Evaluation of Histamine \(H_1\)-, \(H_2\)-, and \(H_3\)-Receptor Ligands at the Human Histamine \(H_4\) Receptor: Identification of 4-Methylhistamine as the First Potent and Selective \(H_4\) Receptor Agonist

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ABSTRACT

The histamine \(H_4\) receptor (\(H_4R\)) 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 \(H_4R\) agonists have not been identified. In the present study, we therefore evaluated the human \(H_4\) receptor (\(hH_4R\)) for its interaction with various known histaminergic ligands. Almost all of the tested \(H_1\) and \(H_2\) receptor antagonists, including several important therapeutics, displaced less than 30% of specific \(^{3}H\)histamine binding to the \(hH_4R\) at concentrations up to 10 \(\mu M\). Most of the tested \(H_2R\) agonists and imidazole-based \(H_3R\) ligands exhibit micromolar-to-nanomolar range \(hH_4R\) affinity, and these ligands exert different intrinsic \(H_4R\) activities, ranging from full agonists to inverse agonists. Interestingly, we identified 4-methylhistamine as a high-affinity \(H_4R\) ligand \((K_i = 50 \text{ nM})\) that has a >100-fold selectivity for the \(hH_4R\) over the other histamine receptor subtypes. Moreover, 4-methylhistamine potently activated the \(hH_4R\) (pEC\(_{50}\) = 7.4 ± 0.1; \(\alpha = 1\)), and this response was competitively antagonized by the selective \(H_4R\) antagonist JNJ 7777120 \([1-[6-(1\text{-chloro}-1\text{-indol}-2\text{-yl})\text{-carbonyl}]-4\text{-methyl}]/\text{piperazine}]) (pA\(_{2}\) = 7.8). The identification of 4-methylhistamine as a potent \(H_4R\) agonist is of major importance for future studies to unravel the physiological roles of the \(H_4R\).

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 \(H_1\) receptor (\(H_1R\)) and \(H_2\) receptor (\(H_2R\)) were pharmacologically identified long before the \(H_3R\) (\(H_3R\) and \(H_4R\)) based on its sequence homology to the \(H_3R\) gene and bioinformatic analysis of human genome databases resulted in identification of the gene encoding the human \(H_4R\) \((hH_4R)\) (Lovenberg et al., 1999), and they have been successful blockbuster targets for more than two decades. The cDNA encoding the histamine \(H_4R\) was cloned more recently (Lovenberg et al., 1999), and bioinformatic analysis of human genome databases resulted in identification of the gene encoding the human \(H_4R\) \((hH_4R)\) based on its sequence homology to the \(H_4R\) gene (37%) (Oda et al., 2000; Liu et al., 2001a; Morse et al., 2001; Nguyen et al., 2001; Zhu et al., 2001). Although the \(H_4R\) is mainly present in the nervous system, the \(H_4R\) is distributed mainly in hematopoietic cells (Oda et al., 2000; Liu et al., 2001a; Morse et al., 2001; Zhu et al., 2001). The \(H_4R\) shows a different pharmacological profile compared with the closely related \(H_3R\), although many \(H_3R\) ligands also interact.
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-κB, are present upstream of the hH₄R gene (Cögé 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 thioperamide was identified as an equally effective H₄R antagonist (Oda et al., 2000; Hough, 2001). High-throughput screening and subsequent medicinal chemistry efforts recently identified the indolylipperazine JNJ 7777120 (Jablonski et al., 2003; Thurmond et al., 2004) and the related benzimidazole analog VUF 6002 (Terzigliu et al., 2004) as selective and potent H₄R antagonists. Studies directed toward 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 imifuranime analog OUP-16, which displays a 15-fold selectivity for the H₄R compared with 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 H₄R agonist (Durant et al., 1975), as a high-affinity H₄R agonist with a more than 100-fold selectivity over the H₂R, H₃R, and H₅R.

### Materials and Methods

**Materials.** Aminopotentidine, amphetamine dihydrobromide, amphetamine dihydrochloride, dimaprit dihydrobromide, histaprodifen dimaleate, homohistamine dihydrobromide, imbutamine dihydrobromide, imetit dihydrobromide, imiprep dihydrobromide, immetridine dihydrobromide, idopropenoprop dihydrobromide, JNJ 7777120, 2,4-bromophenylhistamine dihydrobromide, methimiprep dihydrobromide, 2-pyridylmethyamine (PEA) dihydrochloride, 2-(2-thiazolyl)ethylamine (TEA) dihydrochloride, thiopeptide fumarate, and VUF 8326 were synthesized at the Department of Medicinal Chemistry (Vrije Universiteit Amsterdam, Amsterdam, The Netherlands). Famotidine, ketotifen fumarate, and 5-HT₄-lisuride were purchased from MP Biomedicals (Irvine, CA). Amoxapine, d-chlorpheniramine maleate, clozapine, cimetidine, N-desethyl clozapine, diphenhydramine hydrochloride, doxepin hydrochloride, forskolin, histamine dihydrochloride, imipramine hydrochloride, loxapine, mepryamine (pyrilamine maleate), (R)-α-methylhistamine dihydrochloride, (S)-α-methylhistamine dihydrochloride, N-oxide clozapine, octoclothein, pertussis toxin, polyethyleneimine, ranitidine hydrochloride, tripeptannamine hydrochloride, and triprolidine hydrochloride were purchased from Sigma/RBI (Natick, MA). 2-Nitrophenol-β-d-pyranoside and G418 (geneticin) were from Duichea (The Netherlands); promethazine was from YMPharmacy Amsterdam (Amsterdam, The Netherlands); fexofenadine was from Ultrafine Chemicals (Manchester, UK); tiotidine was from Imperial Chemical Industries PLC (London, UK); and [³H]N-methylhistamine (85 Ci/mmol), [³H]histamine (12.4 Ci/mmol), and [³H]mepyramine (23 Ci/mmol) were from PerkinElmer Life and Analytical Sciences (Boston, MA). [¹²⁵I]Iodoaminopotentidine and [¹²⁵I]Iodoapropenoprop 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 [³HJN]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 Pharmaceuticals, Antwerp, Belgium); cyproheptadine hydrochloride (MSD, Haarlem, The Netherlands); cetirizine hydrochloride and hydroxyzine dihydrochloride (UCB Pharma, Brussels, Belgium); ebastine (Almirall Prodesfarma, Barcelona, Spain); loratidine (Schering Plough, Kenilworth, NJ); mianserin hydrochloride and ORG37770 (Organon NV, Oss, The Netherlands); mifentidine (Instituto De Angeli, Milan, Italy); mizolastine (Synthélabo Recherche, Bagneux, France); proxifen hydrochloride and idopropynaf dine dihydrochloride (Dr. J. A. M. Christiaans; Kovalainen et al., 1999), S(+) and R(−) suproidine (Institute of Pharmacy, Free University Berlin, Berlin, Germany); and R(+) and S(−)-terfenadine carboxylate (Sepracor, Marlborough, MA), 2-methylhistamine dihydrochloride, 4-methylhistamine dihydrochloride, and imipramide hydrochloride (GlaxoSmithKline, Welwyn Garden City, Hertfordshire, UK) are greatly acknowledged.

**Cell Culture.** SK-N-MC cell lines, which stably express either the human H₂R (SK-N-MC/hH₂) or H₄R (SK-N-MC/hH₄) as well as a cAMP-responsive element (CRE)-driven β-galactosidase reporter gene SK-N-MC/hH₂ or SK-N-MC/hH₄ cells (Lovenberg et al., 1992), which were cultured in Eagle’s minimum essential 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/hH₂ cell homogenates were incubated for 40 min at 25°C with approximately 1 nM [³H]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 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 hH₂R 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]Iodoapropenoprop to the hH₄R was performed with the same conditions as described for [³H]histamine. In satura-
tion binding analysis, the nonspecific binding of [3H]histamine or [3H]JNJ 7777120 was determined with 1 μM clobenpropit. The binding analysis of [3H]mepramine and [125I]iodoaminopotentidine binding to human H4R 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-β-galactosidase reporter gene assay was used to determine (inverse) agonistic or antagonistic activity of either the hH4R or hH3R. Approximately 4 million cells/96-well plate of SK-N-MC/hH4 and SK-N-MC/hH3 cells were exposed for 6 h to histaminergic ligands in serum-free Eagle’s minimum essential medium containing 1 μM forskolin. Thereafter, the medium was discarded, the cells were lysed in 100 μl of assay buffer (100 mM sodium phosphate buffer at pH 8.0, 4 mM 2-nitrophenol-β-D-pyranoside, 0.5% Triton X-100, 2 mM MgSO4, 0.1 mM MnCl2, and 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., 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). Analysis of the [3H]histamine saturation binding yielded a Kd value of 1.1 ± 0.0 nM (n = 6) and a Bmax value of 1.8 ± 0.4 pmol/mg protein. Recently, JNJ 7777120 was described as a selective H4R antagonist (Jablonski et al., 2003). In our

Fig. 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 H4R 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, 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 hH_{4}R (pK_{i} = 7.8 ± 0.1 against [\textsuperscript{3}H]histamine) over the hH_{3}R (pK_{i} = 5.3 ± 0.1 against [\textsuperscript{3}H]N\textsuperscript{-}methylhistamine), allowing the use of [\textsuperscript{3}H]JNJ 7777120 to label the H_{3}R (Thurmond et al., 2004). The H_{2}R antagonist [\textsuperscript{3}H]NMK 8146/nM [\textsuperscript{3}H]JNJ 7777120 exhibits a somewhat higher level of nonspecific binding to hH_{2}R expressing SK-N-MC cells, but it also binds saturably and shows an equipotent affinity (K_{D} = 11 ± 3.6 nM; n = 3) and results in a B_{max} value of 1.7 ± 0.4 pmol/mg protein (Fig. 1B). The binding of either 10 nM [\textsuperscript{3}H]histamine (Fig. 1C) or 10 nM [\textsuperscript{3}H]JNJ 7777120 (data not shown) to the hH_{2}R is fully displaced by histamine (pK_{i} = 7.8 ± 0.1), the H_{2A}R antagonist thioperamide (pK_{i} = 6.9 ± 0.1) and the H_{4}R antagonist/H_{3}R antagonist clofenprop (pK_{i} = 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/hH_{4} cells used in this study coexpress a CRE-controlled β-galactosidase reporter gene and can therefore also be used for a functional analysis of H_{4}R ligands. Stimulation of the hH_{4}R with histamine resulted in the inhibition (58 ± 3%; n = 16) of the forskolin-stimulated (1 μM) cAMP-mediated reporter gene transcription with a pEC_{50} value of 7.7 ± 0.1 (n = 16) (Fig. 1D). Treatment of SK-N-MC/ hH_{4} cells with the G_{α_{5,6}} protein inhibitor pertussis toxin (100 ng/ml for 16 h) completely inhibited histamine induced responses, confirming the coupling of the H_{4}R to G_{α_{5,6}} 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), clofenprop acts as a potent partial hH_{4}R agonist with a pEC_{50} value of 7.7 ± 0.1 (n = 3) and an intrinsic activity of 0.8 (Fig. 1D).

Treatment of SK-N-MC/hH_{4} 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 hH_{4}R shows a detectable level of constitutive activity in the SK-N-MC/hH_{4} 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 pEC_{50} value of 7.0 ± 0.1 (Fig. 1D). The inhibition of the constitutive activity of the hH_{4}R by thioperamide was of the same magnitude as observed after treatment with pertussis toxin and thioperamide is referred to as a full inverse hH_{4}R agonist (intrinsic activity α = −1).

In SK-N-MC/hH_{4} cells the cAMP-driven β-galactosidase reporter-gene transcription also can be activated by endogenously expressed G_{α_{i}} protein-coupled β adrenergic receptors (Bahouth et al., 2001). The β_{2} adrenergic receptor agonist fenoterol induced β-galactosidase activity to a similar extent to that of forskolin, with a pEC_{50} value of 6.9 ± 0.1 (n = 6). H_{2}R activation by histamine inhibited the 100 nM fenoterol-induced β-galactosidase activity for 39 ± 3% with a pEC_{50} value of 7.4 ± 0.1 (n = 7). However, inverse agonistic activity of thioperamide, at the hH_{2}R, 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/hH_{4} cells.

All compounds were preliminarily tested as displacers of [\textsuperscript{3}H]histamine binding to the hH_{4}R expressed in SK-N-MC/ hH_{4} cells at a concentration of 10 μM. Compounds inhibiting the specific binding of 10 nM [\textsuperscript{3}H]histamine to the hH_{4}R by ≤30% are expected to have a K_{i} > 10 μM based on the Cheng and Prusoff equation (Cheng and Prusoff, 1973): K_{i} = IC_{50}/(1 + [radioligand]/K_{D}) and were excluded for further testing. Active compounds (displacement ≥30%) were tested more extensively in both [\textsuperscript{3}H]histamine displacement studies and the CRE-β-galactosidase-based functional H_{4}R assay. Most H_{4}R Ligands Are Devoid of H_{3}R Activity. Histamine potently displaces [\textsuperscript{3}H]histamine from the hH_{4}R with a pK_{i} value of 7.8 ± 0.1 (Table 1), whereas H_{3}R 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 loss of affinity. Bulkier substituents at the 2-position (1,1-diphenylpropyl in histaprodifen) even result in a total loss of affinity for the hH_{4}R (Table 1). Agonists, lacking the imidazole ring, such as TEA, PEA, or 8R-lisuride (Bakker et al., 2004), are also not active at the hH_{4}R (Table 1).

After an initial report that the H_{4}R can be labeled with [\textsuperscript{3}H]mepyramine (Nguyen et al., 2001), a large number of H_{4}R antagonists (Table 1), including many clinically relevant drugs, were evaluated for their hH_{4}R affinity as well. Almost all tested H_{4}R antagonists, including mepyramine, showed pK_{i} values <5 (Table 1) and did not show functional activity at 1 and 10 μM at the hH_{4}R (data not shown). Although structurally similar to some tricyclic H_{3}R antagonists devoid of H_{4}R affinity, clozapine binds with moderate potency to the hH_{4}R (pK_{i} = 6.7 ± 0.1) and exerts full agonistic activity at the hH_{4}R with a pEC_{50} value of 6.8 ± 0.1 (n = 5). N-Desmethyl clozapine, a clozapine metabolite, showed a slightly decreased affinity (pK_{i} = 6.5 ± 0.1), whereas N-oxide clonazepine, another clonazepine metabolite, is totally devoid of hH_{4}R affinity. Furthermore, we evaluated clonazepine analogs of therapeutic importance as well. Loxapine and amoxapine showed >10-fold lower affinity (pK_{i} 5.4 ± 0.1 and 5.3 ± 0.1, respectively), whereas octocolsin did not show binding for the hH_{4}R.

Some H_{4}R Ligands Act as H_{3}R Agonists. Within the series of known H_{3}R agonists that we have tested, only some ligands retain H_{4}R activity. Replacement of the imidazole ring of histamine in the selective H_{3}R agonists anathamine and amselamine (Leurs et al., 1994) results in a total loss of hH_{4}R activity at concentrations up to 10 μM. Dimaprit, a H_{3}R agonist/H_{4}R antagonist lacking an imidazole group, binds the hH_{4}R with moderate affinity, showing a pK_{i} value of 6.5 ± 0.1, and exerts partial H_{4}R agonistic activity (Table 1). Impromidine, which was reported to bind to both H_{3}R and H_{4}R, also binds potently to the hH_{4}R with a pK_{i} value of 7.6 ± 0.1 and acts as a partial H_{4}R 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 H_{4}R selective agonist 4-methylhistamine (Durant et al., 1975) is the only known H_{4}R agonist that also acts as full agonist at the H_{4}R (Table 1). 4-Methylhistamine binds two times less potently than histamine to the hH_{4}R, exhibiting a pK_{i} value of 7.3 ± 0.1 (n = 3).

Most tested H_{4}R antagonists, including cimetidine, mfen-tidine, aminopotentidine, ranitidine, famotidine, and tiotidine, only displaced <30% of 10 nM [\textsuperscript{3}H]histamine binding to the hH_{4}R. Only the H_{2A}R ligand burimamide shows a high affinity for the hH_{4}R pK_{i} = 7.4 ± 0.1 (Table 1). Moreover,
various burimamide analogs as H3R antagonists (Vollinga et al., 2001). 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. pEC50 values were determined with [3H]histamine displacement assay; pEC50 values show the inhibition of 1 μM forskolin-induced CRE-β-galactosidase activity in SK-N-MC/hH4 cells.

<table>
<thead>
<tr>
<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>Histamine</td>
<td>4.2 ± 0.1</td>
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<td>7.8 ± 0.1</td>
<td>7.7 ± 0.1</td>
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<td>5.9 ± 0.1</td>
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<tr>
<td>Histaprodifen</td>
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<td>N.D.</td>
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<tr>
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<tr>
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<td>SR-Lisuride</td>
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<td>5.3 ± 0.4</td>
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<tr>
<td>Azatidine</td>
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<td>&lt;5</td>
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<td>N.D.</td>
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<tr>
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<td>5.3 ± 0.1</td>
<td>6.7 ± 0.1</td>
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<td>6.2 ± 0.1</td>
<td>0.8</td>
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<td>N.D.</td>
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<td>Antagonist</td>
<td>&lt;5</td>
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<th>H3R ligand</th>
<th>pK_i at hH3R</th>
<th>H3R Activity</th>
<th>pK_i</th>
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<td>N.D.</td>
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<td>&lt;5</td>
<td>N.D.</td>
<td>N.D.</td>
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</tbody>
</table>

α: intrinsic activity (1 designated for full agonistic, 0 for neutral antagonist, and –1 for full inverse agonistic activity); N.D., not determined; †—, due to non-H4R-mediated effects of SR-lisuride, the pEC50 value was not determined.

Table 1: Activity of H3R and H4R ligands at the hH4R.

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 et al., 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; Fig. 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 (pK_i = 7.6 ± 0.1) is surprisingly observed for VUF 4614. As can be seen in Fig. 2B, VUF 4614 was able to competitively block the H4R 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 hH4R than for the hH4R. However, the hH4R retains some level of stereoselectivity for (R)-α- (pK_i = 6.6 ± 0.1) and (S)-α-methylhistamine (pK_i = 5.4 ± 0.1) (Table 3).
Increasing the spacer length between imidazole and amine group from two carbon atoms (histamine, $pK_i = 7.8 \pm 0.1$) to three carbon atoms (homohistamine, $pK_i = 7.5 \pm 0.1$) slightly decreases the affinity for the hH4R, whereas four carbon atoms (imbutamine, $pK_i = 8.0 \pm 0.1$) 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 ($pK_i = 6.6 \pm 0.1$) (Table 3). Interestingly, besides the affinity, impentamine also looses intrinsic activity for the hH4R ($\alpha = 0$). Previously identified H4R agonists, including immehip, imetit, and VUF 8328 (an imetit analog) (Wieland et al., 2001), also potently bind the hH4R with $pK_i$ values of $7.7 \pm 0.1$, $8.2 \pm 0.1$, and $8.0 \pm 0.1$, respectively. At the hH4R these ligands also act as agonists, but exert somewhat lower intrinsic activity ($\alpha$ 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 ($pK_i = 9.1 \pm 0.1$) binds much less potently to the hH4R ($pK_i = 6.6 \pm 0.1$) and is also not able to fully activate the H4R (Table 3). In agreement with our findings with $N^\alpha$-methylhistamine, the methylated immehip analog methimepip shows a large selectivity for the hH4R ($pK_i = 9.0 \pm 0.1$) over the hH4R ($pK_i = 5.7 \pm 0.1$), 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 cloben- propit and idophenpropit 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 potent partial agonist at the hH4R (Table 3; Fig. 3, A and B). In contrast, iodophenpropit, which also acts as an inverse agonist at the hH4R (Wieland et al., 2001), behaves as a neutral antagonist at the hH4R with a $pK_i$ 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 $pA_2$ value of 8.0 (Fig. 3, C and D), in accordance with its binding affinity. Besides clobenpropit, also the known H2R ligands propranol and the related idopropoxifen show reasonable hH4R affinity with $pK_i$ values of 7.3 ± 0.1 and 7.9 ± 0.1, respectively. As observed for their action at the hH4R, 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 H4R 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 $K_d$ value of 34.4 ± 4.1 nM for [125I]iodophenpropit. The $B_{max}$ 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 (Fig. 4), despite a high level of nonspecific binding of approximately 60% as determined with 10 μM imetit. However, clobenpropit and the related clobenpropit also displaced the nonspecific binding, resulting in a multiple site binding profile. The $pK_i$ values of compounds for the hH4R obtained using [125I]iodophenpropit displacement studies are consistent with their corresponding values obtained using displacement of either [3H]histamine or [3H]JNJ 7777120 binding to the hH4R; only the $pK_i$ value of thioperaemidine obtained using [125I]iodophenpropit displacement studies seems to deviate somewhat form the $pK_i$ 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-methylhistamine for the hH4R, this histamine analog was evaluated in more detail. 4-Methylhistamine not only has high affinity for the hH4R ($pK_i = 7.3 ± 0.1; n = 3$) 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]mepyramine ($K_i = 1.6$ nM), 0.5 nM [125I]iodoaminopotentidine ($K_i = 0.5$ nM) and 1 nM [3H]N*-methylhistamine ($K_i = 2.9$ 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 hH4R, for the mouse and rat H4R with $K_i$ values of 73 and 55 nM, respectively (Fig. 5B). Moreover, 4-methylhistamine exerts full agonistic activity at the hH4R (Fig. 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 hH4R ($pK_i = 5.1 ± 0.1; n = 3$) and hH4R ($pK_i = 5.2 ± 0.1; n = 4$), and partial agonistic hH4R activity (Fig. 5D). The hH4R agonistic effects of 4-methylhistamine can be antagonized by the se-

**TABLE 3**

Activity of H4R ligands at the hH3R and hH4R

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<tr>
<th>Ligand</th>
<th>$pK_i$ (hH3R)</th>
<th>$pEC_{50}$ (hH3R)</th>
<th>$pK_i$ (hH4R)</th>
<th>$pEC_{50}$ (hH4R)</th>
<th>$α$</th>
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<tr>
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<tr>
<td>4-Methylhistamine</td>
<td>5.3 ± 0.1</td>
<td>6.0 ± 0.1</td>
<td>7.8 ± 0.1</td>
<td>7.8 ± 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 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 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.

Fig. 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, whereas 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.

Fig. 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 percentage of specific binding, fit according to a one-site ligand-receptor model. Data shown are from representative experiments, each performed in triplicate.
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 nM (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**

<table>
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<th>Compound</th>
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<th>[3H]JNJ 7777120</th>
<th>[125I]Iodophenpropit</th>
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<td>4-Methylhistamine</td>
<td>7.3 ± 0.1</td>
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<td>Immepip</td>
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<tr>
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<tr>
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<tr>
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<tr>
<td>Kd (nM)</td>
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<td>34.4 ± 4.1*a</td>
</tr>
<tr>
<td>Rmax (pmol/mg protein)</td>
<td>1.8 ± 0.4</td>
<td>1.7 ± 0.4</td>
<td>3.8 ± 0.4</td>
</tr>
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</table>

*a Determined by homologous displacement analysis, using the equation Kd = IC50 - [radioligand].
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 imbu-tamine also act as potent hH4R agonists. Furthermore, the H4R is activated by the H3R/H2R antagonist burimamide, the H3R antagonists clobenpropit, and the H3R agonist iodo-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 hH4R (partial) agonists, a neutral antagonist (VUF 4614; pK\textsubscript{i} = 7.6) and a full inverse agonist (VUF 4742; pK\textsubscript{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\textsubscript{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 ~15
nM affinity of iodophenpropit for the hH4R, we evaluated [125I]iodophenpropit as a potential new H4R radioligand. The hH4R can be labeled to the same extent with both the agonist [3H]histamine and the neutral antagonist [3H]JNJ 7777120. Surprisingly, the B_{max} value determined with [125I]iodophenpropit was twice as much as that determined with either [3H]histamine or [3H]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 H4R (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 H_{3A/B}R ligands, and the pK_{i} 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 H_{3A/B}R 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 a potent H4R agonist will be of major importance for future studies to unravel the physiological roles of the H4R.

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