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RESEARCH PAPER

Pharmacological characterization of the new histamine H_4 receptor agonist VUF 8430

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Background and purpose: We compare the pharmacological profiles of a new histamine H_4 receptor agonist 2-(2-guanidinoethyl)isothiourea (VUF 8430) with that of a previously described H_4 receptor agonist, 4-methylhistamine.
Experimental approach: Radioligand binding and functional assays were performed using histamine H_4 receptors expressed in mammalian cell lines. Compounds were also evaluated *ex vivo* in monocyte-derived dendritic cells endogenously expressing H_4 receptors and *in vivo* in anaesthetized rats for gastric acid secretion activity.
Key results: Both VUF 8430 and 4-methylhistamine were full agonists at human H_4 receptors with lower affinity at rat and mouse H_4 receptors. Both compounds induced chemotaxis of monocyte-derived dendritic cells. VUF 8430 also showed reasonable affinity and was a full agonist at the H_3 receptor. Agmatine is a metabolite of arginine, structurally related to VUF 8430, and was a H_4 receptor agonist with micromolar affinity. At histamine H_3 receptors, agmatine was a full agonist, whereas 4-methylhistamine was an agonist only at high concentrations. Both VUF 8430 and agmatine were inactive at H_1 and H_2 receptors, whereas 4-methylhistamine is as active as histamine at H_2 receptors. *In vivo,* VUF 8430 only caused a weak secretion of gastric acid mediated by H_2 receptors, whereas 4-methylhistamine, dimaprit, histamine and amthamine, at equimolar doses, induced 2.5- to 6-fold higher output than VUF 8430.
Conclusions and implications: Our results suggest complementary use of 4-methylhistamine and VUF 8430 as H_4 receptor agonists. Along with H_4 receptor antagonists, both agonists can serve as useful pharmacological tools in studies of histamine H_4 receptors.
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Keywords: histamine H_4 receptor; agonist; 4-methylhistamine; VUF 8430; agmatine; chemotaxis; gastric acid secretion

Abbreviations: CRE, cAMP responsive element; JNJ 7777120, 1-[(5-chloro-1*H*-indol-2-yl)carbonyl]-4-methylpiperazine; MoDC, monocyte-derived dendritic cell; VUF 8430, 2-(2-guanidinoethyl)isothiourea

Introduction

Histamine is a chemical mediator that controls many physiological functions through the interaction with four histamine receptor subtypes, which are all members of the large multigene family of G-protein coupled receptors (Parsons and Ganellin, 2006). The histamine H_1 and H_2 receptors represent very successful therapeutic targets (Parsons and Ganellin, 2006; receptor nomenclature follows Alexander *et al.*, 2008), while the H_3 receptor and H_4 receptor have emerged as potential targets for future treatment of central nervous system (CNS) disorders (Leurs *et al.*, 2005) and inflammatory diseases (de Esch *et al.*, 2005; Lim *et al.*, 2006b) respectively. The potential therapeutic use is strongly related to the relative selective tissue distribution of both the H_3 receptor and H_4 receptor. Whereas the H_3 receptor is mainly present in the nervous system, the H_4 receptor is primarily localized on haematopoietic cells (Oda *et al.*, 2000; Liu *et al.*, 2001a; Morse *et al.*, 2001; Zhu *et al.*, 2001). The H_4 receptor has been demonstrated to be involved in the chemotaxis of mast cells, eosinophils and monocyte-derived dendritic cells (MoDCs)

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(Hofstra *et al.*, 2003; Ling *et al.*, 2004; Gutzmer *et al.*, 2005), to control mediator production, such as interleukin (IL)-16 release by human $CD8^+$ T cells (Gantner *et al.*, 2002), leukotriene B_4 production by mast cells (Takeshita *et al.*, 2003; Thurmond *et al.*, 2004), and suppression of IL-12p70 production by MoDCs (Gutzmer *et al.*, 2005). Various studies suggest that the H_4 receptor is a potential new drug target for inflammatory diseases, including chronic allergy, asthma, atopic dermatitis and inflammatory bowel diseases (Thurmond *et al.*, 2004; Dunford *et al.*, 2006; 2007). Moreover, the H_4 receptor has been detected in primary synovial culture obtained from rheumatoid arthritis patients and colorectal cancer tissues, suggesting a possible role for H_4 receptors in these diseases as well (Cianchi *et al.*, 2005; Ohki *et al.*, 2007).

Despite a growing body of evidence, validation of the histamine H_4 receptor as a drug target is mandatory. For this purpose, selective and potent agonists and antagonists for this receptor are needed. The H_3 receptor and H_4 receptor proteins are each other's closest relatives and show a relatively high level of homology (Hough, 2001), especially within the seven transmembrane domains, which are thought to bind small molecule agonists and antagonists. As might be expected on the basis of such a high homology, the H_4 receptor binds many imidazole-based H_3 receptor ligands with high affinity (Lim *et al.*, 2005; Gbahou *et al.*, 2006). The previously presumed H_3 receptor-selective, inverse agonist, thioperamide (Arrang *et al.*, 1987) binds to the related H_4 receptor with equal affinity and is therefore now classified as a non-selective H_3/H_4 receptor inverse agonist (Lim *et al.*, 2005). Some pharmaceutical companies have meanwhile started to focus on the discovery of selective non-imidazole H_4 receptor antagonists. This involvement has resulted in a number of patent applications for potent H_4 receptor antagonists (Lim *et al.*, 2006b). Currently, JNJ 7777120 (Jablonowski *et al.*, 2003) and the related VUF 6002 (Terzioglu *et al.*, 2004; Venable *et al.*, 2005) should be considered as prototypic non-imidazole H_4 receptor antagonists.

Considering their high value in research, we have focused on the discovery of selective non-imidazole H_4 receptor agonists, as well. Previously, we have described the histamine analogue, 4-methylhistamine (Durant *et al.*, 1975) as a potent H_4 receptor agonist (Lim *et al.*, 2005). Moreover, we recently reported on the synthesis and initial structure–activity relationships (SAR) of the non-imidazole H_4 receptor agonist 2-(2-guanidinoethyl)isothiourea (VUF 8430) (Lim *et al.*, 2006a). In the present paper, we compare the potential of the new H_4 receptor agonist VUF 8430 as a pharmacological tool, with the standard H_4 receptor agonist 4-methylhistamine and other histamine receptor ligands. Our results clearly indicate the usefulness of VUF 8430 as a complementary pharmacological tool in dissecting the functions of the histamine H_4 receptor.

Methods

Cell culture and transfection

SK-N-MC cell lines, which stably express either the human H_3 or H_4 receptors as well as a cAMP responsive element (CRE)-driven β-galactosidase reporter gene (Lovenberg *et al.*, 1999; Liu *et al.*, 2001a), were cultured in Eagle's minimal essential medium (EMEM) supplemented with 5% fetal calf serum, 0.1 $mg \cdot mL^{-1}$ streptomycin, 100 $\mu \cdot mL^{-1}$ penicillin and 600 $\mu g \cdot mL^{-1}$ G418 at 37°C in 5% CO_2 and 95% humidity. COS-7 and HEK 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% and 10%, respectively, fetal calf serum, 0.1 $mg \cdot mL^{-1}$ streptomycin, and 100 $\mu \cdot mL^{-1}$ penicillin. Approximately 10^6 COS-7 cells were seeded in a 10 cm dish, 1 day prior to transfection. Plasmid DNA was mixed in 0.9% NaCl solution, wherafter 25 kDa polyethyleneimine (PEI) solution (1 $mg \cdot mL^{-1}$, pH 7.0) was added to obtain a 2:1 mass ratio PEI : DNA. The mixture was incubated for 10 min, and it was then added to the COS-7 cell monolayer.

Radioligand binding assays

Cell homogenates of SK-N-MC cells expressing human H_3 receptors were incubated for 40 min at 25°C with approximately 1 $nmol \cdot L^{-1}$ [^{3}H]N^α-methylhistamine in 25 $mmol \cdot L^{-1}$ potassium phosphate buffer and 140 $mmol \cdot L^{-1}$ NaCl (pH 7.4 at room temperature), whereas cell homogenates of SK-N-MC expressing human H_4 receptors were incubated 1 h at room temperature with 10 $nmol \cdot L^{-1}$ [^{3}H]histamine in 50 $mmol \cdot L^{-1}$ Tris-HCl (pH 7.4 at 37°C), with or without competing ligands. Bound radioligand was collected on 0.3% PEI-pretreated 96-well GF/C filters, which were washed three times with 3 mL of ice-cold washing buffer (4°C) containing 25 $mmol \cdot L^{-1}$ Tris-HCl and 140 $mmol \cdot L^{-1}$ NaCl (pH 7.4 at 4°C) for the H_3 receptor and 50 $mmol \cdot L^{-1}$ Tris-HCl (pH 7.4 at 4°C) for the H_4 receptor). The binding analysis for mouse and rat H_4 receptors were performed as described above for human H_4 receptors. The binding analysis of [^{3}H]mepyramine and [^{125}I]iodoaminopotentidine binding to human H_1 receptors and human H_2 receptors, respectively, was performed according to Bakker *et al.* (2004). The binding data were analysed with Prism 4.0 (Graphpad Software, Inc.) and data are presented as mean ± SEM.

Reporter gene assay

A CRE-β-galactosidase reporter gene assay was employed to determine the activity of the tested ligands at either the human H_3 or H_4 receptor. Approximately 4×10^6 cells per 96-well plate of SK-N-MC cells were exposed for 6 h to the tested compounds in serum-free EMEM medium containing 1 $\mu mol \cdot L^{-1}$ forskolin. Thereafter, the medium was discarded, the cells were lysed in 100 µL assay buffer [100 $mmol \cdot L^{-1}$ sodium phosphate buffer at pH 8.0, 4 $mmol \cdot L^{-1}$ 2-nitrophenol-β-D-pyranoside (ONPG), 0.5% Triton X-100, 2 $mmol \cdot L^{-1}$ $MgSO_4$, 0.1 $mmol \cdot L^{-1}$ $MnCl_2$, 40 $mmol \cdot L^{-1}$ 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). To measure activity of the compounds at H_2 receptors, approximately 4×10^6 resuspended HEK 293T cells were transiently cotransfected with a mixture containing 2.5 µg CRE-β-galactosidase reporter gene and 2.5 µg cDNA of the human H_2 receptor, and 35 µL of 1 $mg \cdot mL^{-1}$ 25 kDa linear PEI, and transferred into a 96-well plate (4×10^4 cells per well). After incubation of 24 h, the cells were exposed with the tested ligands for 6 h. The β-galactosidase activity was

determined as described above. For the H_1 receptor, HEK 293T cells were contransfected with NFAT-luciferase reporter gene and cDNA of the human H_1 receptor, with a method as described above, and incubated 24 h before the cells were exposed with the tested compounds for 6 h. Subsequently, the luciferase was determined as described previously (Bakker *et al*., 2004). The intrinsic activity of agonists was determined relative to the activity of histamine.

Generation of monocyte-derived dendritic cells

Peripheral blood mononuclear cells (PBMC) were separated from heparinized buffy coats by density gradient centrifugation on Lymphoprep (Fresenius Kabi Norge AS, Norway). Adherent cells were obtained by plastic adherence: 1×10^8 PBMC were plated in 80 cm^2 culture flasks (Nuclon™Δ, Nunc GmbH & Co AG, Wiesbaden, Germany) in Iscove medium supplemented with 5% v/v AB serum, 1% w/v non-essential salts, 2 mmol·L^{-1} L-glutamine, 100 µ·mL^{-1} penicillin, 100 mg·mL^{-1} streptomycin and 0.5% w/v gentamycin (all from Biochrom AG, Berlin, Germany) for 1 h (37°C, 5% CO_2, humidified atmosphere). The non-adherent cells were removed by vigorous washing with phosphate buffered saline and visual inspection. The adherent cells (enriched monocytes, purity at least 85%) were further cultured in RPMI 1640 medium supplemented with 5% v/v FCS, 12 mmol·L^{-1} HEPES, 2 mmol·L^{-1} L-glutamine, 100 U·mL^{-1} penicillin, 100 mg·mL^{-1} streptomycin, interleukin-4 (10 ng·mL^{-1}, R&D Systems, Wiesbaden, Germany) granulocyte-monocyte-colony stimulating factor (50 ng·mL^{-1}, Berlex Pharmaceutical Company, Montville, USA). Half of the medium was replaced by fresh medium on days 3 and 5 of culture. Non-adherent cells were harvested at day 7 of culture and considered as MoDC as described previously (Gutzmer *et al*., 2005).

Chemotaxis assay

Chemotaxis of MoDC was measured over polycarbonate membranes with 5 µm pore diameter (Corning Inc., Costar, NY, USA). CCL2 (positive control, 0.8 nmol·L^{-1}), histamine, 4-methylhistamine and VUF 8430 (at 10 µmol·L^{-1}) were used as a chemoattractant in the lower chamber. The upper chamber with the membrane was filled with 250 µL medium containing 10^6 MoDCs. Chemotaxis was allowed for 1.5 h and the number of transmigrated cells was counted after staining with Trypan blue in a Burker-Türk counting chamber.

Gastric acid secretion in vivo

All animal procedures were in accordance with international guidelines governing animal experimentation, which was approved by the Ethics Committee of the University of Parma. Experiments were carried out with adult male Wistar rats (7–9 weeks, 200–250 g), purchased from Harlan-Italy (MI) and housed at constant temperature (20°C) and humidity (50–55%), with alternating 12-h light and dark cycles, and fed with standard laboratory chow and tap water. Gastric secretion in anaesthetized rats was measured by the stomach-lumen perfusion technique of Bertaccini *et al*. (1968) with minor modifications. The rats were used after 18-h fasting with free access to water. After anaesthesia with ethyl-urethane (1.25 g·kg^{-1} intraperitoneally), the stomach was perfused (60 mL·h^{-1}) through an oesophageal cannula with warm saline (NaCl 154 mmol·L^{-1}, 37°C, pH 5.5) at a rate of 1 mL·min^{-1}, using a peristaltic pump. The perfusion fluid was collected in 10-min periods via a duodenal cannula, and titrated to pH 7 with 10 mmol·L^{-1} NaOH, using an automatic titration system (Radiometer, Copenhagen). In separate experiments, the histamine receptor ligands, amthamine (0.1–1000 µmol·kg^{-1}), histamine (0.01–1000 µmol·kg^{-1}), 4-methylhistamine (3–300 µmol·kg^{-1}), dimaprit (3–300 µmol·kg^{-1}) or VUF 8430 (30–300 µmol·kg^{-1}) were injected intravenously (i.v.) to investigate the effects on basal acid secretion. The H_2 receptor inverse agonist ranitidine (3 mg·kg^{-1} or 8.6 µmol·kg^{-1} i.v.) or the H_4 receptor antagonist JNJ 7777120 (10 mg·kg^{-1} or 36 µmol·kg^{-1} i.v.) was given 30 min before secretagogues. Acid responses to secretory compounds were calculated for each rat by subtracting basal acid output (average of two collection periods before the stimulant injection) from the maximal acid response (average of two collection periods) and expressed as ΔµEq HCl·kg^{-1}·min^{-1}. Amthamine, dimaprit, histamine, 4-methylhistamine, ranitidine, ethyl-urethane and VUF 8430 were dissolved in saline. DMSO 100% was used as the vehicle to dissolve JNJ 7777120. Each agent was prepared immediately before use and administered in a volume of 0.1 mL per 100 g body weight. Control animals received the vehicle in place of the active agent.

Data analysis

Results are expressed as the means ± SEM of 5–8 rats per group. Comparisons between two groups were made by using the Student's *t*-test for unpaired data. A value of $P < 0.05$ was considered statistically significant. The software package Prism GraphPad 4.0 (GraphPad Software Inc., San Diego, CA) was used to process data.

Materials. Amthamine dihydrobromide, dimaprit dihydrobromide, JNJ 7777120, 4-methylhistamine dihydrochloride, thioperamide fumarate and VUF 8430 [*S*-(2-guanidylethyl)-isothiourea dihydrobromide] were synthesized at the Department of Medicinal Chemistry, Vrije Universiteit Amsterdam, while [^{125}I]iodoaminopotentidine was labelled at the Department of Nuclear Medicine and PET Research, Vrije Universiteit Medical Centre, Amsterdam. Forskolin, histamine dihydrochloride, mepyramine (pyrilamine maleate), N^{α}-methylhistamine dihydrochloride, *Pertussis* toxin, 750 kDa PEI, ranitidine hydrochloride, agmatine sulfate, putrescine dihydrochloride, spermine, spermidine and Trypan blue were purchased from Sigma RBI (USA). ONPG and G418 were purchased from Duchefa (The Netherlands), and [^{3}H]N^{α}-methylhistamine (85 Ci·$mmol^{-1}$), [^{3}H]histamine (12.4 Ci·$mmol^{-1}$), [^{3}H]mepyramine (23 Ci·$mmol^{-1}$) were from Perkin-Elmer Life Science, Inc. (USA), and 25 kDa PEI (for transfection) was from Polyscience, Inc. (Germany). Gifts of SK-N-MC cell lines expressing human H_3 receptors, human H_4 receptors, mouse H_4 receptors, rat H_3 receptors and rat H_4 receptors from Dr Lovenberg are greatly acknowledged (Liu *et al*., 2001a,b).

Results

Binding of VUF 8430 and related compounds to human and rodent H_4 receptors

In radioligand binding studies using [^{3}H]histamine and SK-N-MC cells stably expressing the human H_4 receptor (Liu *et al*., 2001a), the newly discovered VUF 8430 (Lim *et al*., 2006a) was almost as potent as histamine and the known histamine H_4 receptor agonist, 4-methylhistamine (Lim *et al*., 2005) in binding to the H_4 receptor (Figure 1A, Table 1). Moreover, the structurally related H_2 receptor agonist dimaprit was approximately 100-fold less effective than VUF 8430 in displacing [^{3}H]histamine binding to the human H_4 receptor (Figure 1A, Table 1).

The endogenous amine, agmatine, has a clear structural resemblance to VUF 8430 (Table 1) and is formed by decarboxylation of L-arginine by arginine decarboxylase (ADC) and hydrolyzed by agmatinase to putrescine. Agmatine binds to several receptors in the brain and has been proposed as a novel neurotransmitter (Li *et al*., 1994; Reis and Regunathan, 2000). In our experiments, agmatine showed a micromolar affinity for the human H_4 receptor (Figure 1A, Table 1). As can be seen in Figure 1A, its amino acid precursor, arginine, was totally inactive at the H_4 receptor, whereas its metabolite, putrescine, still showed moderate affinity (Figure 1B, Table 1). Following this observation, we tested a variety of other polyamines (Figure 1B) and found that the putrescine homologue cadaverine, spermine and spermidine all showed

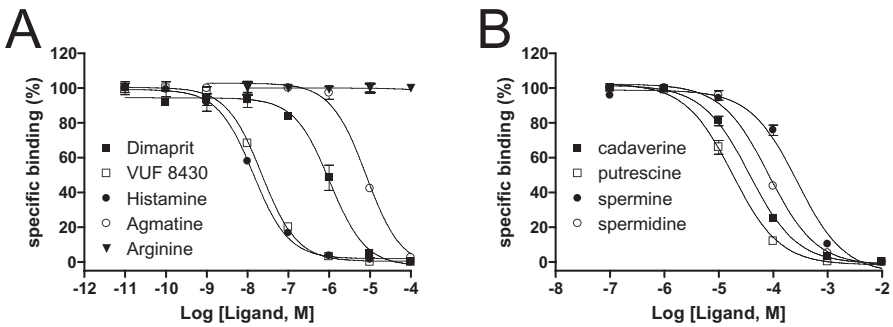
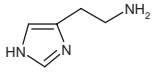
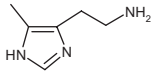
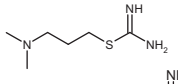
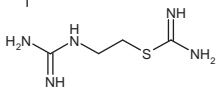
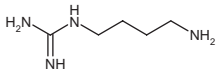
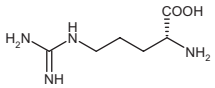
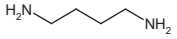


Figure 1 Binding of a variety of amines to the human H_4 receptor. (A) Histamine, dimaprit, VUF 8430 and agmatine, but not arginine, and (B) the polyamines putrescine, cadaverine, spermidine and spermine dose-dependently displaced binding of [^{3}H]histamine to the human H_4 receptor stably expressed in SK-N-MC cells.

Table 1 Affinity (pK_i) of H_4 receptor ligands for different species variants of H_4 receptor stably expressed in SK-N-MC cells

Ligand	*Structure*	*Human*	*Mouse*	*Rat*
Histamine		7.9 ± 0.1	7.4 ± 0.1	7.3 ± 0.1
4-methyl-histamine		7.6 ± 0.1	7.2 ± 0.1	6.7 ± 0.1
Dimaprit		6.8 ± 0.1	6.2 ± 0.1	6.0 ± 0.1
VUF 8430		7.5 ± 0.1	7.0 ± 0.1	6.9 ± 0.1
Agmatine		5.6 ± 0.1	5.1 ± 0.1	5.1 ± 0.2
L-arginine		<3	n.d.	n.d.
Putrescine		4.9 ± 0.1	n.d.	n.d.
Cadaverine		4.7 ± 0.1	n.d.	n.d.
Spermidine	NH_2-$(CH_2)_4$-NH-$(CH_2)_3$-NH_2	3.7 ± 0.1	n.d.	n.d.
Spermine	NH_2-$(CH_2)_3$-NH-$(CH_2)_4$-NH-$(CH_2)_3$-NH_2	4.3 ± 0.1	n.d.	n.d.

Data shown are mean ± SEM of at least three independent experiments.
n.d., not determined.

moderate to weak affinities for the human H_4 receptor (Figure 1B, Table 1).

Agonist activities of VUF 8430 and agmatine at human histamine receptors

We subsequently evaluated the functional activities of histamine, VUF 8430, 4-methylhistamine and agmatine at the four human histamine receptor subtypes. As reported previously (Lim *et al.*, 2005; 2006a), both histamine ($\alpha = 1$ by definition), 4-methylhistamine ($\alpha = 1$) and VUF 8430 ($\alpha = 1$) act as full agonists at the human H_4 receptor as measured by the G_i-protein mediated inhibition of the forskolin-induced (1 μmol·L^{-1}) CRE-mediated transcription of β-galactosidase in SK-N-MC cells, stably expressing the human H_4 receptor (B_{max} = 1.8 pmol·mg^{-1} protein) and a CRE-β-galactosidase reporter gene (Figure 2A). In this assay, the endogenous agonist histamine was slightly more potent ($pEC_{50} = 7.7 \pm 0.1$, $n = 16$) compared with VUF 8430 ($pEC_{50} = 7.3 \pm 0.1$, $n = 6$) or 4-methylhistamine ($pEC_{50} = 7.4 \pm 0.1$, $n = 5$). Agmatine, under these experimental conditions, was a partial agonist ($\alpha = 0.65 \pm 0.05$, $n = 4$) with a pEC_{50} value of 5.4 ± 0.1 ($n = 4$). At the related human H_3 receptor (Figure 2B), both histamine ($\alpha = 1$ by definition) and VUF 8430 ($\alpha = 1$) were full agonists, as measured by the inhibition of the 1 μmol·L^{-1} forskolin-induced CRE-mediated transcription of β-galactosidase in SK-N-MC cells stably expressing the human H_3 receptor (B_{max} = 475 fmol·mg^{-1} protein) and a CRE-β-galactosidase reporter gene. Agmatine also acts as a full agonist ($\alpha = 1$) at the H_3 receptor (Figure 2B) with a pEC_{50} value of 6.1 ± 0.1 ($n = 3$); 4-methylhistamine was clearly less effective at the human H_3 receptor (Figure 2B) and was a partial agonist ($\alpha = 0.38 \pm 0.12$, $n = 3$) with a pEC_{50} value of 6.1 ± 0.1 ($n = 3$).

VUF 8430 did not interact with the histamine H_1 and H_2 receptor subtypes. Histamine displayed a pEC_{50} value of 7.0 ± 0.1 ($n = 4$) at human H_1 receptors, measured using HEK 293T cells transiently expressing the human H_1 receptor and a NFAT-luciferase reporter gene (Figure 2C). In the same experimental set-up, 4-methylhistamine, VUF 8430 and agmatine showed no agonist activity at the H_1 receptor at concentrations up to 100 μmol·L^{-1} (Figure 2C). At human H_2 receptors, histamine displayed a pEC_{50} of 7.2 ± 0.1 ($\alpha = 1$, $n = 4$), measured using COS-7 cells transiently expressing the human H_2 receptor and a CRE-β-galactosidase reporter gene. In this assay, 4-methylhistamine ($\alpha = 1$) exhibited a pEC_{50} of 6.8 ± 0.1 ($n = 4$), whereas VUF 8430 and agmatine only weakly activated the human H_2 receptor at 100 μmol·L^{-1} (Figure 2D).

Affinity of H_4 receptor agonists at rodent histamine receptors

Previously, significant species differences were reported for the binding of histamine to rodent H_4 receptors (Liu *et al.*, 2001b). We therefore tested VUF 8430 and agmatine for their affinity at mouse and rat H_4 receptors (Figure 3A,B, Table 1). As previously reported (Liu *et al.*, 2001b), histamine showed an three- to fivefold lower affinity for the mouse and rat H_4 receptor compared with the human H_4 receptor, measured by displacement of [^{3}H]histamine binding to homogenates of SK-N-MC cells stably expressing the various H_4 receptor orthologs (Figure 3A, Table 1). Similarly, both VUF 8430 and

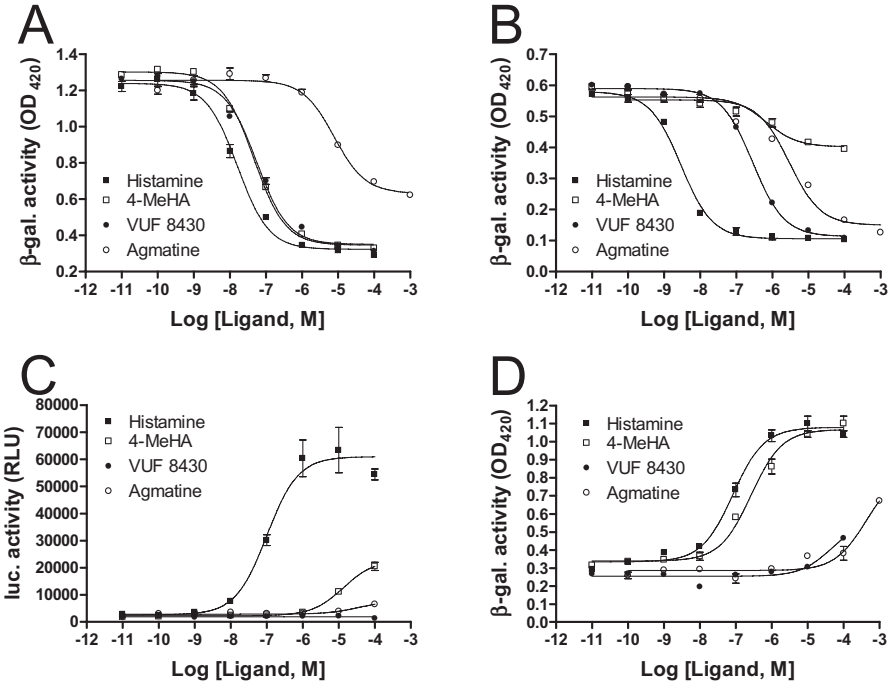


Figure 2 Functional activity of histamine, 4-methylhistamine (4-MeHA), VUF 8430 and agmatine at the human H_4 receptor (A), human H_3 receptor (B) expressed in SK-N-MC cells and human H_1 receptor (C) and human H_2 receptor (D) expressed in HEK 293T cells. The NFAT-luciferase reporter gene assay was used to measure H_1 receptor activity, while the CRE-β-galactosidase was employed to measure activation of cAMP production by H_2 receptor or inhibition of forskolin-induced cAMP generation by the H_3 and H_4 receptors.

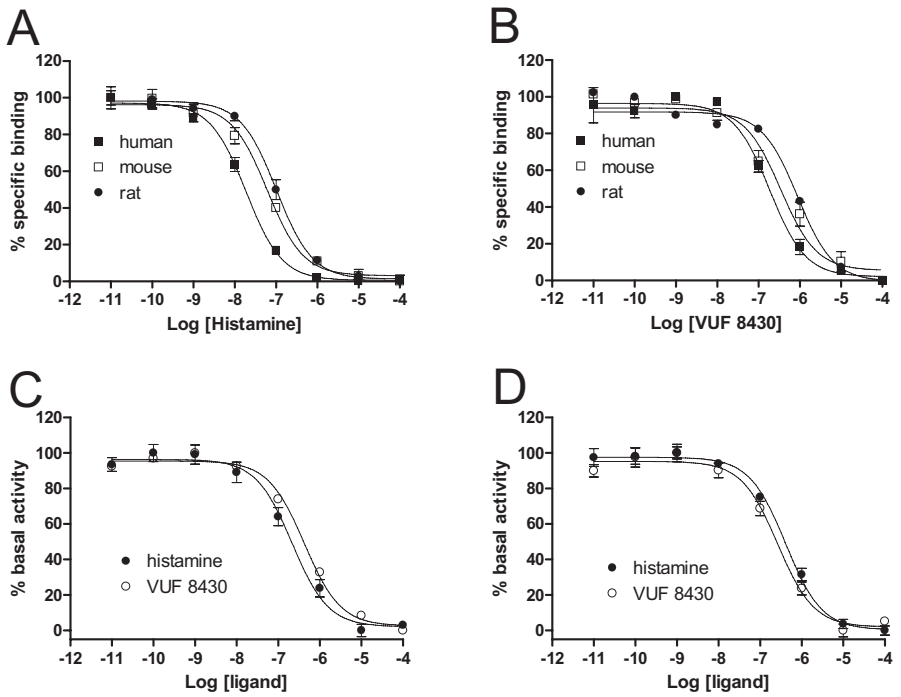


Figure 3 Interaction of VUF 8430 with the H_4 receptor species variants. Histamine (A) and VUF 8430 (B) inhibits binding of [^{3}H]histamine to human, mouse and rat H_4 receptors stably expressed in SK-N-MC cells. VUF 8430 inhibited forskolin-induced cAMP responsive element (CRE) through mouse H_4 receptor (C) and rat H_4 receptor (D), to the same extent as histamine.

Table 2 Affinity (pK_i) of H_4 receptor ligands at rat histamine receptors

Ligand	*pK_i at rat histamine receptor*			
	H_1	*H_2*	*H_3*	*H_4*
Histamine	4.5 ± 0.2	4.3 ± 0.1	8.1 ± 0.1	7.4 ± 0.1
4-methylhistamine	<4	4.1 ± 0.1	5.2 ± 0.1	6.8 ± 0.1
Dimaprit	4.4 ± 0.2	4.5 ± 0.3	6.3 ± 0.1	6.1 ± 0.1
VUF 8430	<4	3.7 ± 0.1	6.5 ± 0.1	6.9 ± 0.1
Thioperamide	4.1 ± 0.1	3.7 ± 0.1	7.8 ± 0.1	7.2 ± 0.1
JNJ 7777120	5.1 ± 0.1	4.9 ± 0.1	5.3 ± 0.1	7.8 ± 0.1
VUF 6002	4.7 ± 0.1	4.5 ± 0.1	5.3 ± 0.1	6.8 ± 0.1

Data shown are mean ± SEM of at least three independent experiments.

agmatine showed a somewhat lower affinity at the rodent receptor (Figure 3B, Table 1). At both the mouse (Figure 3C) and rat H_4 receptor (Figure 3D), VUF 8430 was as effective as histamine in inhibiting the 1 μmol·L^{-1} forskolin-induced CRE activation, displaying pEC_{50} values of 6.5 ± 0.1 ($\alpha = 1$, $n = 3$) and 6.4 ± 0.1 ($\alpha = 1$, $n = 4$) respectively. Furthermore, agmatine equipotently and partially activates the mouse and rat H_4 receptors with a pEC50 value of 4.6 ± 0.1.

To understand the action of the various H_4 receptor agonists in *in vivo* rodent models we have also tested histamine, VUF 8430 and 4-methylhistamine at the four different rat histamine receptors (Table 2). As expected, both 4-methylhistamine and VUF 8430 do not show significant affinity ($pK_i < 4$) for the rat H_1 receptor and each display a similar affinity for the rat H_2 receptor compared with histamine (Table 2). As observed for the human H_3 receptor, VUF 8430 binds with higher affinity to rat H_3 receptors, compared with 4-methylhistamine, whereas both H_4 receptor agonists show a similar affinity for rat H_4 receptors (Table 2). In the same set of experiments, we also tested the H_4 receptor antagonists thioperamide, JNJ 7777120 (Jablonowski *et al.*, 2003) and VUF 6002 (Terzioglu *et al.*, 2004; Venable *et al.*, 2005) at the four rat histamine receptors. As observed previously for the human histamine receptors (Lim *et al.*, 2005), thioperamide was slightly more active at the rat H_3 receptor ($pK_i = 7.8$) compared with the rat H_4 receptor ($pK_i = 7.2$), whereas JNJ 7777120 and VUF 6002 showed a good selectivity (>100 fold) for the rat H_4 receptor (Table 2).

In vivo *evaluation of VUF 8430 as a pharmacological tool for studies of H_4 receptors*

In view of the difference in agonist activity at H_2 receptors, of 4-methylhistamine and VUF 8430 (Figure 2D) we compared

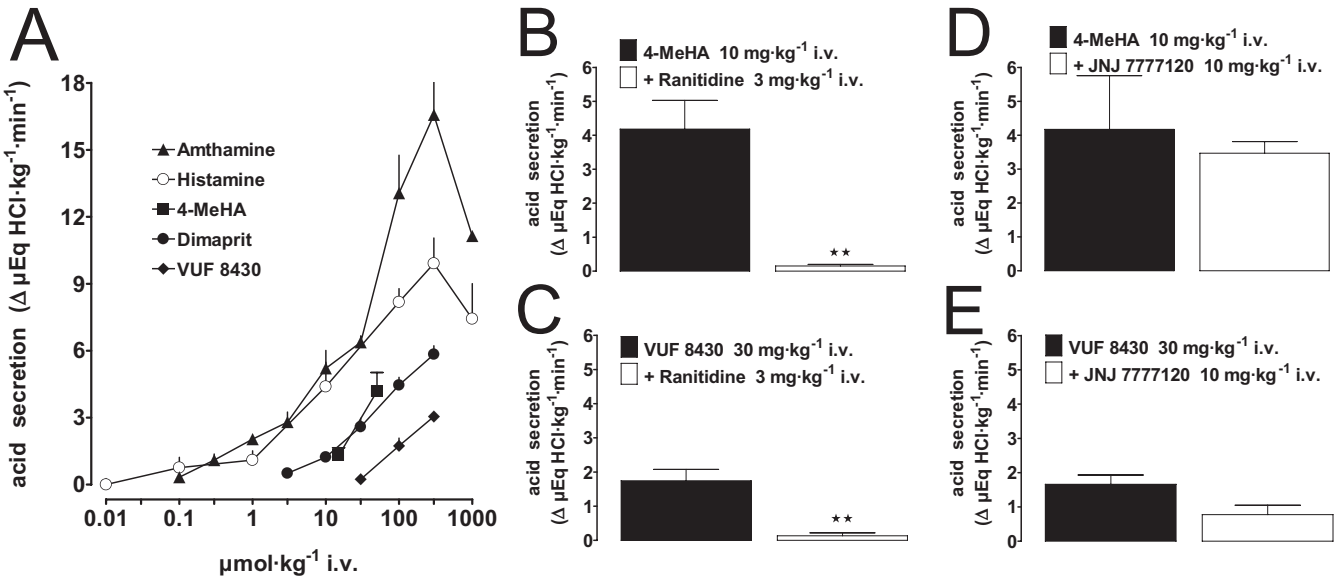


Figure 4 Analysis of gastric acid secretion in anaesthetized rats. The histamine H_2 receptor agonists amthamine and 4-methylhistamine (4-MeHA) induced acid secretion, but VUF 8430 only showed minimal effects at high doses (A). The effects of 4-methylhistamine (30 mg·kg^{-1} or 93 µmole·kg^{-1} i.v.) (B) or VUF 8430 (30 mg·kg^{-1} or 50 µmole·kg^{-1} i.v.) (C) were inhibited by ranitidine, but no significant inhibition by JNJ 7777120 (D and E) was observed. Values represent the mean ± SEM of responses in 6–8 animals for each experimental group. Comparisons between two groups were made by using the Student's *t*-test for unpaired data. $^{**}P < 0.01$ versus 4-methylhistamine (B) or VUF 8430 (C).

VUF 8430 and 4-methylhistamine for their potential to stimulate H_2 receptor function *in vivo*. We therefore measured gastric acid secretion in unconscious rats after application of histamine, the H_2 receptor agonists dimaprit and amthamine, VUF 8430, or 4-methylhistamine. Both histamine and amthamine are potent inducers *in vivo* of gastric acid secretion in the rat (Figure 4A). Moreover, dimaprit and 4-methylhistamine also induce gastric acid secretion, although higher doses were needed, whereas VUF 8430 only marginally induced gastric acid secretion at the highest tested doses (Figure 4A). These data are in good accordance with our *in vitro* findings in transfected cells (Figure 2D). Gastric acid secretion induced by VUF 8430 (30 mg·kg^{-1} or 50 µmole·kg^{-1}, i.v.) and 4-methylhistamine (10 mg·kg^{-1} or 93 µmole·kg^{-1}, i.v.) was suppressed by administration of the selective H_2 receptor antagonist ranitidine (3 mg·kg^{-1}, i.v.) (Figure 4B). The H_4 receptor antagonist JNJ 7777120 was not able to reduce 4-methylhistamine or VUF 8430-induced gastric acid secretion (Figure 4B).

Ex vivo *evaluation of VUF 8430*

We also evaluated the H_4 receptor-mediated effects of VUF 8430 in an *ex vivo* assay of migration of human MoDCs. The H_4 receptor has been shown to be expressed on the MoDCs and plays a role in histamine-induced chemotaxis (Gutzmer *et al.*, 2005). Histamine-induced migration of MoDCs as efficaciously as the chemokine, CCL2 and this effect was also mimicked by H_4 receptor agonists 4-methylhistamine and VUF 8430 (Figure 5).

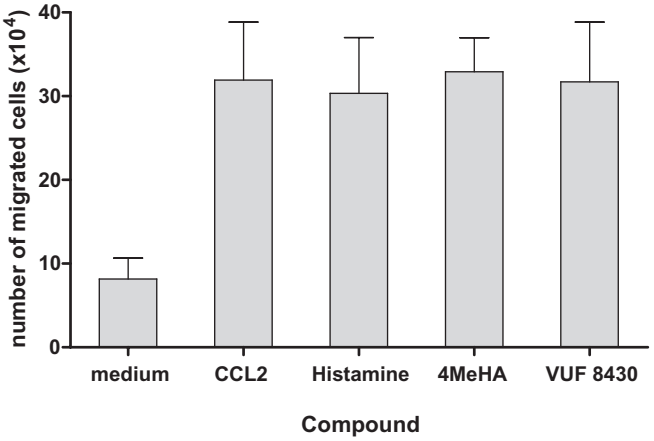


Figure 5 Chemotaxis of human monocyte-derived dendritic cells (MoDCs). MoDCs were loaded in upper wells of migration chambers and allowed to migrate towards chemotactic agents in the lower well through polycarbonate membrane with 5-µm pore diameter. The positive control (0.8 nmol·L^{-1} CCL2) and the tested compounds [histamine, 4-methylhistamine (4-MeHA), and VUF 8430, all at 10 µmol·L^{-1}] were diluted in cell culture medium as described in *Methods*.

Discussion and conclusions

In this study, we evaluated VUF 8430 as a pharmacological tool for the study of histamine H_4 receptors, using *in vitro, ex vivo and in vivo* models. VUF 8430 was originally synthesized in a receptor programme looking for close analogues of the H_2 receptor agonist dimaprit (Durant *et al.*, 1977). However, with the discovery of new histamine receptor subtypes (H_3 and H_4 receptors), we have recently tested a large number of histamine receptor ligands for their activity at the human H_4 receptor (Lim *et al.*, 2005). This detailed evaluation resulted in the discovery of the moderately potent H_2 receptor agonist, 4-methylhistamine, as a potent H_4 receptor agonist (Lim *et al.*, 2005). Moreover, we also observed that dimaprit

showed reasonable H_4 receptor agonist activity (Lim *et al.*, 2005) and subsequently described a new efficient synthetic pathway and an initial SAR of the related VUF 8430 (Lim *et al.*, 2006a). This dimaprit derivative shows high affinity at the human H_4 receptor and acts as a full agonist (Lim *et al.*, 2006a; this study). As shown in this study, both 4-methylhistamine and VUF 8430 also act as H_4 receptor agonists at endogenously expressed H_4 receptors in MoDCs. Expression of H_4 receptors was detected in MoDCs at both the mRNA and protein level and these receptors were involved in the activation of the transcription factor AP-1, the inhibition of IL-12p70 secretion and chemotaxis (Gutzmer *et al.*, 2005). We found that both VUF 8430 and 4-methylhistamine induced chemotaxis of MoDCs as effectively as histamine or the chemokine CCL2.

The discovery of VUF 8430 as an H_4 receptor agonist led us to investigate whether agmatine is an H_4 receptor ligand as well. Agmatine is a metabolite of L-arginine, occurs naturally in the body and has been considered to act as an important chemical messenger at other sites such as imidazoline receptors and α_2-adrenoceptors (Li *et al.*, 1994; Reis and Regunathan, 2000; Raasch *et al.*, 2001). In our study, we showed that replacement of the isothiourea group of VUF 8430 with an amine function, as in agmatine, resulted in a sharp drop in binding affinity for H_4 receptors. Nevertheless, in contrast to its precursor, L-arginine, agmatine binds to the H_4 receptor with a K_i value of 2 $\mu mol \cdot L^{-1}$. In the reporter gene assay, agmatine showed partial agonist activity at the human H_4 receptor and full agonism at human H_3 receptors. Micromolar concentrations of agmatine are considered to have physiological importance (Lortie *et al.*, 1996; Raasch *et al.*, 2001) and are found in the brain, kidney and other tissues (Li *et al.*, 1994; Lortie *et al.*, 1996; 2004). Hence, agmatine may be one of the endogenous ligands for the H_3 and H_4 receptor. These data also show the importance of the isothiourea group of VUF 8430 in the binding to H_4 receptors. Previously, we reported that the isothiourea group most likely forms hydrogen bonds with residues E5.46 (1.82 Å) and S6.52 (2.21 Å) in the receptor protein (Jongejan *et al.*, 2008). The resulting H-bonding network is identical to the pattern observed for the imidazole ring of histamine, and an essential structural feature for the binding of histamine to the H_4 receptor (Lim *et al.*, 2005).

Besides agmatine, we also observed that other polyamines bind to H_4 receptors; putrescine, cadaverine, spermidine and spermine all showed affinity for H_4 receptors. Cadaverine is commonly found in microorganisms, while putrescine, spermidine and spermine are metabolites of arginine endogenously found in the human body. Like agmatine, concentrations of the polyamines are tightly controlled and they increase under certain conditions, such as liver regeneration, sepsis, brain ischaemia and acute excitotoxic brain damage (Anehus *et al.*, 1986; Gilad *et al.*, 1996; Noguchi *et al.*, 1996; Vivo *et al.*, 2002). The relevance of this action of polyamines on H_4 receptor remains to be elucidated.

A detailed *in vitro* comparison of the actions of VUF 8430, agmatine and the earlier identified H_4 receptor agonist 4-methylhistamine, indicate that all three compounds, like histamine (Liu *et al.*, 2001b), show a somewhat reduced affinity at the rat and mouse H_4 receptors compared with the human H_4 receptor. In radioligand binding and functional studies, VUF 8430 also shows reasonable affinity and full agonist efficacy at the related H_3 receptors. Similarly, agmatine acts as an efficacious agonist at the H_3 receptor, whereas 4-methylhistamine only shows some agonistic action at H_3 receptors, at high concentrations. Both VUF 8430 and agmatine are very weakly active at the H_2 receptor and inactive at the H_1 receptor. In contrast, despite the relatively low affinity of 4-methylhistamine at the H_2 receptor, 4-methylhistamine is as active as histamine at this receptor. Interestingly, the differential effects of 4-methylhistamine and VUF 8430 at the H_2 receptor were also observed *in vivo*. In anaesthetized rats, we observed that 4-methylhistamine was effective in stimulating gastric acid secretion *in vivo*, thus confirming a considerable activity at the H_2 receptor (Durant *et al.*, 1975). Compared with 4-methylhistamine, VUF 8430 is considerably less potent and less efficacious in triggering gastric acid secretion in the rat. Only at very high dosages, VUF 8430 stimulated gastric acid secretion and then only to a very small extent. The stimulatory effect of both H_4 receptor agonists was effectively blocked by the H_2 receptor inverse agonist ranitidine, but not by the H_4 receptor antagonist JNJ 7777120. Based on these data, we conclude that 4-methylhistamine *in vivo* shows considerable H_2 receptor agonistic activities at dosages >3 $mg \cdot kg^{-1}$ i.v. For VUF 8430 only at considerable higher dosages (30 $mg \cdot kg^{-1}$ i.v.) some minor H_2 receptor-mediated effects were observed *in vivo*.

In conclusion, our search for new ligands for the histamine H_4 receptor has resulted in the discovery of the potent H_4 receptor agonist VUF 8430, which shows a pharmacological profile clearly different from that of the other high affinity H_4 receptor agonist, 4-methylhistamine. Whereas 4-methylhistamine still showed considerable H_2 receptor agonistic activity (*in vitro* and *in vivo*), but was devoid of H_1 receptor activity and only weakly activated the H_3 receptor, VUF 8430 on the other hand showed a limited selectivity towards the H_3 receptor, but was very selective with respect to the H_1 or H_2 receptors. Consequently, both high affinity H_4 receptor agonists are complementary and together with the available selective H_4 receptor antagonists can serve as pharmacological tools in future studies to validate the H_4 receptor as a new drug target. Furthermore, identification of VUF 8430 also led to the discovery of agmatine as potential endogenous H_4 receptor agonist. The physiological relevance of agmatine and the polyamines as histamine H_4 receptor ligands remains to be elucidated.

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Conflict of interest

None.

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