Pharmacological characterization of oxime agonists of the histamine H₄ receptor

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Abstract: The histamine H₁ receptor (H₁,R) 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 H₁,R antagonists. Using this as a starting point we have identified new indole and benzimidazole oxime piperidines as ligands for the H₁,R. These compounds have a high affinity for the human H₁,R with Kᵢ values ranging from 17–53 nM. They also have high to moderate affinity for the H₁,R from mouse, rat, guinea pig, and monkey, but poor affinity for the dog homologue. In addition to the high affinity for the H₁,R, 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 H₁,R 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 H₁,R, 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: H₁ receptor, eosinophils, mast cells, pruritus

Interoduction

Histamine is a biogenic amine that functions as a messenger for many physiological and pathalogical 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 H₁ receptor (H₁,R) primarily mediates allergic responses. Antihistamines specifically targeting the H₁,R have been widely used to treat allergic disorders such as seasonal or perennial allergic rhinitis and chronic urticaria. The H₂ receptor (H₂,R) enhances gastric acid secretion in the stomach and H₂,R antagonists have been used in the treatment of dyspepsia. The H₃ receptor (H₃,R) regulates the release of histamine and neurotransmitters from the neurons. The newest member of the histamine receptor family, the H₄ receptor (H₄,R), has been implicated in inflammatory and pruritic responses and data indicate that the receptor may be crucial to understanding
allergic and inflammatory disorders such as atopic dermatitis, where traditional \( H_3R \) antagonists are commonly prescribed but are generally considered ineffective.

Despite all of the promising early work, many of the mechanisms mediated by the \( H_3R \) are still unclear. One of the key elements for helping to understand the role of the \( H_3R \) 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 \( H_3R \) function both \textit{in vitro} and \textit{in vivo}.

Here we describe oxime-containing agonists of the \( H_3R \) that have excellent potency and selectivity for the \( H_3R \) 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-trimethylsilyloxymethyl 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\(^6\) afforded the \( N \)-methylated piperidine 6. Subjecting 6 to hydroxylamine hydrochloride in pyridine produced a 1:1 mixture of \( E \)- and \( Z \)-regioisomeric oximes 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.\(^1\) 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 \( Z \)-regioisomeric oximes 13a–c and 14a–c respectively. The regioisomers were separated via SFC conditions using the same conditions as for the benzimidazoles.

**Mice**

\( H_3R \)-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

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**Scheme 1** Preparation of the benzimidazole oxime.\(^a\)

\(^a\)Conditions and reagents: a) \( \text{NaN}_3 (60\% \text{ in mineral oil})\), \( \text{DMF-SEM-CL} \), 0 °C → room temp, 3.5 h, 92%. b) \( n-BuLi (2.5 \text{ M in hexanes})\), THF, –78 °C, 2 h, then add the Weinreb amide 3b, 2 h at room temp, 62%. (c) 6N HCl, EtOH, 80 °C, 3 h, 79%. d) \( \text{Formic Acid, formaldehyde 37\%, 70 °C, 8 h, 28%–64\%} \). (e) \( \text{NH}_2\text{OH-HCl, pyridine, 60 °C, 4.5 h, 89\%} \). f) Separate the \( E/Z \)-oxime regioisomers: Chiracel AD column, SFC Conditions, IPA/CO\(_2\).

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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. The stably transfected SK-N-MC cells were generated as previously described. SK-N-MC cells stably transfected with the mouse H3R were generated as previously described for the human H3R cell line. 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 H3R where mouse brain homogenates were used. Binding assays for the human, mouse and rat H3R, the human H1R and the human H2R were carried out as previously described. Binding assays for the monkey and dog H1R were conducted as previously described. The guinea pig H1R binding assay utilized SK-N-MC cells as previously described. Binding to the human H2R was carried out according to the method previously described. Binding to the mouse H3R was determined in membranes from SK-N-MC cells stably transfected with the mouse H3R. 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 [3H]pyrilamine (Kd = 3 nM) for 45 minutes at 25°C. Nonspecific binding was defined using 100 µM unlabeled triprolidine. Binding to the mouse H3R was determined using mouse brain homogenates as previously described for the rat H3R. For all studies, the Kd values were calculated based on an experimentally determined appropriate Kd value according to the Cheng and Prusoff equation. All Kd 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 H3R 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. The luciferase assays for all species were carried out as previously described for the human and dog H3R. 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 (EC50) values using Prism (GraphPad Software, San Diego, CA) and the values are given as the average EC50 of at least triplicate determinations.

Selectivity panel

A panel of 50 different biogenic amine receptors, neupeptide receptors, ion channel binding sites, and neurotransmitter transporter binding assays were carried out as previously described. The assays were run at 1 µM of the H3R 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. 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\(^6\) 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 autofluorescence 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 minutes 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\(_4\)R antagonists JNJ 7777120 and JNJ 10191584 (Figure 1), we subsequently observed that replacing the piperazine ring in either of these compounds with a piperidine ring afforded a moderately potent H\(_4\)R antagonist when the benzimidazole core was retained, but lost H\(_4\)R-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\(_4\)R-agonist activity. Moreover, the H\(_4\)R-agonist activity resided only in the Z-geometric isomer of the oxime as the E-geometric isomer was essentially devoid of H\(_4\)R activity. Motivated by the potential value of specific H\(_4\)R agonists in the investigation of H\(_4\)R 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](https://www.dovepress.com/)

Figure 1 Chemical structures of JNJ 7777120 A) and JNJ 10191584 B).
The binding affinity of these compounds was determined using cells expressing the H₄R from different species. As shown in Table 1, all four compounds had high affinity for the human H₄R and good selectivity over other histamine receptors. These compounds also demonstrated high binding affinity for the H₁R homologues from different species, although their binding to the dog H₁R was very weak. These compounds also showed good selectivity against the mouse histamine H₁R and H₂R. For comparison the Kᵢ values for histamine, the H₄R 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 H₂R. 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 H₄R 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 H₄R. As for histamine, the oxime compounds all act as agonists at both the human and mouse H₄R. Histamine, compound 8 and compound 14b all have similar potency at the human H₄R, 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 H₄R 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 H₄R.

To further explore the agonist potential of these compounds, a second transfection system was used consisting of HEK-293 cells expressing the H₄R, a chimeric Gqi protein and an SRE-luciferase reporter construct. Under these conditions, H₄R signaling was directed to the Gqi pathway by the chimeric Gqi protein and activation of the H₄R 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 H₄R in this system, but were still full agonists at the mouse H₄R (Table 3). The agonist potential was also explored at the rat and monkey H₄R. 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 H₄R. Both the potency and the agonist activity at the human H₄R has about the same rank order of potency (Figure 3). Both the potency and the

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

<table>
<thead>
<tr>
<th>Species</th>
<th>Histamine²⁷</th>
<th>10191584</th>
<th>Cmpd 8</th>
<th>Cmpd 14a</th>
<th>Cmpd 14b</th>
<th>Cmpd 14c</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₄ Human</td>
<td>13 ± 0.2</td>
<td>5 ± 0.1²⁷</td>
<td>32 ± 0.4²⁷</td>
<td>17 ± 8</td>
<td>49 ± 2</td>
<td>31 ± 1</td>
</tr>
<tr>
<td>Mouse</td>
<td>79 ± 1.1</td>
<td>4 ± 0.5²⁷</td>
<td>126 ± 2²⁷</td>
<td>8 ± 4</td>
<td>105 ± 35</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>Rat</td>
<td>70 ± 1</td>
<td>4 ± 0.5²⁷</td>
<td>50 ± 0.7²⁷</td>
<td>16 ± 3</td>
<td>345 ± 86</td>
<td>20 ± 6</td>
</tr>
<tr>
<td>Monkey</td>
<td>16 ± 0.2</td>
<td>32 ± 1²⁷</td>
<td>200 ± 3²⁷</td>
<td>60 ± 7</td>
<td>40 ± 6</td>
<td>25 ± 2</td>
</tr>
<tr>
<td>Dog</td>
<td>63 ± 0.9</td>
<td>79 ± 1²⁷</td>
<td>661 ± 10²⁷</td>
<td>1228 ± 256</td>
<td>5464 ± 2577</td>
<td>8253 ± 1687</td>
</tr>
<tr>
<td>Guinea Pig</td>
<td>10 ± 0.1</td>
<td>1000 ± 17²⁷</td>
<td>1585 ± 27²⁷</td>
<td>123 ± 19</td>
<td>485 ± 33</td>
<td>46 ± 7</td>
</tr>
<tr>
<td>H₁ Human</td>
<td>ND</td>
<td>&gt;10,000³⁹</td>
<td>&gt;10,000³⁹</td>
<td>&gt;1000</td>
<td>&gt;10,000³⁹</td>
<td>&gt;10,000³⁹</td>
</tr>
<tr>
<td>Mouse</td>
<td>ND</td>
<td>&gt;10,000³⁹</td>
<td>&gt;10,000³⁹</td>
<td>ND</td>
<td>&gt;10,000³⁹</td>
<td>&gt;10,000³⁹</td>
</tr>
<tr>
<td>H₂ Human</td>
<td>ND</td>
<td>&gt;30,000³⁹</td>
<td>ND</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>H₃ Human</td>
<td>ND</td>
<td>5125 ± 1087³⁹</td>
<td>&gt;10,000³⁹</td>
<td>&gt;100000</td>
<td>2691 ± 717</td>
<td>&gt;100000</td>
</tr>
<tr>
<td>Mouse</td>
<td>ND</td>
<td>10,000</td>
<td>ND</td>
<td>491 ± 88</td>
<td>1097 ± 153</td>
<td>3601 ± 194</td>
</tr>
</tbody>
</table>

**Abbreviations:** ND, not determined; SEM, standard error of mean.
Table 2 Selectivity of oxime agonists

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Compound</th>
<th>8</th>
<th>14a</th>
<th>14b</th>
<th>14c</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>73</td>
<td>28</td>
<td>36</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>M3</td>
<td>82</td>
<td>54</td>
<td>47</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>5-HT2a</td>
<td>42</td>
<td>74</td>
<td>19</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>5-HT2b</td>
<td>ND</td>
<td>74</td>
<td>63</td>
<td>ND</td>
<td></td>
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</tbody>
</table>

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

The 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_{1R} 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_{1R} can induce a calcium influx in these cells. 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. 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. 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. 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

<table>
<thead>
<tr>
<th>Reporter</th>
<th>Species</th>
<th>Value</th>
<th>Histamine</th>
<th>Cmpd 8</th>
<th>Cmpd 14b</th>
<th>Cmpd 14c</th>
</tr>
</thead>
<tbody>
<tr>
<td>cAMP</td>
<td>Human</td>
<td>EC_{50} (nM)</td>
<td>42 ± 4</td>
<td>50 ± 2</td>
<td>26 ± 8</td>
<td>515 ± 46</td>
</tr>
<tr>
<td></td>
<td></td>
<td>α</td>
<td>0.70 ± 0.01</td>
<td>0.46 ± 0.02</td>
<td>1.00 ± 0.02</td>
<td></td>
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<tr>
<td></td>
<td>Mouse</td>
<td>EC_{50} (nM)</td>
<td>337 ± 20</td>
<td>9.0 ± 0.1</td>
<td>2.0 ± 0.1</td>
<td>174 ± 63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>α</td>
<td>1.00 ± 0.01</td>
<td>0.84 ± 0.01</td>
<td>1.08 ± 0.01</td>
<td></td>
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<tr>
<td>Luciferase</td>
<td>Human</td>
<td>EC_{50} (nM)</td>
<td>77 ± 9</td>
<td>73 ± 20</td>
<td>37 ± 13</td>
<td>109 ± 14</td>
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<tr>
<td></td>
<td></td>
<td>α</td>
<td>0.58 ± 0.03</td>
<td>0.67 ± 0.02</td>
<td>0.74 ± 0.05</td>
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<tr>
<td></td>
<td>Mouse</td>
<td>EC_{50} (nM)</td>
<td>5316 ± 101</td>
<td>69 ± 8</td>
<td>11 ± 4</td>
<td>180 ± 91</td>
</tr>
<tr>
<td></td>
<td></td>
<td>α</td>
<td>1.02 ± 0.06</td>
<td>1.08 ± 0.12</td>
<td>1.16 ± 0.12</td>
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</tr>
<tr>
<td>Rat</td>
<td>EC_{50} (nM)</td>
<td>171 ± 14</td>
<td>182 ± 21</td>
<td>9 ± 3</td>
<td>477 ± 204</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>α</td>
<td>0.86 ± 0.03</td>
<td>1.10 ± 0.16</td>
<td>0.86 ± 0.19</td>
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<tr>
<td>Monkey</td>
<td>EC_{50} (nM)</td>
<td>48 ± 7</td>
<td>692 ± 382</td>
<td>139 ± 70</td>
<td>1393 ± 638</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>α</td>
<td>1.39 ± 0.89</td>
<td>0.62 ± 0.09</td>
<td>0.77 ± 0.19</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: cAMP, cyclic adenosine monophosphate; EC_{50}, half-maximum effective concentration; SEM, standard error of mean.
for highly selective well-characterized agonists of the H₄R 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 H₄R-binding affinity (ranging from 1.5-fold to 32-fold) in several animal species over the benzimidazole derivative JNJ 10191584. The presence of the piperazine 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 piperazine 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 H₄R-binding affinity (Kᵢ > 10,000 nM), whereas, the benzimidazole piperidine analog 6, provided moderate human H₄R-binding affinity (Kᵢ = 81 nM). The loss of hH₄R-binding affinity in 12b is not completely explained by the loss of the amide functionality since compound 12c still retains moderate H₄R-binding affinity (Kᵢ = 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 H₄R 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 H₄R 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 2  Agonist activity at the human and mouse receptor. Functional agonism of the human A) and mouse B) H₄R 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.
Figure 3 Eosinophil shape change. The H₄R 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 H₄R 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 H₄R-binding affinity is similar with the 5-chloro analog being more potent in all species than the 5-methyl analog.
All of the oxime agonists that we have evaluated display excellent affinity for the human H₄R, although they have lower affinity than that of JNJ 7777120 and more closely match JNJ 10191584. They also have high affinity for the mouse H₄R, 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 H₄R. 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 H₄R, but little if any affinity for the dog H₄R. This variability in ligand affinity among the different species homologues of the H₄R 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 H₄R. 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. The cAMP system uses G₁₅ G-proteins, whereas the luciferase system uses a G₁₅ 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. 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 H₄R 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 H₄R 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

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**Figure 4** Calcium mobilization in mast cells. The H₄R agonists induce mouse bone marrow-derived mast cell (BMMC) calcium mobilization. A. Dose response of H₄R 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 H₄R-deficient (red squares) mice. The results show the average of triplicates and the error bars represent SEM. Abbreviation: 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$_4$R. 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 H4R especially in mouse systems. All of the compounds have high affinity and selectivity for the H4R. At the mouse H4R 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 H4R-deficient mice. As described above, the two primary H4 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 H4R 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 H4R pharmacology.

Acknowledgments

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References


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### Table S1 Selectivity of oxime agonists

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**Notes:** % inhibition at 1 µM; -, inhibition <10%.

**Abbreviation:** ND, not determined.