

Themed Issue: Histamine Pharmacology Update

RESEARCH PAPER

Profiling of histamine H₄ receptor agonists in native human monocytes

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Keywords

4-methylhistamine; clobenpropit;
histamine H₄ receptor; histamine;
ST-1006; UR-PI376; VUF8430

Received

29 October 2012

Revised

27 February 2013

Accepted

10 April 2013

BACKGROUND AND PURPOSE

Since the identification of the histamine H₄ receptor, several ligands activating this receptor have been described and more compounds are in development. These ligands are well characterized in pharmacological assays, including radioligand competition binding studies, GTPγS and GTPase assays. In most cases, these experiments are performed in transfected cell lines, expressing unnaturally high levels of target receptors and G-protein signalling components. In this study we investigated the specific properties of H₄ receptor ligands in native cells.

EXPERIMENTAL APPROACH

Histamine and five different H₄ receptor agonists – 4-methylhistamine, UR-PI376, clobenpropit, VUF8430 and ST-1006 – were characterized in freshly isolated human monocytes. The ligands (10 nM–10 μM) were tested as inhibitors of IL-12p70 secretion from human monocytes and the effects of the H₂ receptor antagonist ranitidine and the H₄ receptor antagonist JNJ7777120 on their action was investigated.

KEY RESULTS

Histamine and all the tested agonists reduced IL-12p70 secretion into monocyte supernatants by 40–70%. The potencies varied with pEC₅₀ values ranging from 5.7 to 6.9, depending on the agonist used. All potencies were lower than those determined in the original investigations of the compounds. Pretreatment of monocytes with H₂ or H₄ receptor antagonists showed that some H₄ receptor ligands also had low activity at the H₂ receptor.

CONCLUSIONS AND IMPLICATIONS

Our study demonstrates discrepancies between the potencies obtained from assays in transfected cell lines and assays in native human cells, indicating the importance of evaluating H₄ receptor ligands in native cells.

LINKED ARTICLES

This article is part of a themed issue on Histamine Pharmacology Update. To view the other articles in this issue visit <http://dx.doi.org/10.1111/bph.2013.170.issue-1>

Abbreviations

PTX, *Pertussis* toxin

Introduction

The histamine H₄ receptor subtype is the most recently identified (Nakamura *et al.*, 2000; Oda *et al.*, 2000) of the four known G-protein-coupled histamine receptor subtypes

(receptor nomenclature follows Alexander *et al.*, 2011). The H₄ receptor is expressed in many different tissue types and particularly in those of the haematopoietic system. Although the main role of the H₄ receptor is thought to be in inflammatory diseases such as asthma, atopic dermatitis,

inflammatory pain and allergic rhinitis (Engelhardt *et al.*, 2009; Gutzmer *et al.*, 2011), newer studies suggest that H₄ receptors could also be involved in pruritus, cancer, neuropathic pain, vestibular disorders and type 2 diabetes (Gutzmer *et al.*, 2011; Kiss and Keseru, 2012). Because of its widespread expression and its potential as a target for therapeutic interventions, the function of the H₄ receptor needs to be thoroughly characterized.

To accomplish this goal, several H₄ receptor ligands have been developed in recent years for assessment *in vitro*, in animal models and as potential therapeutic agents. These H₄ receptor agonists have been rigorously tested for their binding properties and potencies in binding-assays in cell lines (Lim *et al.*, 2005; 2006; Igel *et al.*, 2009; Sander *et al.*, 2009). For these studies human neuroblastoma cells lines (Lim *et al.*, 2005) or insect cells (Igel *et al.*, 2009; Sander *et al.*, 2009) are used, after transfection with the H₄ receptor gene from different species and the relevant G-protein signalling components in order to induce signalling in response to receptor stimulation. With the use of these cell lines, histamine displacement assays and functional assays, for example GTPase assays, give information on the binding affinity and efficacy of H₄ receptor ligands at this receptor derived from different species. These pharmacological assays allow the comparison of different ligands with a standardized and robust assay system. However, cell lines expressing high amounts of one single receptor and its signalling components represent artificial systems, which probably do not completely represent the situation in native cells. When comparing different H₄ receptor ligands in *in vitro* assays on native cells, we and others have observed variations in their activity that were not predicted from the data derived from the cell-line assays. Therefore we decided to perform a more extensive study comparing the effect of histamine with a panel of different H₄ receptor agonists, which have been described by different research groups: 4-methylhistamine (Lim *et al.*, 2005), UR-PI376 (Igel *et al.*, 2009), clobenpropit (Lim *et al.*, 2005), VUF8430 (Lim *et al.*, 2006) and ST-1006 (Sander *et al.*, 2009). As a robust functional readout we selected the secretion of the cytokine IL-12p70 from human monocytes, which has been shown to be down-regulated by histamine and dependent on the activation of histamine H₄ and H₂ receptors (Gutzmer *et al.*, 2005; Dijkstra *et al.*, 2008).

Our study focused on the importance of evaluating H₄ receptor ligands in a native cellular system, as distinct from a transfected cell line. After testing various H₄ receptor ligands at several concentrations in such a native system, we found that the potency in down-regulating IL-12p70 production varied between the ligands and was somewhat less than their potency in mediating G-protein signalling in the cell-line assays. Furthermore the H₄ receptor agonists seem to exhibit a different proportion of binding at H₂ and H₄ receptors, as identified by the corresponding receptor antagonists.

Methods

Isolation, culture, and stimulation of human monocytes

Buffy coats, after thrombocyte preparation from whole blood, were obtained from the local blood bank. According to the

guidelines for blood donation, the anonymous donors were healthy and had not taken any medication for 4 weeks before giving blood. Peripheral blood mononuclear cells (PBMC) were separated from the buffy coats by density centrifugation on lymphoprep (Fresenius Kabi Norge AS, Oslo, Norway), and erythrocytes were removed by incubation with Gey's lysis buffer. 1×10^8 PBMC were placed in a Petri dish (Ø 15 cm) for 1 h (37°C, 5% CO₂, humidified atmosphere) and non-adherent cells were removed by vigorous washing with PBS. Adherent cells, which were monocytes with a purity of at least 85%, were cultured in RPMI 1640, supplemented with 2 mM l-glutamine, 100 mg·mL⁻¹ penicillin/streptomycin, 12 mM HEPES and 5% v/v FCS (PAN-Biotech, Aidenbach, Germany; all other media components from Biochrom, Berlin, Germany) at 37°C in a humidified atmosphere containing 5% CO₂. The monocytes were used within 24 h after isolation for the experiments.

Determination of pEC₅₀ values

Monocytes were stimulated with different concentrations of histamine receptor agonists (10 nM–10 µM) for 24 h, the time shown in previous studies to be optimal for observing histamine-induced decrease in the production of cytokines of the IL-12 family on subsequent activation (Gutzmer *et al.*, 2005; Gschwandtner *et al.*, 2012). Then the cells were activated by addition of IFN-γ (200 ng·mL⁻¹) and 2 h later LPS (50 ng·mL⁻¹); this 2 h time gap was shown to be very effective in the induction of IL-12 (Wittmann *et al.*, 1999). Supernatants were taken after 24 h and the secretion of IL-12p70 was measured by ELISA.

Histamine receptor blocking experiments

Blocking experiments were performed as described previously (Gschwandtner *et al.*, 2012). Monocytes were treated with 10 µM of the receptor antagonists ranitidine and/or JNJ7777120. 30 min later monocytes were stimulated with histamine receptor agonists for 24 h, and then the cells were activated by addition of IFN-γ and 2 h later LPS. Supernatants were taken after 24 h and the secretion of IL-12p70 was measured by ELISA.

ELISA

Cell free supernatants were taken after a total stimulation time of 48 h and the amount of IL-12p70 was determined with ELISA according to the manufacturer's instructions (eBioscience, San Diego, CA, USA).

Statistical analysis

Data are represented as mean ± SEM and the program GraphPad Prism version 5 (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis. For calculation of *P*-values, we used one-way ANOVA with Bonferroni post test (Figures 1 and 3). The pEC₅₀ values were calculated by non-linear regression analysis from dose-response curves (Figure 2). A *P*-value < 0.05 was regarded as significant.

Materials

Various H₄ receptor agonists were used in this study: histamine (Alk-Scherax, Wedel, Germany), 4-methylhistamine (Tocris Bioscience, Bristol, UK), described in (Lim *et al.*,

2005)), UR-PI376 (Department of Pharmaceutical/Medicinal Chemistry, University, of Regensburg, Germany; (Igel *et al.* (2009)), clobenpropit (Sigma-Aldrich, Deisenhofen, Germany), described in Lim *et al.* (2005)), VUF8430 (Tocris Bioscience), described in Lim *et al.* (2006)) and ST-1006 (Institute of Pharmaceutical Chemistry, ZAFES/NeFF/OSF/CMP, Johann Wolfgang Goethe University Frankfurt/Main, Germany; (Sander *et al.*, 2009)). Table 1 gives a summary of the chemical properties and characteristics of the H₄ receptor agonists.

For blocking of histamine receptors, the following antagonists were used: ranitidine (selective H₂ receptor antagonist; Biomol, Hamburg, Germany) and JNJ7777120 (selective H₄ receptor antagonist, also shows low activity at H₂ receptors (Jablonowski *et al.*, 2003); Sigma-Aldrich). If not stated otherwise, histamine receptor ligands were used at a concentration of 10 µM. Other reagents for stimulation of human monocytes were used as follows: human recombinant IFN-γ (Peprotech Hamburg, Germany), LPS (*Escherichia coli* 055:B5; Sigma-Aldrich) and Pertussis toxin (PTX; Calbiochem, Darmstadt, Germany).

Results

All H₄ receptor agonists tested down-regulate IL-12p70 production in monocytes

As described previously (Wittmann *et al.*, 1999; Pflanz *et al.*, 2002; Gschwandtner *et al.*, 2012), stimulation of monocytes with sequential IFN-γ and LPS increased the secretion of IL-12 many-fold (Figure 1). Here we used six different H₄ receptor agonists (Table 1) for pre-stimulation of monocytes and evaluated the secretion of IL-12p70 in response to IFN-γ and LPS stimulation performed 24 h later. We used the ligands at a concentration known to induce effects in monocyte-derived dendritic cells, for some of the ligands, without inducing cellular toxicity (Gutzmer *et al.*, 2005; Dijkstra *et al.*, 2008). Pre-stimulation with all the tested H₄ receptor agonists resulted in a significant decrease in IL-12p70 secretion, ranging from 40 to 70% suppression, depending on the agonist used. In contrast to the agonists, the H₄ receptor antagonist JNJ7777120 (Thurmond *et al.*, 2004) did not modulate the expression of IL-12p70 (Figure 1).

Differences in potency between the tested H₄ receptor agonists

In order to calculate the potencies of the different H₄ receptor agonists, we performed dose-response experiments over a range of concentrations (10 nM – 10 µM). The sigmoidal dose curves in Figure 2 showed that the different H₄ receptor ligands exhibited different potencies, which were lower than the corresponding values determined in assays in transfected cell lines (Table 1 and Table 2).

Specificity of H₄ receptor agonists

We used the established H₄ receptor antagonist JNJ7777120 (Thurmond *et al.*, 2004), to show specificity of the agonists for this histamine receptor. JNJ7777120 was used at 10 µM, a concentration shown previously to completely block cellular response to a H₄ receptor agonist (Reher *et al.*, 2012). The

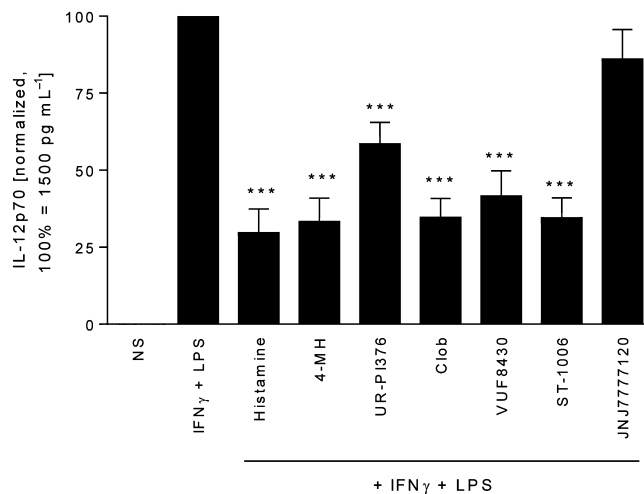


Figure 1

H₄ receptor agonists down-regulate IL-12p70 production in human monocytes. Monocytes were stimulated for 24 h with histamine, followed by activation with IFN-γ and LPS for 24 h. The IL-12p70 content in the cell supernatant was determined by ELISA. Histamine and the tested H₄ receptor ligands decreased IFN-γ and LPS-induced IL-12p70, while JNJ7777120 did not affect IL-12p70 production. The absolute IL-12p70 production in IFN-γ and LPS stimulated monocytes was in the range of 70–5561 pg mL⁻¹ with a mean of 1522 pg mL⁻¹. Data shown are means ± SEM of eleven independent experiments. **P* < 0.05, significantly different from IFN-γ + LPS alone. 4-MH, 4-methylhistamine; Clob, clobenpropit.

action of one out of six ligands – ST-1006 – was completely blocked by JNJ7777120 and IL-12p70 expression was around 100% (similar to the non-stimulated controls). In contrast, JNJ7777120 only partially blocked down-regulation of IL-12p70 induced by the other H₄ receptor agonists tested (Figure 3A).

As some H₄ receptor ligands are known to also bind to the H₂ receptor (Sterk *et al.*, 1986; Lim *et al.*, 2005), we tested in addition the effect of the H₂ receptor antagonist ranitidine (Daly *et al.*, 1981) on the action of the H₄ receptor ligands. Ranitidine partially blocked the effect of histamine and 4-methylhistamine and did not modulate the decreased secretion of IL-12p70 in response to UR-PI376, clobenpropit, VUF8430 and ST-1006 (Figure 3B). The antagonists alone did not modulate IL-12p70 production (data not shown).

To investigate the potential involvement of G-protein-independent signalling pathways, we performed experiments in the presence of PTX (100 ng·mL⁻¹). IL-12p70 secretion from monocytes was significantly reduced by PTX alone (Figure 3c). In combination with the agonists, no further significant down-regulation of IL-12p70 was observed (Figure 3c). The concentration of PTX used in these experiments was not toxic to the cells, as the secretion of the chemokine CXCL10, as a control cytokine unrelated to IL-12, was not down-regulated by PTX (data not shown).

Discussion and conclusions

In the present study we compared the potencies of different H₄ receptor agonists on a robust H₄ receptor effect in freshly

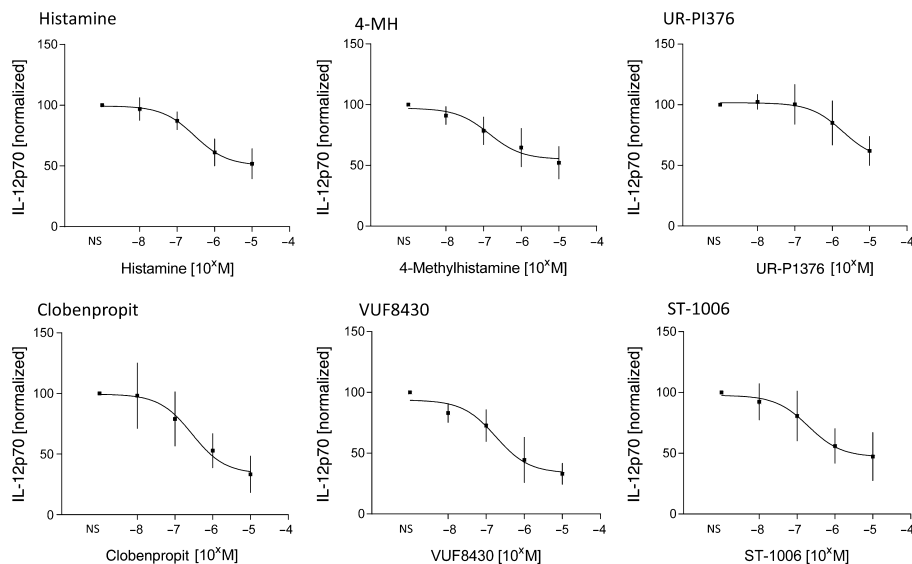


Figure 2

Dose titration and pEC_{50} determination for different H_4 receptor agonists. Monocytes were stimulated for 24 h with H_4 receptor agonists at the concentrations shown, followed by activation with IFN- γ and LPS for 24 h, and determination of IL-12p70 content in the cell supernatant by ELISA. EC_{50} values are shown in Table 2 and were calculated by non-linear regression analysis from the sigmoidal dose response curves. The absolute IL-12p70 production in IFN- γ and LPS stimulated monocytes was in the range of 125–1697 $pg\ mL^{-1}$ with a mean of 554 $pg\ mL^{-1}$. Data shown are means \pm SEM of six independent experiments. 4-MH, 4-methylhistamine.

Table 2

Comparison of the properties of H_4 receptor ligands in native human monocytes

	Histamine	4-Methylhistamine	UR-PI376	Clobenpropit	VUF8430	ST-1006
IL-12 down-regulation	✓	✓	✓	✓	✓	✓
pEC_{50} published	7.7 ± 0.10	7.4 ± 0.10	7.5 ± 0.01	7.7 ± 0.10	7.3 ± 0.10	8.95 ± 0.29
pEC_{50} determined in monocyte assay (see Figure 2 for data)	6.5 ± 0.18	6.9 ± 0.24	5.7 ± 0.32	6.5 ± 0.28	6.8 ± 0.18	6.7 ± 0.29
IL-12 down-regulation blocked by antagonism of						
H4R	Partially	Partially	Partially	Partially	Partially	✓
H2R	Partially	Partially	X	X	X	X

The potency of H_4 receptor ligands with regard to IL-12p70 down-regulation and the modulation of their effect by H_2 receptor (H2R) and H_4 receptor (H4R) antagonists are shown.

Potencies are shown as pEC_{50} values and are expressed as means \pm SEM.

isolated, native human monocytes. All the tested agonists induced down-regulation of IL-12p70 secretion from monocytes in a magnitude similar to the endogenous ligand histamine. However, we found lower values for their potency (pEC_{50}), compared with those from assays in transfected cell lines, performed with these agonists (Table 2). Plasma protein binding capacity of the ligands was not responsible for the discrepancy in potency between the GTP γ S assay and monocyte assay (A. Buschauer, pers. comm.). Moreover, the observation was made for all tested H_4 receptor agonists as well as for histamine, indicating that the lower potency values did not depend on the nature/structure of the various H_4 receptor

ligands, but probably resulted from differences between the present native test system, and that in the transfected cell lines used for the primary evaluation of the compounds (Lim *et al.*, 2005; Igel *et al.*, 2009; Sander *et al.*, 2009). Possible differences in the test systems that might explain the variation in potency are:

- The cell types used in the earlier studies were either derived from neoplastic cells (Lim *et al.*, 2005) or represent insect cell lines (Igel *et al.*, 2009; Sander *et al.*, 2009). These cell lines are *per se* different from native human cells with regard to their characteristics, e.g. expression of different

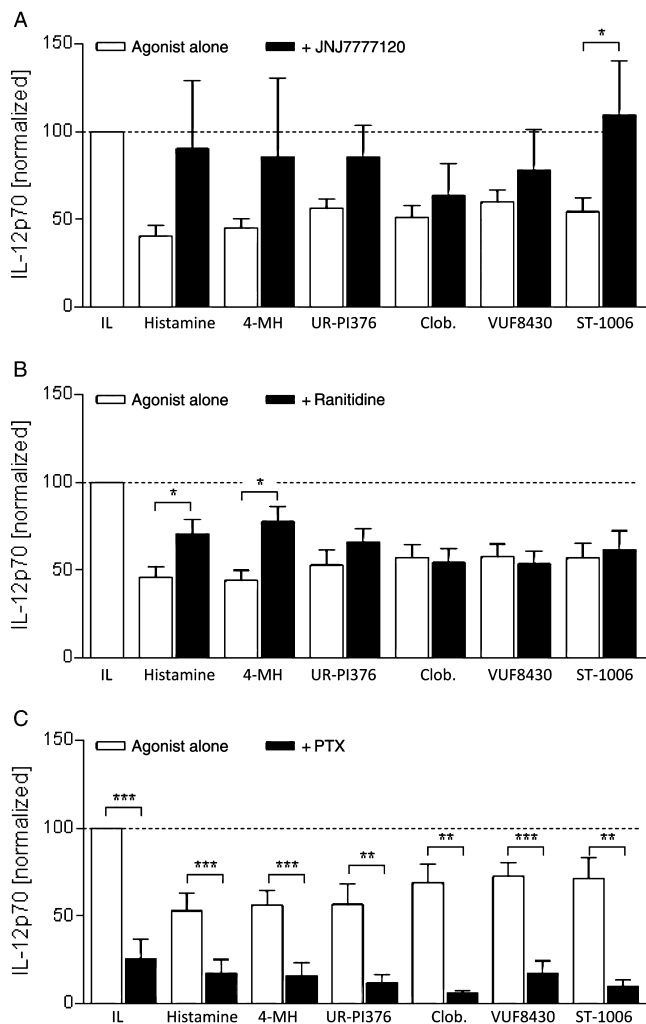


Figure 3

H₄ receptor and H₂ receptor antagonists and PTX have different actions on the effect of H₄ receptor agonists. Monocytes were stimulated with 10 μ M JNJ777120 (H₄ receptor antagonist, A) or ranitidine (H₂ receptor antagonist, B) for 30 min or with 100 ng mL⁻¹ PTX for 20 min (C) and thereafter with H₄ receptor agonists (10 μ M) for 24 h, followed by activation with IFN- γ and LPS for 24 h. The IL-12p70 content in the cell supernatant was determined by ELISA. The absolute IL-12p70 production in IFN- γ and LPS stimulated monocytes was in the range of 334–1236 pg mL⁻¹ with a mean of 728 pg mL⁻¹. Data shown are means \pm SEM of 19, 16 and 7 independent experiments in a, b and c respectively. * P < 0.05, ** P < 0.01, *** P < 0.001, significantly different from agonist alone. IL: IFN- γ and LPS; 4-MH, 4-methylhistamine; Clob, clobenpropit.

proteins, changed proliferative potential and growth at lower temperatures (especially insect cells).

- Some pharmacological testing was performed with cell membranes (Igel *et al.*, 2009) and not on intact cells. Clearly with cell membrane preparations, some potentially relevant influences present in the whole cell might be lost, especially in terms of the signalling cascade. This cascade is terminated at the membrane-bound G-proteins and cannot continue all the way into the nucleus in order to reach the relevant targets, as in whole cells.

- The cell lines used in the earlier assays were usually selectively transfected to express high levels of one type of receptor and a limited range of G-protein subtypes. In contrast, native cells express lower levels of the desired receptor and in addition they express other types of receptors, as well as signalling components that might interfere with binding of a compound to the desired receptor.

Interestingly, the difference between the potency values obtained from our native test system in comparison with the cell-line assays is larger for H₄ receptor agonists tested in Sf9 insect cells (UR-PI376 and ST-1006) than for other agonists tested in SK-N-MC cells. This difference might be explained by the fact that in the insect cell system, additional G-protein and regulators of G-protein signalling (RGS) fusions are required, which probably result in a further over-estimation of ligand potency. Therefore, when choosing a cellular system for functional assays one should always use a system with as few modifications as possible, relative to the native cells. In general, cell lines used for pharmacological assays represent an ideal model system, which helps in the initial characterization of pharmacologically active compounds. However, the results derived from these cells probably cannot be transferred without modification to native cells.

Recently another study investigated the effects of UR-PI376 on human native eosinophils (Reher *et al.*, 2012). In this study, the potency of the H₄ receptor agonist varied with the assay used. Thus for chemotaxis an EC₅₀ of 8 nM (pEC₅₀ of 8.1) was measured, a potency higher than that derived from the cell-line assays (pEC₅₀ of 7.5) whereas in a Ca²⁺ influx assay, an EC₅₀ of 320 nM (pEC₅₀ of 6.5) was determined. In the same Ca²⁺ influx assay, histamine was also tested and exhibited a EC₅₀ of 1 μ M (pEC₅₀ of 6.0). These potency values obtained from the Ca²⁺ influx assay are in the same range as the values determined in our monocyte assay. This study together with ours shows that the potency values of agonists in different native cellular systems are in a similar range, although there is, of course, some variation depending on the cellular context. Moreover, it has to be noted that compared with cell-line assays, the variation of results between different experiments is greater when working with native cells, due to the individual variations between the different human donors, whose blood was used for isolation of the monocytes.

Another important observation from the comparison of the two studies is that the potency seems to be similar for a readout that is measured within seconds after receptor stimulation (i.e. Ca²⁺ influx, pEC₅₀ of 6.0 for histamine (Reher *et al.*, 2012)) and for a readout that is measured 48 h after receptor stimulation (i.e. IL-12p70 secretion measured in this study, pEC₅₀ of 6.5 for histamine).

Based on the cell-line assays, the H₄ receptor ligands were classified either as partial agonists (clobenpropit and ST-1006 (Lim *et al.*, 2005; Sander *et al.*, 2009)) or as full agonists (4-methylhistamine and VUF8430 (Lim *et al.*, 2005; 2006)). This classification is based on the magnitude of their effect in relation to histamine, which is defined as a full agonist with maximum effectiveness. According to our assays, we would classify UR-PI376 as a partial agonist, because its maximum effect is half the effect of histamine. The other H₄ receptor agonists tested show a slightly lower response than

that of histamine, but are still comparable in effectiveness. The classification as partial or full agonist might be dependent on the readout system and probably is different in biological assays, where a submaximal activation of G-protein signalling by a ligand might still result in a full response in the final readout, such as cytokine secretion.

Some of the ligands that we used in our assays were shown to have also affinities to and activities at the histamine H₂ receptor, such as 4-methylhistamine (Lim *et al.*, 2005), VUF8430 (Sterk *et al.*, 1986) and, of course, the endogenous ligand histamine, which binds to all histamine receptors. This is of particular importance because the H₂ receptor is also involved in the down-regulation of IL-12p70 secretion from monocytes by histamine (Gutzmer *et al.*, 2005). Therefore, we tested in our assay the effect of the H₄ receptor antagonist JNJ777120 (Thurmond *et al.*, 2004) and the H₂ receptor antagonist ranitidine (Daly *et al.*, 1981) on the action of the agonists. The action of histamine and 4-methylhistamine was partially blocked by both the H₄ receptor and the H₂ receptor antagonist, whereas the effect of VUF8430 was partially blocked by the H₄ receptor antagonist, but not by the H₂ receptor antagonist. These results confirm that these substances have binding potential at both the H₂ and H₄ receptors.

Recently a study suggested that H₄ receptor signalling can also proceed in a G-protein independent manner by the recruitment of β -arrestin to the H₄ receptor, potentially initiating a signalling cascade, distinct from the G-protein mediated pathway (Rosethorn and Charlton, 2011). However, it is technically difficult to analyze β -arrestin recruitment in native primary cells. As an alternative approach, we performed experiments in presence of the G-protein uncoupling agent PTX. However, we observed that PTX alone down-regulated IL-12p70 production in monocytes, as has already been described (Bagley *et al.*, 2002; Spensieri *et al.*, 2006). Combination of PTX together with the H₄ receptor agonists did not result in any greater inhibition of the secretion of IL-12p70, compared with PTX alone. Because of these difficulties when working with PTX with IL-12p70 as a readout, we can still only speculate that apart from G_{i/o}-dependent signalling, an alternative G_{i/o}-independent pathway might also be involved in histamine-mediated down-regulation of IL-12p70 in monocytes.

As shown by this study as well as others (Reher *et al.*, 2012) there might be some paradoxical effects of H₄ receptor ligands in native cells, which should be kept in mind. As already suggested (Seifert *et al.*, 2011), the open and unbiased analysis of the effects of H₄ receptor ligands in recombinant as well as native systems is necessary to fully understand H₄ receptor signalling and the pathophysiological function of this histamine receptor. Moreover, an important aim in the future should be the development and functional characterization of H₄ receptor ligands in systems closer to native cells and tissues. This would greatly improve the characterization of H₄ receptor ligands as well as the understanding of the biological relevance of the H₄ receptor in various tissues and diseases.

Acknowledgements

We thank Prof. Armin Buschauer from the Department of Pharmaceutical and Medicinal Chemistry II at the University

of Regensburg, Germany, for critical discussions and for sharing his knowledge on H₄ receptor ligand binding to plasma proteins. This study was funded by grants from the Deutsche Forschungsgemeinschaft (DFG): Gu434/5-2 (RG and TW), the Austrian Science Fund (FWF): T545-B19 (MG) and the project Translational Research Innovation – Pharma (TRIP) (HS). We acknowledge the support from the European Community COST Action BM0806 (Recent Advances in histamine H₄ receptor research).

Conflicts of interest

The authors declare no conflict of interest.

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