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ORIGINAL RESEARCH

Pharmacological characterization of oxime agonists of the histamine H_4 receptor

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Abstract: The histamine H_4 receptor (H_4R) has generated excitement as a potential target for the development of novel anti-inflammatory therapies. However, many of its physiological functions are still being uncovered and the development of new pharmacological tools is crucial to help facilitate this work. Previously, indole and benzimidazole piperazines have been described as potent and selective H_4R antagonists. Using this as a starting point we have identified new indole and benzimidazole oxime piperidines as ligands for the H_4R. These compounds have a high affinity for the human H_4R with K_i values ranging from 17–53 nM. They also have high to moderate affinity for the H_4R from mouse, rat, guinea pig, and monkey, but poor affinity for the dog homologue. In addition to the high affinity for the H_4R, these compounds also exhibit excellent selectivity against other histamine receptors as well as many other receptor targets. These oxime ligands act as agonists of the human H_4R in transfected reporter systems, although the degree of agonism depends on the system utilized. Agonistic activity was also observed in human eosinophils as evidenced by their ability to induce a shape change in these cells, although the degree of agonism ranges from full agonist to partial agonist depending on the test conditions. In contrast to their activity at the human H_4R, all of the oxime compounds act as full agonists at the mouse receptor regardless of the test system including the ability to induce a calcium response in mouse bone marrow-derived mast cells. Finally the most selective compound, JNJ 28610244, was shown to induce scratching in mice indicating that it can also function as an agonist *in vivo*.

Keywords: H_4 receptor, eosinophils, mast cells, pruritus

Introduction

Histamine is a biogenic amine that functions as a messenger for many physiological and pathological activities such as allergic inflammation, gastric acid secretion, and neurotransmission. Histamine exerts its effect through a family of histamine receptors which includes four members.[1] The four histamine receptors all belong to the G-protein coupled receptor family and activate specific G-proteins. The effects of histamine have been extensively studied. The H_1 receptor (H_1R) primarily mediates allergic responses. Antihistamines specifically targeting the H_1R have been widely used to treat allergic disorders such as seasonal or perennial allergic rhinitis and chronic urticaria. The H_2 receptor (H_2R) enhances gastric acid secretion in the stomach and H_2R antagonists have been used in the treatment of dyspepsia. The H_3 receptor (H_3R) regulates the release of histamine and neurotransmitters from the neurons. The newest member of the histamine receptor family, the H_4 receptor (H_4R), has been implicated in inflammatory and pruritic responses and data indicate that the receptor may be crucial to understanding

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allergic and inflammatory disorders such as atopic dermatitis, where traditional H_1R antagonists are commonly prescribed but are generally considered ineffective.

Despite all of the promising early work, many of the mechanisms mediated by the H_4R are still unclear. One of the key elements for helping to understand the role of the H_4R is to have selective ligands that target the receptor. Such ligands are, of course, potentially useful as therapeutics as seen with the highly successful H_1R- and H_2R-targeted antihistamines. Meanwhile, these ligands are also very useful as research tools for investigating H_4R function both *in vitro* and *in vivo*. Here we describe oxime-containing agonists of the H_4R that have excellent potency and selectivity for the H_4R and thus serve as excellent pharmacological tools for investigating the receptor function.

Materials and methods

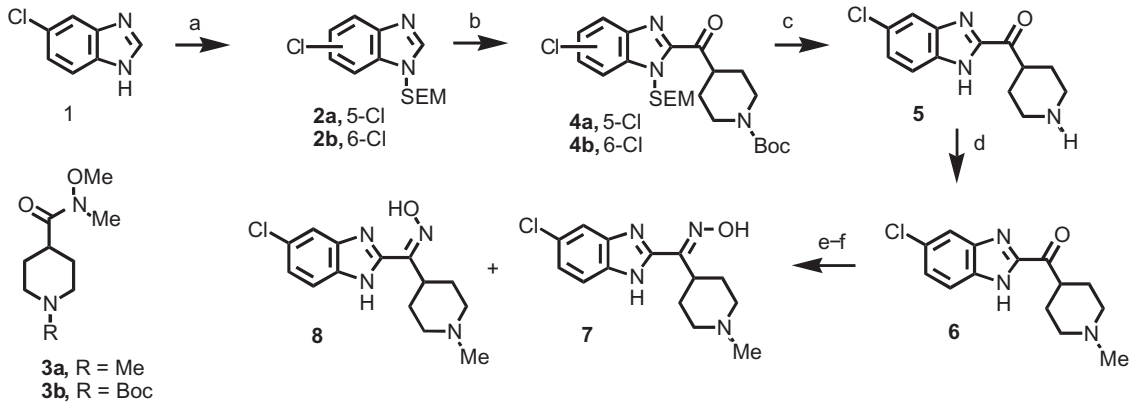
Chemistry

The targeted Z-regioisomeric oxime compounds were synthesized as outlined in Schemes 1 and 2. The commercially available 5-chlorobenzimidazole **1** was protected as its 2-trimethylsilylethoxymethyl derivative[2] which provided a 1:1 mixture of N1 and N3 regioisomers **2a**, **b**. Metalation at the 2-position of the benzimidazole ring using *n*-butyllithium in tetrahydrofuran at low temperature followed by trapping with the known Weinreb amide[3–5] (**3b**) afforded the desired keto-adduct **4a**, **b**. Removal of both Boc protecting groups under acidic conditions provided the unmasked amine **5**. Treatment of **5** under Eschweiler–Clarke conditions[6,7] afforded the N-methylated piperidine **6**. Subjecting **6** to hydroxylamine hydrochloride in pyridine produced a 1:1 mixture of E- and Z-oxime regioisomers (**7** and **8**, respectively) that were inseparable under normal chromatography conditions. Separation of the oxime regioisomers was accomplished using a chiral chromatography column under supercritical fluid conditions (SFC).[8] Under these chromatography conditions, the E-oxime was always eluted first from the column followed by the Z–oxime. The geometry of the oximes was confirmed by nuclear magnetic resonance spectroscopy[9] and by X-ray analysis.

The preparation of the indole analogs was carried out as illustrated in Scheme 2. The commercially available 5-substituted indoles **9a–c** were protected as their sulfonamide derivatives[10] to afford the corresponding intermediates **10a–c** in high yield. Subsequent metalation at the C-2 position of indoles **10a–c** was done in similar fashion to that as described for the benzimidazoles giving rise to the keto-adducts **11a–c** after trapping with the Weinreb amide **3a**.[3] The phenylsulfone moiety was removed under basic conditions and the resulting product was treated with hydroxylamine hydrochloride as noted above to provide a 1:1 mixture of E- and Z-regioiomeric oximes **13a–c** and **14a–c** respectively. The regioisomers were separated via SFC conditions using the same conditions as for the benzimidazoles.

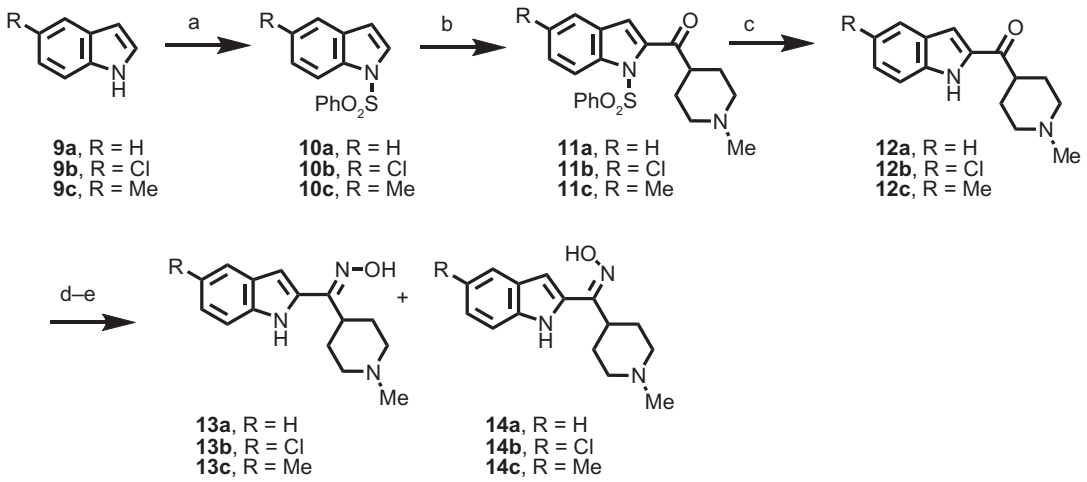
Mice

H_4R-deficient mice were generated as previously described [11] and crossed on to BALB/c background for at least ten generations. Wild type BALB/c mice were obtained from Charles River Laboratories (Wilmington, MA). Age-matched animals were used in all experiments. Mice were housed in community cages on a 12-hour light cycle and fed mouse chow and water ad libitum. All procedures were performed according to the internationally accepted guidelines for the care and use



Scheme 1 Preparation of the benzimidazole oxime.[a]

[a]Conditions and reagents: **a**) NaH (60% in mineral oil), DMF, SEM-CL, 0 °C → room temp, 3.5 h, 92%. **b**) *n*-BuLi (2.5 M in hexanes), THF, –78 °C, 2 h, then add the Weinerb amide **3b**), 2 h at room temp, 62%, (**c**) 6N HCl, EtOH, 80 °C, 3 h, 79%, **d**) Formic Acid, formaldehyde 37%, 70 °C, 8 h, 28%–64%, (**e**) NH_2OH-HCl, pyridine, 60 °C, 4.5 h, 89%, **f**) Separate the E/Z-oxime regioisomers; Chiracel AD column, SFC Conditions, IPA/CO_2.



Scheme 2 Preparation of the indole oxime.[b]

[b]Conditions and Reagents: **a**) *n*-BuLi (2.5 M in hexanes), THF, –78 °C, 30 min, then, $PhSO_2Cl$, 1 h, raise to room temp 2 h, 95%, **b**) Lithium diisopropylamide, THF, –78 °C, 30 min, then add the Weinreb amide **3a**), 2 h at room temp, 42%, **c**) KOH, MeOH, 50 °C, 2 h, 89%, **d**) NH_2OH-HCl, pyridine, 60 °C, 16 h, **e**) Separate the E/Z-oxime regioisomers; Chiracel AD column, SFC conditions, IPA/CO_2.

of laboratory animals in research and were approved by the local Institutional Animal Care and Use Committee.

Materials

JNJ 7777120 was synthesized as previously described.[12] The stably transfected SK-N-MC cells were generated as previously described.[13–16] SK-N-MC cells stably transfected with the mouse H_1R were generated as previously described for the human H_1R cell line.[13] All of the radiolabeled ligands were purchased from PerkinElmer Life Sciences (Boston, MA).

Binding assays

Binding assays were conducted using membranes from cells transfected with the receptor of interest except for the mouse H_3R where mouse brain homogenates were used. Binding assays for the human, mouse and rat H_4R, the human H_1R and the human H_3R were carried out as previously described.[13] Binding assays for the monkey and dog H_4R were conducted as previously described.[14] The guinea pig H_4R binding assay utilized SK-N-MC cells as previously described.[16] Binding to the human H_2R was carried out according to the method previously described.[17] Binding to the mouse H_1R was determined in membranes from SK-N-MC cells stably transfected with the mouse H_1R. Cell pellets were homogenized in 50 mM Tris pH 7.5 containing 5 mM EDTA, and supernatants from an 800 g spin were collected and recentrifuged at 30,000 g for 30 min. Pellets were rehomogenized in 50 mM Tris pH 7.5 containing 5 mM EDTA. The resulted cell membranes were incubated with 6 nM [^{3}H]pyrilamine (K_d = 3 nM) for 45 minutes at 25 °C. Nonspecific binding was defined using 100 μM unlabeled triprolidine. Binding to the mouse H_3R was determined using mouse brain homogenates as previously described for the rat H_3R.[13] For all studies, the K_i values were calculated based on an experimentally determined appropriate K_d value according to the Cheng and Prusoff equation.[18] All K_i results are given as the average of at least triplicate determinations.

Cell-based functional assays

The cyclic adenosine monophosphate (cAMP) assay for the human and mouse H_4R was carried out in SK-N-MC cell lines that express β-galactosidase under the control of cAMP-responsive elements and were carried out as previously described.[13] The luciferase assays for all species were carried out as previously described for the human and dog H_4R.[14] The data from each concentration-response curve for either reporter assays were fitted to a sigmoidal dose-response curve to obtain half-maximum effective concentration (EC_{50}) values using Prism (GraphPad Software, San Diego, CA) and the values are given as the average EC_{50} of at least triplicate determinations.

Selectivity panel

A panel of 50 different biogenic amine receptors, neuropeptide receptors, ion channel binding sites, and neurotransmitter transporter binding assays were carried out as previously described.[13] The assays were run at 1 μM of the H_4R agonist compounds, and the percentage of inhibition is given as the average of triplicate determinations.

Measurement of eosinophil shape change using flow cytometry

The human polymorphonuclear leukocytes (PMNL) were prepared and the gated autofluorescence/forward scatter assay (GAFS) was conducted as previously described.[19] Briefly, human PMNL were resuspended in assay buffer (phosphate-buffered saline [PBS] containing 10 mM Ca^{2+} and Mg^{2+}, 10 mM HEPES, 10 mM glucose and 0.1% bovine serum albumin [BSA], pH 7.2–7.4). Aliquots of cells (5×10^5 PMNL) were incubated with ligands in 1.2-mL polypropylene cluster tubes (Costar, Cambridge, MA) in a final volume of 100 µL. The tubes were placed in a 37 °C water bath for 10 minutes, after which they were transferred to an ice-water bath, and 250 µL of ice-cold fixative (2% paraformaldehyde in PBS) was added to terminate the reaction and to maintain the cell shape change. The cell shape change was analyzed with the flow cytometer (Becton Dickinson, Mountain View, CA). Eosinophils in PMNL were gated based on their high autoflourescence relative to that of neutrophils. Cell shape change was monitored in forward scatter signals.

Mouse bone marrow-derived mast cell assays

Mast cells were differentiated from bone marrow derived from BALB/c mice as previously described.[11,13] The calcium mobilization assay was run using the FLIPR Calcium 4 Assay Kit (Molecular Devices, Sunnyvale, CA). The assay buffer was Dulbecco's modified Eagle's medium (DMEM)/F12 medium without phenol red which contains 0.5% BSA. Mast cells were suspended to 2×10^6/mL in assay buffer and mixed with calcium 4 media prepared in assay buffer. Probenecid (2.5 mM) and pluronic acid (0.08%) were added to the cell suspension and the cells were plated in the biocoat poly-D-Lysine black plate (Becton Dickinson, Franklin Lakes, NJ) and incubated for 60 minures at 37 °C. Ligands were added to the cells right before the calcium measurements. The fluorescence intensity was calculated as the maximum minus the minimum fluorescence over a two-minute period. All data points are given as the average of triplicate measurements.

Induction and assessment of itch responses

JNJ 28610244 (**2c**) was diluted from 10 mM DMSO stock solution into PBS. JNJ 7777120 was made up in a vehicle of 20% (w/v) hydroxypropyl-β-cyclodextran/water. There were five mice per group. The itch response induction was conducted as previously described.[20] Briefly, the hair of the mice was clipped over the rostral part of the back 24 hours before intradermal injection of 100 nmol JNJ 28610244. Where noted, animals received a 30 mg/kg oral dose of JNJ 7777120 20 min before injection of JNJ 28610244 or PBS intradermally. Immediately after intradermal injection, the animals were returned to an acrylic cage (approximately 15 cm diameter × 30 cm high), to which they had been acclimated for at least one hour before the experiment, for observation of itch responses. Itch responses were measured as previously described.[20]

Results

Following the discovery of the potent and selective H_4R antagonists JNJ 7777120 and JNJ 10191584 (Figure 1), we subsequently observed that replacing the piperizine ring in either of these compounds with a piperidine ring afforded a moderately potent H_4R antagonist when the benzimidazole core was retained, but lost H_4R-binding affinity with the indole core (data not shown). In addition, we found that conversion of the carbonyl unit into an oxime moiety provided compounds that possessed potent and unexpected H_4R-agonist activity. Moreover, the H_4R-agonist activity resided only in the Z-geometric isomer of the oxime as the E-geometric isomer was essentially devoid of H_4R activity. Motivated by the potential value of specific H_4R agonists in the investigation of H_4R biological functions, we synthesized a series of indole and benzimidazole oxime analogs. Four of these compounds (**8**, **14a**, **14b**, and **14c**) were selected for further characterization.

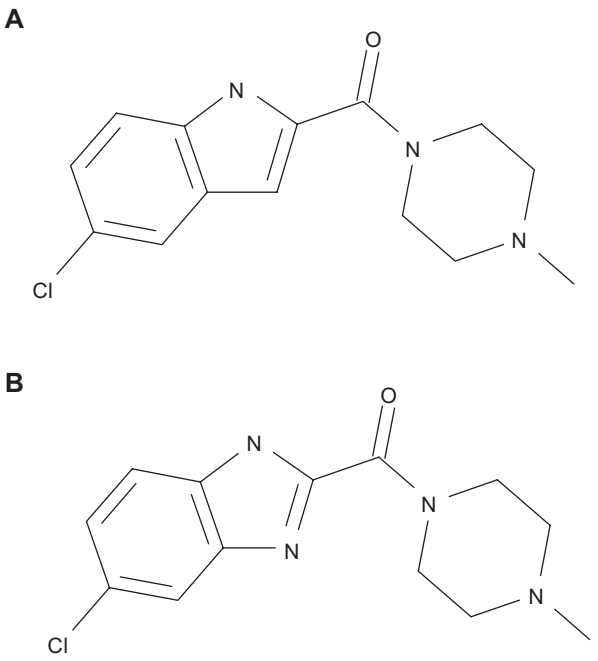


Figure 1 Chemical structures of JNJ 7777120 **A**) and JNJ 10191584 **B**).

The binding affinity of these compounds was determined using cells expressing the H_4R from different species. As shown in Table 1, all four compounds had high affinity for the human H_4R and good selectivity over other histamine receptors. These compounds also demonstrated high binding affinity for the H_4R homologues from different species, although their binding to the dog H_4R was very weak. These compounds also showed good selectivity against the mouse histamine H_1R and H_3R. For comparison the K_i values for histamine, the H_4R antagonists JNJ 7777120 and JNJ 10191584 are shown.

In order to further explore the selectivity of these compounds, they were tested in radioligand-binding assays against a panel of other molecular targets including GPCRs, ion channels and transporters. All compounds were tested at 1 μM concentration, which is at least 20-fold higher than their affinities for the human H_4R. Using 50% inhibition as a cut-off for selectivity, compound **14c** was selective against all of the targets tested, compound **14b** only cross-reacted with the 5-HT2b receptor, compound **8** with the muscarinic M1 and M3 receptors and compound **14a** with the muscarinic M3, 5-HT2a and 5-HT2b receptors (Table 2 and supplementary Table S1). This result demonstrated the excellent specificity of these compounds for the H_4R with **14c** being the most selective.

The functional agonist activity of the oxime compounds was determined using a cell-based cAMP reporter assay. Histamine inhibits forskolin-induced cAMP production in a dose-dependent manner in SK-N-MC cells expressing the H_4R. As for histamine, the oxime compounds all act as agonists at both the human and mouse H_4R. Histamine, compound **8** and compound **14b** all have similar potency at the human H_4R, but the latter two compounds are only partial agonists as suggested by an α value of less than 1 (Figure 2a and Table 3). Compound **14c** is less potent, but is as efficacious as histamine in this assay (ie, it is a full agonist as suggested by an α value of around 1). At the mouse H_4R compounds **8** and **14b** are both more potent than histamine, whereas compound **14c** and histamine have about the same potency (Figure 2b and Table 3). In this case all three compounds act as full agonists at the mouse H_4R.

To further explore the agonist potential of these compounds, a second transfection system was used consisting of HEK-293 cells expressing the H_4R, a chimeric G_{qi} protein and an SRE-luciferase reporter construct. Under these conditions, H_4R signaling was directed to the G_q pathway by the chimeric G_{qi} protein and activation of the H_4R by an agonist causes an increase in luciferase levels. Consistent with the cAMP system, the oxime analogues all showed agonist activity with the luciferase system following the same rank order of potency (Table 3). These compounds were all partial agonists at the human H_4R in this system, but were still full agonists at the mouse H_4R (Table 3). The agonist potential was also explored at the rat and monkey H_4R. All three compounds were found to be full agonists at the rat receptor, however only compound **8** displayed full agonist activity at the monkey receptor (Table 3).

Histamine is known to induce a rapid and transient eosinophil shape change that can be detected by the GAFS assay that is mediated by the H_4R[19] and, therefore, the oxime agonists presented here were tested for agonistic activity in human eosinophils. The results showed that all three compounds were able to induce eosinophil shape change. In this assay histamine was the most potent agonist followed by compounds **8**, **14b** and then **14c** (Figure 3). Both the potency and the

Table 1 *In vitro* K_i (nM ± SEM) for histamine receptor binding

	Species	Histamine[27]	7777120	10191584	Cmpd 8	Cmpd 14a	Cmpd 14b	Cmpd 14c
H_4	Human	13 ± 0.2	5 ± 0.1[27]	32 ± 0.4[27]	17 ± 8	49 ± 2	31 ± 1	53 ± 14
	Mouse	79 ± 1.1	4 ± 0.5[27]	126 ± 2[27]	8 ± 4	105 ± 35	8 ± 1	21 ± 3
	Rat	70 ± 1	4 ± 0.5[27]	50 ± 0.7[27]	16 ± 3	345 ± 86	20 ± 6	184 ± 23
	Monkey	16 ± 0.2	32 ± 1[27]	200 ± 3[27]	60 ± 7	40 ± 6	25 ± 2	189 ± 13
	Dog	63 ± 0.9	79 ± 1[27]	631 ± 10[27]	1228 ± 256	5464 ± 2577	8253 ± 1687	10146 ± 1398
	Guinea Pig	10 ± 0.1	1000 ± 17[27]	1585 ± 27[27]	123 ± 19	485 ± 33	46 ± 7	317 ± 85
H_1	Human	ND	>10,000[13]	>10,000	>1000	>10,000	>10,000	>10,000
	Mouse	ND	>10,000[13]	>10,000	ND	>10,000	>10,000	>10,000
H_2	Human	ND	>30,000[13]	ND	>1000	>1000	>1000	>1000
H_3	Human	ND	5125 ± 1087[13]	>10,000[30]	>10000	2691 ± 717	>10000	>10000
	Mouse	ND	10,000	ND	491 ± 88	1097 ± 153	3601 ± 194	2657 ± 237

Abbreviations: ND, not determined; SEM, standard error of mean.

Table 2 Selectivity of oxime agonists

Receptor	Compound			
	8	**14a**	**14b**	**14c**
M1	73	28	36	15
M3	82	54	47	23
5-HT2a	42	74	19	35
5-HT2b	ND	74	63	ND

Note: % inhibition at 1 μM.
Abbreviation: ND, not determined.

efficacy of the compounds appeared to vary depending upon the donor used. In one donor (Figure 3A) histamine was very potent with an EC_{50} of 95 nM and all three compounds appear to be full agonists. However, in a second donor (Figure 3B) histamine was less potent (EC_{50} = 400 nM) and in this case all of the compounds displayed partial agonism. The efficacy of the compounds may be related to the expression levels of the H_4R on the cell surface, which is also reflected in the potency of histamine.

Mouse bone marrow-derived mast cells were used to determine if the compounds were agonists in mouse primary cells. It has previously been described that histamine acting via the H_4R can induce a calcium influx in these cells.[11] As for histamine, the three oxime compounds are all able to induce a calcium signal in these cells in a dose-dependent manner (Figure 4A). Compounds **14b** (EC_{50} = 49 ± 18 nM) and **8** (EC_{50} = 33 ± 2 nM) were more potent than histamine (EC_{50} = 314 ± 41 nM), whereas compound **14c** (EC_{50} = 430 ± 49 nM) had a potency similar to histamine. The calcium response induced by compound **14c** is solely due to activation of the H_4R since it is absent in cells from H_4R-deficient mice (Figure 4B). As for the other mouse functional assays, all of the compounds exhibited full agonist activity for inducing a calcium response.

In addition to being excellent research tools for the investigation of histamine and histamine receptors *in vitro*, potent and specific H_4R agonists can greatly facilitate *in vivo* studies. For example, it has been shown that the H_4R can mediate pruritus in mice.[20,21] Indeed both histamine and 4-methylhistamine can induce scratching when injected into the skin of wild-type mice, but not in H_4R-deficient mice.[20] Compound **14c** (JNJ 28610244) was tested for its ability to induce scratching in mice since it was the most selective of the agonists presented here. Intradermal injection of JNJ 28610244 induced scratching in wild-type mice, but not in H_4R-deficient mice (Figure 5A). In addition the scratching induced by JNJ 28610244 could be reduced by oral administration of the selective H_4R antagonist JNJ 7777120 (Figure 5B). These results indicate that JNJ 28610244 (**14c**) can act as an H_4R-selective agonist *in vivo* in the mouse.

Discussion

The histamine H_4 receptor is the latest histamine receptor to be described and it has been implicated in various immune responses.[22] Most of these studies have relied on the specific H_4R antagonist JNJ 7777120 and H_4R-deficient mice. The most useful tools for deciphering receptor function are selective agonist and antagonist pairs. Since the selectivity of any ligand is very difficult to know completely, the combination of an agonist and an antagonist often provides the most confidence that an observed effect can be attributed to

Table 3 *In vitro* EC_{50} (nM ± SEM) for histamine receptor activity

Reporter	Species	Value	Histamine	Cmpd 8	Cmpd 14b	Cmpd 14c
cAMP	Human	EC_{50} (nM)	42 ± 4	50 ± 2	26 ± 8	515 ± 46
		α		0.70 ± 0.01	0.46 ± 0.02	1.00 ± 0.02
	Mouse	EC_{50} (nM)	337 ± 20	9.0 ± 0.1	2.0 ± 0.1	174 ± 63
		α		1.00 ± 0.01	0.84 ± 0.01	1.08 ± 0.01
Luciferase	Human	EC_{50} (nM)	77 ± 9	73 ± 20	37 ± 13	109 ± 14
		α		0.58 ± 0.03	0.67 ± 0.02	0.74 ± 0.05
	Mouse	EC_{50} (nM)	5316 ± 1201	69 ± 8	11 ± 4	180 ± 91
		α		1.02 ± 0.06	1.08 ± 0.12	1.16 ± 0.12
	Rat	EC_{50} (nM)	171 ± 14	182 ± 21	9 ± 3	477 ± 204
		α		0.86 ± 0.03	1.10 ± 0.16	0.86 ± 0.19
	Monkey	EC_{50} (nM)	48 ± 7	692 ± 382	139 ± 70	1393 ± 638
		α		1.39 ± 0.89	0.62 ± 0.09	0.77 ± 0.19

Abbreviations: cAMP, cyclic adenosine monophosphate; EC_{50}, half-maximum effective concentration; SEM, standard error of mean.

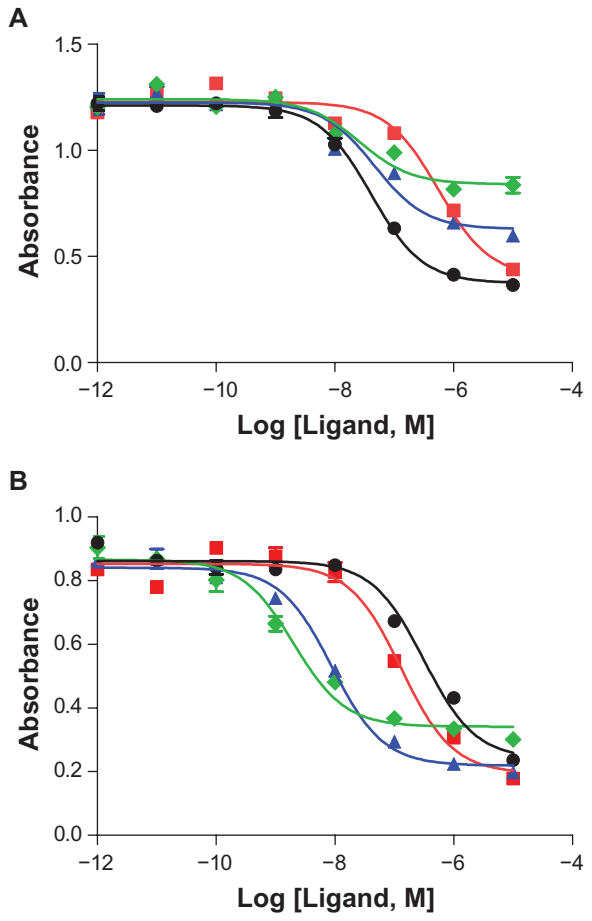


Figure 2 Agonist activity at the human and mouse receptor. Functional agonism of the human **A**) and mouse **B**) H_4R in the cAMP reporter system. Histamine (black circles), compound **14c** (red squares), compound **8** (blue triangles) and compound **14b** (green diamonds) were tested. The results show the average of triplicates and the error bars represent SEM.
Abbreviation: SEM, standard error of mean.

the receptor in question. That is, a response is most likely mediated by a particular receptor if an agonist that targets the receptor induces the response and this can be completely reversed by an antagonist. Today there are good antagonist tools for the H_4R with the most useful being JNJ 7777120, but this is not true for agonists. Histamine has been the most widely used agonist, but, of course, this biogenic amine has cross-reactivity with all four histamine receptors and perhaps other transporters as well.[23] A histamine analogue, 4-methylhistamine, has also been used extensively as a selective H_4R agonist.[24] This ligand has 100-fold selectivity for the H_4R compared to other histamine receptors, however, it does show H_2 receptor agonist activity *in vivo* and its potency at the H_2 receptor is similar to that of histamine.[24,25] Furthermore, other agonists of the H_4R have shown non-specific activity on T cells that may limit their usefulness.[26] Given the limitations of the existing agonists there is a need for highly selective well-characterized agonists of the H_4R for use as pharmacological tools.

JNJ 7777120 and JNJ 10191584 are structurally related analogs, the only difference being that the former contains an indole ring and the latter contains a benzimidazole ring system. This minor distinction, however, affords the indole analog JNJ 7777120 greater H_4R-binding affinity (ranging from 1.5-fold to 32-fold) in several animal species over the benzimidzole derivative JNJ 10191584. The presence of the piperizine ring in the above compounds allows the nitrogen atom adjacent to the carbonyl to participate in conjugation giving rise to a resonance species that contains a partial negative charge on the oxygen atom of the carbonyl and a partial positive charge on the amide nitrogen in the piperazine ring. In contrast, when the piperizine ring is replaced with a piperidine ring (ie, JNJ 7777120 and **12b**), the amide functionality is lost, and thus, conjugation with the carbonyl cannot occur. Interestingly, the piperidine indole derivative **12b**, was found to be devoid of human H_4R-binding affinity ($K_i > 10{,}000$ nM), whereas, the benzimidazole piperidine analog **6**, provided moderate human H_4R-binding affinity ($K_i = 81$ nM). The loss of hH_4R-binding affinity in **12b** is not completely explained by the loss of the amide functionality since compound **12c** still retains moderate H_4R-binding affinity ($K_i = 127$ nM).

The ketone functionality present in **6** and **12** could be transformed into their corresponding oxime derivatives as a mixture of E- and Z-geometric isomers (ie, **7** and **8**, and **13** and **14**, Schemes 1 and 2). Separation of the E- and Z-geometric isomers was accomplished by chiral high-pressure liquid chromatography. The Z-isomers in each case were more potent ligands for the human H_4R than the corresponding E-isomers. There are two possible hypotheses for this observation. First, the E-oximes are directed toward the piperidine ring. This orientation can perturb the piperidine ring into a conformation that is not optimal for binding to the receptor. In contrast, the Z-oximes are directed towards the heterocyclic ring (indole or benzimidazole) and do not invoke steric interactions with the piperidine ring, thus allowing the piperidine ring an opportunity to adopt the most preferred conformation for receptor binding. Alternatively, one can hypothesize that the orientation of the oxime itself is primarily responsible for the observed H_4R activity. That is, in the E-conformation the oxime may experience steric interactions with the receptor leading to poorer binding affinity. Whereas in the Z-conformation, the oxime may encounter desirable interactions with the receptor resulting in greater binding affinity. In either case, for the series of molecules that we examined, the Z-isomers always

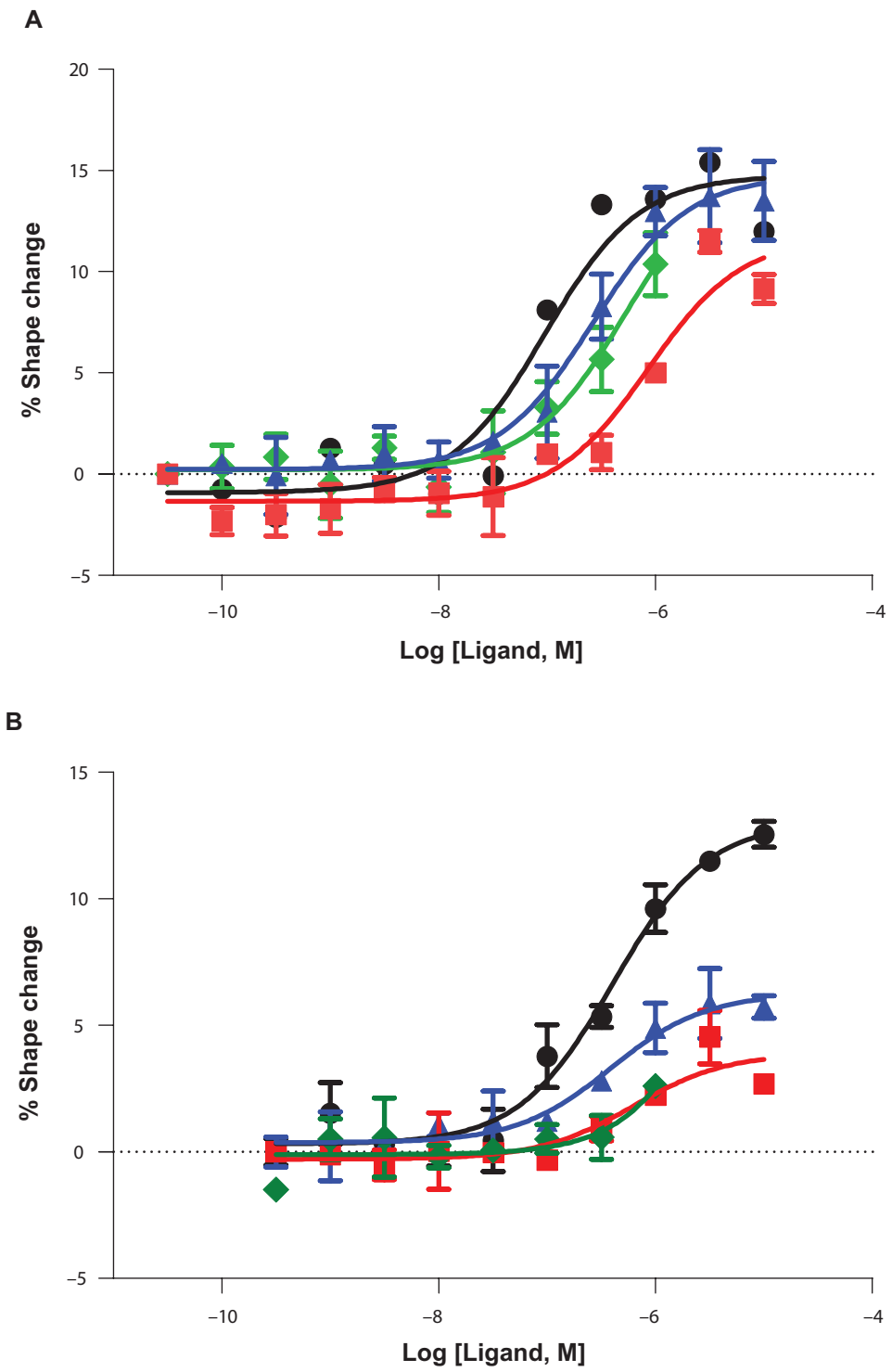


Figure 3 Eosinophil shape change. The H_4R agonists induce eosinophil shape change in two donors (**A** and **B**). Histamine (black circles), compound **14c** (red squares), compound **8** (blue triangles) and compound **14b** (green diamonds) were tested. The results show the average of triplicates and the error bars represent SEM.
Abbreviation: SEM, standard error of mean.

displayed higher binding affinities. Lastly, the data for the unsubstituted indole oxime (**14a**) versus the 5-methyl indole oxime (**14c**) showed greater variations in H_4R binding depending upon the species examined. However, comparing the 5-methyl indole (**14c**) with the 5-chloro indole (**14b**) shows that the selectivity trend for H_4R-binding affinity is similar with the 5-chloro analog being more potent in all species than the 5-methyl analog.

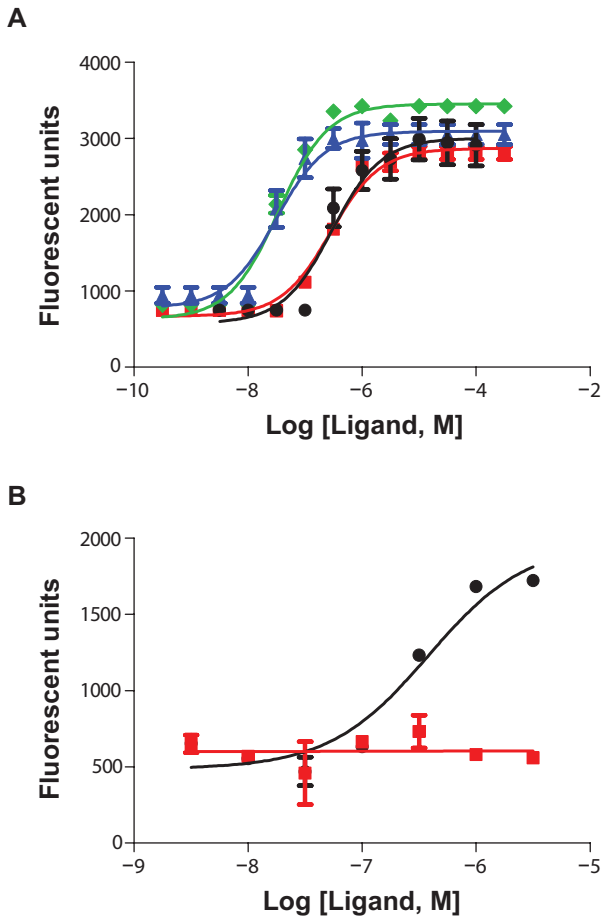


Figure 4 Calcium mobilization in mast cells. The H_4R agonists induce mouse bone marrow-derived mast cell (BMMC) calcium mobilization. **A**), Dose response of H_4R ligands for inducing calcium mobilization. Histamine (black circles), compound **14c** (red squares), compound **8** (blue triangles) and compound **14b** (green diamonds) were tested. **B**), Compound **14c** induced calcium mobilization in cells from wild-type (black circles) and H_4R-deficient (red squares) mice. The results show the average of triplicates and the error bars represent SEM.
Abbreviation: SEM, standard error of mean.

All of the oxime agonists that we have evaluated display excellent affinity for the human H_4R, although they have lower affinity than that of JNJ 7777120 and more closely match JNJ 10191584. They also have high affinity for the mouse H_4R, and for compounds **14b** and **8** the affinity is even higher than for the human homologue. Compounds **14b** and **8** also exhibit high affinity for the rat and monkey H_4R. Whereas compound **14a** exhibits high affinity for the monkey receptor, but lower affinity for rat receptor and compound **14c** has moderate affinity for both the rat and monkey receptors. As opposed to JNJ 7777120 and JNJ 10191584, all of the compounds described here have moderate to high affinity for the guinea pig H_4R, but little if any affinity for the dog H_4R. This variability in ligand affinity among the different species homologues of the H_4R has been described previously for other ligands and is thought to be related to the differences in sequence homology between the species.[14,25,27]

Three of the four compounds were followed-up with a detailed determination of their agonist potential. In transfected cell systems all three compounds were able to act as agonists of the human H_4R. In general the rank order of agonist potency matched the order of compound affinity as one might expect. Consequently, compounds **8** and **14b** displayed similar potencies and compound **14c** was found to be the least potent agonist. Additionally, the efficacy of the compounds was found to vary. For example, when cAMP was used as the reporter system, compound **14c** was as efficacious as histamine and therefore was designated as a full agonist. However, the other two compounds were identified as partial agonists. These same two compounds were also partial agonists when the luciferase reporter system was employed as was compound **14c**, contrary to its full agonist behavior in the cAMP reporter system. Therefore, the characterization of the agonists as partial or full agonists depends on the functional system utilized. There are several possible explanations for this. First, the efficacy of various compounds can depend on the downstream signaling events and in particular to the G-protein coupling.[28] The cAMP system uses G_i G-proteins, whereas the luciferase system uses a G_{qi} chimeric G-protein and this difference may account for the different behavior observed with the compounds. Secondly, the level of receptor expression can also determine the degree of efficacy. Lower expression levels of the receptor can lead to lower partial agonist activity.[28] While the difference is not great, histamine does appear less potent in the luciferase system suggesting that the receptor levels are lower. This may result in compounds like **14c** behaving as a partial agonist in this system.

Given the discrepancies and artificial nature of transfection systems, it is best to study agonist potential in primary cells. To this end the compounds were tested for agonist potential in human eosinophils. In general any chemotactic signal induces reorganization of actin leading to a change in cell shape that can be detected by changes in forward scattering by flow cytometry.[29] Previously, it has been shown that histamine acting via the H_4R induces chemotaxis and shape change in eosinophils.[19] As in the transfected systems the three compounds presented here can induce shape change in eosinophils. The rank order of potency closely matched the measured affinities at the H_4R with histamine being the most potent followed by compounds **8**, **14b**, and then **14c**. However, the efficacy appeared to vary depending upon the donor. In some cases the compounds

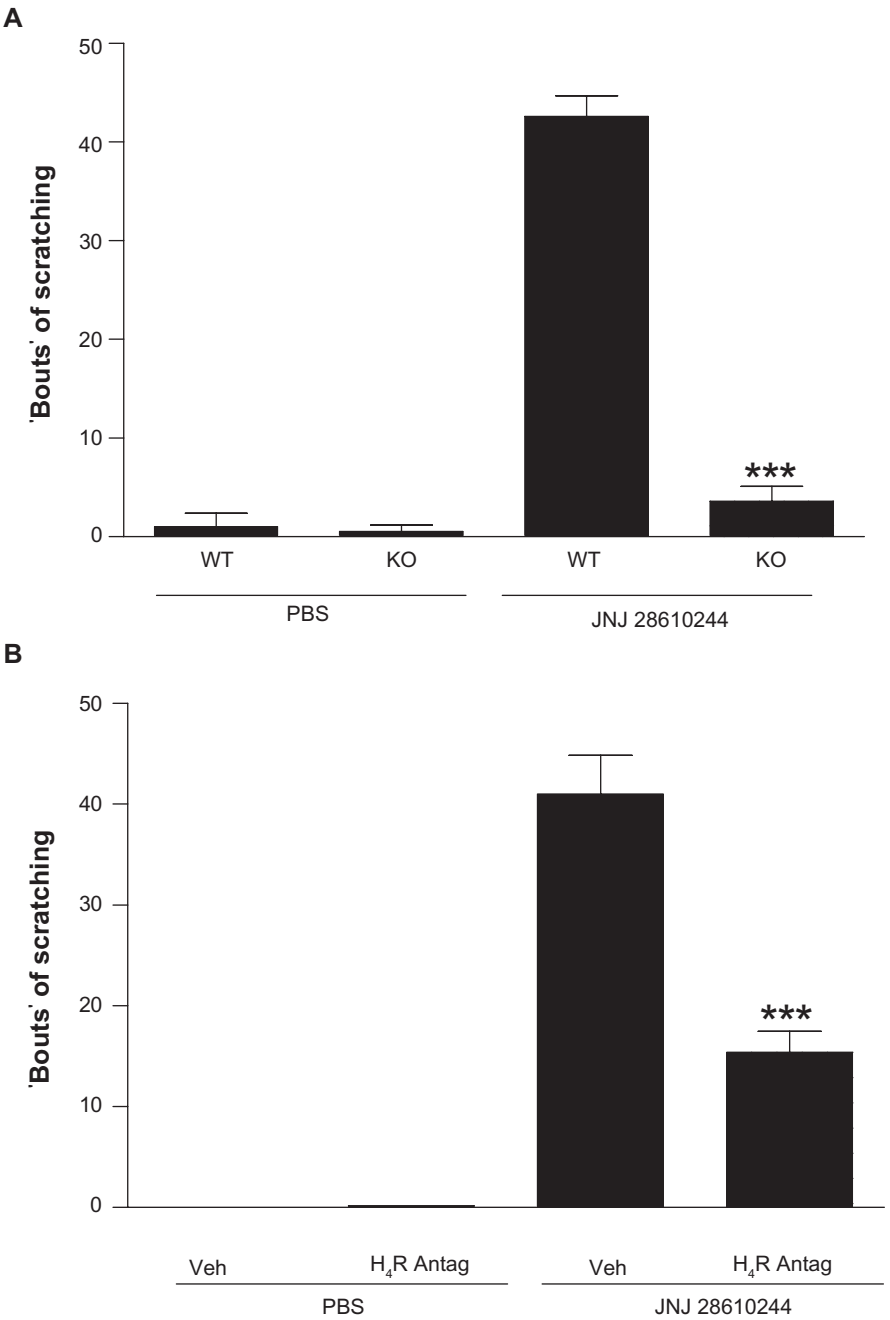


Figure 5 JNJ 28610244 induces pruritus. The H_4R agonist JNJ 28610244 (**14c**) induces pruritic response in BALB/c mice. **A**) The pruritic response was measured after injection of 100 nmol JNJ 28610244 (**14c**) at in either wild-type (WT) or H_4R-deficient mice (KO). **B**) PBS or 100 nmol JNJ 28610244 (**14c**) was injected into wild-type mice with or without pretreatment with the H_4R antagonist JNJ 7777120 (30 mg/kg) and the pruritic response was measured as described. Five mice per group were used and error bars represent the SEM.

Notes: ***$P < 0.001$ by one-way ANOVA with post-hoc Bonferroni's test compared to wild-type (WT) or vehicle (Veh) control.

Abbreviations: ANOVA, analyses of variance; PBS, phosphate-buffered saline; SEM, standard error of mean.

exhibited full agonist capability (Figure 3A), whereas in others they were weak partial agonists (Figure 3B). As for the transient transfection systems this may also be due to the receptor expression levels. The higher the expression level (as judged by the EC_{50} of histamine) the higher the efficacy of partial agonists.

The story appears to be less complicated at the mouse H_4R. Once again in both the cAMP and luciferase reporter systems, the rank order of the compounds in terms of potency closely matches the measured affinity of the compounds, although both histamine and compound **8** seem to be less potent than expected in the luciferase system.

Therefore, compound **14b** was the most potent agonist and histamine is the least potent agonist under these conditions. In general this same order of potency is found for inducing calcium responses in mouse bone marrow-derived mast cells. As opposed to the human systems, all three compounds appear to be full agonists at the mouse receptor in all of the systems studied.

The three compounds presented here appear to be very useful tools as agonists of the H_4R especially in mouse systems. All of the compounds have high affinity and selectivity for the H_4R. At the mouse H_4R the compounds act as full agonists in a variety of cell systems including bone marrow-derived mast cells. Furthermore, the compounds appear to act as agonists *in vivo* as evidenced by the scratching induced by **14c** (JNJ 28610244) in wild-type mice that was not present in H_4R-deficient mice. As described above, the two primary H_4R agonists currently used as tools are histamine and 4-methylhistamine. The discovery of the oxime agonists reported herein has several advantages over the historical tool compounds. First, both histamine and 4-methylhistamine have a lower affinity for the mouse H_4R than for the human homologue,[24] whereas the ligands described here have similar affinities in both species. Furthermore, the oxime agonists have demonstrated improved selectivity versus other histamine receptors and against many other receptors in general. The combination of high affinity and high selectivity make these compounds excellent new tools for further exploring and understanding H_4R pharmacology.

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Supplementary Table S1

Table S1 Selectivity of oxime agonists

Target	Compound			
	8	14a	14b	14c
A1 (h)	–	16	–	–
A2A (h)	–	–	14	–
A3 (h)	–	–	–	–
alpha 1 (nonselective)	–	–	–	–
alpha 2 (nonselective)	–	16	18	–
beta 1 (h)	–	13	15	–
AT1 (h)	–	–	–	–
BZD (central)	–	10	–	–
B2 (h)	–	–	–	–
CCKA (h) (CCK1)	–	–	–	–
D1 (h)	–	28	11	–
D2S (h)	–	–	–	–
ETA (h)	–	–	–	–
GABA (nonselective)	–	–	–	–
GAL2 (h)	–	11	–	–
IL-8B (h) (CXCR2)	–	–	–	–
CCR1 (h)	–	–	–	–
H1 (h)	15	11	–	–
H2 (h)	–	–	–	–
MC4 (h)	–	–	–	–
ML1	–	–	–	–
M1 (h)	73	28	36	15
M2 (h)	27	–	–	–
M3 (h)	82	54	47	23
NK2 (h)	17	–	–	–
NK3 (h)	11	–	–	–
Y1 (h)	–	–	15	–
Y2 (h)	–	–	–	–
NT1 (h) (NTS1)	–	–	–	–
delta 2 (h) (DOP)	–	–	–	–
kappa (KOP)	–	10	–	25
mu (h) (MOP)	–	11	21	–
ORL1 (h) (NOP)	–	–	10	–
5-HT1A (h)	–	10	–	–
5-HT1B	11	–	–	–
5-HT2A (h)	42	74	19	35
5-HT2B (h)	ND	74	63	ND
5-HT3 (h)	30	16	11	–
5-HT5A (h) (5-ht5A)	–	13	–	–
5-HT6 (h)	–	15	10	–
5-HT7 (h)	31	43	20	34
sst (nonselective)	–	–	–	–
VIP1 (h) (VPAC1)	–	–	–	12
V1a (h)	–	–	10	–
Ca^{2+} channel (L, verapamil site)	13	–	–	–
K+V channel	–	–	–	–
SK+Ca channel	–	–	–	–
Na^{+} channel (site 2)	11	11	26	–
Cl^{-} channel	–	–	–	–
NE transporter (h)	19	49	–	–
DA transporter (h)	–	42	–	–

Note: % inhibition at 1 μM; –, inhibition <10%.
Abbreviation: ND, not determined.