepip (Kitbunnadaj et al., 2005) or non-imidazole
H3R antagonists, like 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 H3R. In the series of tested H3R ligands, we have identified iodophenpropit as potent neutral H4R antagonist and the burimamide analog VUF 4742 as the second identified H4R inverse agonist. From the screening of H2R ligands we have identified 4-methylhistamine as the first high affinity H4R agonist (Ki = 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.
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Novel, Potent, and Highly Selective Histamine H₃ Receptor Agonist. *J Med Chem* 47:2414-2417


FOOTNOTES

a) Unnumbered footnotes:

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* These authors contributed equally (HDL and RMvR).

b) Reprint requests to: Dr. R. Leurs, Leiden/Amsterdam Center for Drug Research, Department of Medicinal Chemistry, Vrije Universiteit Amsterdam, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands. Tel: ++31-20-4447600. Fax: ++31-20-4447610. E-mail: r.leurs@few.vu.nl
Figure 1. The cell line SK-N-MC/hH₄ stably expresses functional hH₄R and CRE-control β-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 µM forskolin-induced CRE activation as measured by a β-galactosidase reporter gene, while the inverse agonist thioperamide dose-dependently blocks the hH₄R constitutive activity (D). Data shown are from representative of experiments, each performed in triplicate.

Figure 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, VUF 4686), neutral H₄R antagonist (VUF 4614), or inverse H₄R agonist (VUF 4742). (B) The 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.
Figure 3. Effects of H₃R ligands at the H₄R. (A) Effects of histamine, thioperamide, clobenpropit, and iodophenpropit at the hH₃R expressed in SK-N-MC cells, as determined by modulation of the forskolin-induced CRE-mediated β-galactosidase activity. (B) Histamine and clobenpropit dose-dependently inhibit forskolin-induced responses activity in SK-N-MC/hH₄ cells, while thioperamide acts as inverse agonist at the hH₄R. Iodophenpropit does not change forskolin-induced responses activity, acting as a neutral antagonist. (C) Iodophenpropit (IPP) antagonizes the effects of histamine at the hH₄R, resulting in a rightward shift of histamine dose-response curve. (D) Schild plot analysis of iodophenpropit-mediated antagonism responses. Data shown are from representative experiments, each performed in triplicate.

Figure 4. Displacement of [¹²⁵I]iodophenpropit binding to the hH₄R by different concentrations of H₃/₄ ligands. The nonspecific activity was determined with 1 µM imetit. The data, shown as % of specific binding, fit according to a one-site ligand-receptor model. Data shown are from representative experiments, each performed in triplicate.

Figure 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]iodoaminopotentidine, 1 nM [³H]N⁶-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) in comparison to 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.

**Figure 6.** Physiological effects of 4-methylhistamine on hematopoietic cells *in vitro.* (A) Effects of 4-methylhistamine on human eosinophil shape change, (B) the effect of 4-methylhistamine on human eosinophil shape change is inhibited by the H4R antagonist JNJ 7777120, but not by H1R or H2R antagonist (mepyramine and ranitidine, respectively), (C) 4-methylhistamine induction of migration of murine bone marrow mast cells (BMMC), and (D) the 4-methylhistamine-induced murine BMMC migration is completely inhibited by the H4R-selective antagonist JNJ 7777120 in a dose-dependent manner. Data shown are from representative experiments, each performed either in triplicate (A and C) or in duplicate (B and D).
Table 1. Activity of H₁R and H₂R ligands at the hH₄R. The ligands were tested as described in Methods and Materials. Data shown are mean ± standard error of mean of at least three independent experiments, each performed in triplicate.

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<td>antagonist</td>
<td>7.4 ± 0.1</td>
<td>7.7 ± 0.1</td>
<td>0.7</td>
</tr>
<tr>
<td>Cimetidine</td>
<td>6.2 ± 0.2²</td>
<td>antagonist</td>
<td>&lt;5</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Famotidine</td>
<td>7.8 ± 0.1²</td>
<td>antagonist</td>
<td>&lt;5</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Mifentidine</td>
<td>-</td>
<td>antagonist</td>
<td>&lt;5</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Ranitidine</td>
<td>7.1 ± 0.1²</td>
<td>antagonist</td>
<td>&lt;5</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Tiotidine</td>
<td>7.8 ± 0.2²</td>
<td>antagonist</td>
<td>&lt;5</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

¹ pKi values were determined with [³H]histamine displacement assay.

² pEC₅₀ values show the inhibition of 1 µM forskolin-induced CRE-β-galactosidase activity in SK-N-MC/hH₄ cells.
α is intrinsic activity, 1 designated for full agonistic, 0 for neutral antagonist, and -1 for full inverse agonistic activity.

d The H₁R antagonists Astemizole, Chlorpheniramine, Cyproheptadine, Desipramine, Dexchlorpheniramine, Diphenhydramine, Doxepine, Imipramine, Ketotifen, Mianserine, Octoclothepin, ORG-3770, Promethazine, S(-)-terfenadine, Tripelennamine, and Triprolidine also have pKᵢ values < 5.

d due to non H₄R-mediated effects of 8R-lisuride, the pEC₅₀ value was not determined.

pKᵢ values were determined with [³H]mepyramine displacement assay (Bakker et al., 2001).

pKᵢ values were determined with [³H]mepyramine displacement assay (Bakker et al., 2004).

pKᵢ values were determined with [³H]mepyramine displacement assay (Govoni et al., 2003).

pKᵢ values were determined with [³H]mepyramine displacement assay (Gillard et al., 2002).

Potencies as inverse H₁R agonists determined with Receptor Selection and Amplification Technology (R-SAT) assay (Bakker, 2003).

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

n.d. = not determined.
Table 2. Activity of burimamide analogs at the hH₄R. The ligands were tested as described in Methods and Materials. Data shown are mean ± standard error of mean of at least three independent experiments, each performed in triplicate.

<table>
<thead>
<tr>
<th>ligand</th>
<th>n</th>
<th>R</th>
<th>pKᵢ at hH₄R</th>
<th>pKᵢᵃ</th>
<th>pEC₅₀ᵇ</th>
<th>αᶜ</th>
</tr>
</thead>
<tbody>
<tr>
<td>burimamide</td>
<td>4</td>
<td>methyl</td>
<td>5.4 ± 0.2ᵉ</td>
<td>7.4 ± 0.1</td>
<td>6.8 ± 0.1</td>
<td>0.7</td>
</tr>
<tr>
<td>VUF 4681</td>
<td>4</td>
<td>ethyl</td>
<td>7.6 ± 0.1</td>
<td>7.0 ± 0.1</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>VUF 4682</td>
<td>4</td>
<td>n-propyl</td>
<td>8.0 ± 0.1</td>
<td>7.2 ± 0.1</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>VUF 4683</td>
<td>4</td>
<td>isopropyl</td>
<td>8.1 ± 0.1</td>
<td>7.7 ± 0.1</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>VUF 4686</td>
<td>4</td>
<td>benzyl</td>
<td>7.3 ± 0.1</td>
<td>7.1 ± 0.3</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>VUF 4687</td>
<td>4</td>
<td>phenylethyl</td>
<td>7.2 ± 0.1</td>
<td>5.9 ± 0.3</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>VUF 4614</td>
<td>5</td>
<td>ethyl</td>
<td>5.0 ± 0.1ᶠ</td>
<td>7.6 ± 0.1</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>VUF 4616</td>
<td>5</td>
<td>isopropyl</td>
<td>5.0 ± 0.1ᶠ</td>
<td>7.9 ± 0.1</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>VUF 4617</td>
<td>5</td>
<td>cyclohexyl</td>
<td>5.4 ± 0.1ᶠ</td>
<td>7.9 ± 0.1</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>VUF 4742</td>
<td>5</td>
<td>4-chlorobenzyl</td>
<td>5.8 ± 0.2ᶠ</td>
<td>6.9 ± 0.1</td>
<td>7.2 ± 0.1</td>
<td>-1</td>
</tr>
</tbody>
</table>

ᵃpKi values were determined with [³H]histamine displacement assay.

ᵇpEC₅₀ values show the inhibition of 1 µM forskolin-induced CRE-β-galactosidase activity in SK-N-MC/hH₄ cells.

cα is intrinsic activity, 1 designated for full agonistic, 0 for neutral antagonist, and -1 for full inverse agonistic activity.

dThese compounds exerted a biphasic response: a slight decrease of 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, as it cannot be blocked by H₄R antagonist JNJ 7777120.

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

ᶠpKi values were determined with [¹²⁵I]iodoaminopotentidine displacement assay (Vollinga et al., 1995).
n.d. = not determined.
Table 3. Activity of H₄R ligands at the hH₃R and the hH₄R. The ligands were tested as described in Methods and Materials. Data shown are mean ± standard error of mean of at least three independent experiments, each performed in triplicate.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>hH₃R</th>
<th>hH₄R</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pKᵢ</td>
<td>pEC₅₀</td>
</tr>
<tr>
<td>Histamine</td>
<td>8.0 ± 0.1</td>
<td>8.3 ± 0.1</td>
</tr>
<tr>
<td>Nα-methylhistamine</td>
<td>8.4 ± 0.1</td>
<td>9.4 ± 0.1</td>
</tr>
<tr>
<td>(R)-α-methylhistamine</td>
<td>8.2 ± 0.1</td>
<td>9.5 ± 0.1</td>
</tr>
<tr>
<td>(S)-α-methylhistamine</td>
<td>7.2 ± 0.1</td>
<td>8.0 ± 0.1</td>
</tr>
<tr>
<td>Homohistamine</td>
<td>7.3 ± 0.1</td>
<td>7.4 ± 0.2</td>
</tr>
<tr>
<td>Imbutamine</td>
<td>8.4 ± 0.1</td>
<td>9.2 ± 0.1</td>
</tr>
<tr>
<td>Impentamine</td>
<td>8.3 ± 0.1</td>
<td>8.4 ± 0.1</td>
</tr>
<tr>
<td>Immepip</td>
<td>9.3 ± 0.1</td>
<td>10.4 ± 0.1</td>
</tr>
<tr>
<td>Methimeneip</td>
<td>9.0 ± 0.1</td>
<td>9.5 ± 0.2</td>
</tr>
<tr>
<td>Immethridine</td>
<td>9.1 ± 0.1</td>
<td>9.8 ± 0.1</td>
</tr>
<tr>
<td>Imetit</td>
<td>8.8 ± 0.1</td>
<td>9.9 ± 0.1</td>
</tr>
<tr>
<td>VUF 8328</td>
<td>8.5 ± 0.1</td>
<td>9.3 ± 0.2</td>
</tr>
<tr>
<td>Clobenpropit</td>
<td>8.6 ± 0.1</td>
<td>9.4 ± 0.1</td>
</tr>
<tr>
<td>Iodopenpropit</td>
<td>8.2 ± 0.1</td>
<td>8.5 ± 0.1</td>
</tr>
<tr>
<td>Impromidine</td>
<td>6.8 ± 0.1</td>
<td>6.5 ± 0.3</td>
</tr>
<tr>
<td>Burimamide</td>
<td>7.9 ± 0.1</td>
<td>7.0 ± 0.1</td>
</tr>
<tr>
<td>Thioperamide</td>
<td>7.3 ± 0.1</td>
<td>7.5 ± 0.1</td>
</tr>
<tr>
<td>Proxyfan</td>
<td>7.9 ± 0.1</td>
<td>8.5 ± 0.1</td>
</tr>
<tr>
<td>Iodoproxyfan</td>
<td>9.2 ± 0.1</td>
<td>10.3 ± 0.1</td>
</tr>
<tr>
<td>JNJ 7777120</td>
<td>5.3 ± 0.1</td>
<td>6.0 ± 0.1</td>
</tr>
</tbody>
</table>

* a pKᵢ values were determined with [³H]histamine displacement assay.

* b pKᵢ values were determined with [³H]Nα-methylhistamine displacement assay.

* c pEC₅₀ values show the inhibition of 1 µM forskolin-induced CRE-β-galactosidase activity in SK-N-MC/hH₃ or SK-N-MC/hH₄ cells.

* d α is intrinsic activity, 1 designated for full agonistic, 0 for neutral antagonist, and -1 for full inverse agonistic activity.
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>
</tr>
</thead>
<tbody>
<tr>
<td>Histamine</td>
<td>7.8 ± 0.1</td>
<td>7.7 ± 0.1</td>
<td>7.6 ± 0.2</td>
</tr>
<tr>
<td>4-Methylhistamine</td>
<td>7.3 ± 0.1</td>
<td>7.6 ± 0.1</td>
<td>7.4 ± 0.1</td>
</tr>
<tr>
<td>Immepip</td>
<td>7.7 ± 0.1</td>
<td>7.9 ± 0.1</td>
<td>7.7 ± 0.1</td>
</tr>
<tr>
<td>Clozapine</td>
<td>6.7 ± 0.1</td>
<td>6.4 ± 0.1</td>
<td>6.5 ± 0.2</td>
</tr>
<tr>
<td>Clobenpropit</td>
<td>8.1 ± 0.1</td>
<td>7.9 ± 0.1</td>
<td>7.8 ± 0.1</td>
</tr>
<tr>
<td>Iodophenpropit</td>
<td>7.9 ± 0.1</td>
<td>7.7 ± 0.1</td>
<td>7.7 ± 0.1</td>
</tr>
<tr>
<td>Thioperamide</td>
<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 ± 0.2</td>
<td>7.8 ± 0.1</td>
</tr>
</tbody>
</table>

K_d (nM)         | 20.9 ± 1.6    | 11.1 ± 3.6      | 34.4 ± 4.1^a         |
B_max (pmol/mg protein) | 1.8 ± 0.4   | 1.7 ± 0.4       | 3.8 ± 0.4            |

^a Determined by homologous displacement analysis, employing equation K_d = IC_{50} – [radioligand].
Figure 2

A

- △ thioperamide
- ▽ VUF 4742
- ○ VUF 4614
- ○ VUF 4686
- ◆ burimamide
- □ VUF 4683
- ▼ histamine

Log [Ligand, M] vs. OD$_{420}$

B

- ■ [VUF 4616]
- □ 300 nM
- ● 1 μM
- ◆ 3 μM
- ▽ 10 μM

Log [Histamine, M] vs. OD$_{420}$
Figure 4

% Specific Binding

- Immeipip
- Clobenpropit
- Histamine
- Iodophenpropit
- Thioperamide
- Clozapine

Log [Ligand, M]

(-11, -10, -9, -8, -7, -6, -5, -4)
Figure 5

A

B

C

D

E

% of specific binding

Log [4-Methylhistamine, M]

% of specific binding

Log [4-Methylhistamine, M]

Log [Ligand, M]

% of specific binding

Log [Ligand, M]

OD<sub>400</sub>

Log [Ligand, M]

Log [4-Methylhistamine, M]
Figure 6

A

% shape change vs. Log [Ligand, M]
- Histamine
- 4-Methylhistamine

B

% shape change vs. Log [4-Methylhistamine, M]
- -
- + 10 μM Ranitidine
- + 10 μM Mepyramine
- + 10 μM JNJ 7777120

C

migrated cells vs. Log [4-Methylhistamine, M]

D

migrated cells vs. +JNJ 7777120 (nM)

- Control (medium)
- 20 μM 4-Methylhistamine