Molecular Pharmacology Fast Forward. Published on January 25, 2011 as doi:10.1124/mol.111.071266
This article has not been copyedited and formatted. The final version may differ from this version.

MOL #71266

Title page

Paradoxical stimulatory effects of the "standard" histamine H_4-receptor antagonist JNJ7777120: The H_4-receptor joins the club of 7TM receptors exhibiting functional selectivity

Roland Seifert, Erich H. Schneider, Stefan Dove, Irena Brunskole, Detlef Neumann, Andrea Strasser and Armin Buschauer

Institute of Pharmacology, Medical School of Hannover, Hannover, Germany (R.S. and D.N.)

Department of Pharmacology and Toxicology, University of Regensburg, Regensburg, Germany (E.H.S.)

Institute of Pharmaceutical and Medicinal Chemistry II, University of Regensburg, Regensburg, Germany (I.B., S.D., A.S. and A.B.)

Molecular Pharmacology Fast Forward. Published on January 25, 2011 as DOI: 10.1124/mol.111.071266
This article has not been copyedited and formatted. The final version may differ from this version.

Running title page

Running title: Functional selectivity of the H_4R

Correspondence address:

Dr. Roland Seifert

Institute of Pharmacology

Medical School of Hannover

Carl-Neuberg-Str. 1

D-30625 Hannover, Germany

Phone: +49-511-532-2805

Fax: +49-511-532-4081

Email: seifert.roland@mh-hannover.de

Number of text pages: 25

Number of Tables: 1

Number of Figures: 3

Number of references: 40

Number of words in the abstract: 205

Number of words in the main text (introduction and discusssion): 4092

Abbreviations: ERK, extracellular signal-regulated kinase; H_xR, histamine H_1- H_2-, H_3- or H_4-receptor; c, canine, h, human; m, mouse, r, rat; GRK, G-protein-coupled receptor kinase; JNJ7777120, 1-[(5-chloro-1*H*-indol-2-yl)carbonyl]-4-methylpiperazine; PTX, pertussis toxin; 7TM receptor, receptor with 7 transmembrane domains, also referred to as G-protein-coupled receptor (GPCR), although G-protein-independent pathways can also be activated.

Molecular Pharmacology Fast Forward. Published on January 25, 2011 as DOI: 10.1124/mol.111.071266
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

The histamine H_4-receptor (H_4R) is expressed in several cell types of the immune system and is assumed to play an important pro-inflammatory role in various diseases including bronchial asthma, atopic dermatitis and pruritus. Accordingly, H_4R antagonists have been suggested to provide valuable drugs for the treatment of the afore-mentioned diseases. Over the past decade, the indole derivative, 1-[(5-chloro-1*H*-indol-2-yl)carbonyl]-4-methylpiperazine (JNJ7777120), has become the "standard" H_4R antagonist and has been extensively used to assess the pathophysiological role of the H_4R. However, the situation has now become more complicated by recent data (Rosethorne and Charlton, *Mol Pharmacol* (2011), in press; Schnell et al., *Naunyn-Schmiedeberg's Arch Pharmacol* (2011), in press) showing that JNJ7777120 can also activate β-arrestin in a supposedly G_i-protein-independent (pertussis toxin-insensitive) manner and that at certain H_4R species orthologs, JNJ7777120 exhibits partial agonist efficacy with respect to G_i-protein activation (steady-state high-affinity GTPase activity). These novel findings can be explained within the concept of *functional selectivity* or *biased signalling*, assuming unique ligand-specific receptor conformations with distinct signal transduction capabilities. Thus, great caution must be exerted when interpreting *in vivo* effects of JNJ7777120 as H_4R antagonism. We discuss future directions to get out of the dilemma that, currently, there is no "standard" H_4R antagonist available to the scientific community.

Molecular Pharmacology Fast Forward. Published on January 25, 2011 as DOI: 10.1124/mol.111.071266
This article has not been copyedited and formatted. The final version may differ from this version.

Histamine (Fig. 1) is an important neurotransmitter and local mediator (Hill et al., 1997). A decade ago, several groups, independently of each other, identified a novel member of the H_xR family with unique pharmacological properties, the H_4R (for review, see e.g., Hough, 2001; Thurmond et al., 2008; Leurs et al., 2009). The H_4R is a G_i-protein-coupled receptor, causing inhibition of adenylyl cyclase and, in cells of the immune system, activation of phospholipase C *via* release of Gβγ-complexes (Fig. 2A). In cell membranes, activation of G_i-proteins by the H_4R can be monitored by histamine-stimulated [^{35}S]GTPγS binding to, or [γ-^{32}P]GTP hydrolysis by, G_i-proteins (Schneider et al., 2009). The discovery of the H_4R was highlighted in *Molecular Pharmacology* by a *Perspective* by Hough in 2001. Already in this very first review-type publication on this topic, it was noted that the pharmacological properties of the H_4R, although clearly distinct from other H_xRs, differed from each other in various studies. Most notably, one group reported on relatively high affinity of H_4R for H_1R antagonists, but other groups found no interaction of the H_4R with H_1R antagonists (Hough, 2001; Nguyen et al., 2001). Despite considerable efforts, these early-noted discrepancies have not been satisfactorily explained (Deml et al., 2009). And now, again, the H_4R causes headache concerning its pharmacological properties.

The H_4R is expressed in several cell types of the immune system including mast cells, eosinophils, dendritic cells and T lymphocytes. Based on this localization of the receptor and studies with the H_4R knock-out mouse, it has been suggested that the H_4R plays a pro-inflammatory role in bronchial asthma, atopic dermatitis and pruritus and that H_4R antagonists could be useful drugs for the treatment for these conditions (see e.g., Thurmond et al., 2008; Leurs et al., 2009). This suggestion has been corroborated by the finding that the indole derivative JNJ7777120 (Fig. 1), a potent H_4R antagonist (Jablonowski et al., 2003; Venable et al., 2005), exhibits anti-inflammatory effects in mouse asthma model (Dunford et al., 2006). Moreover, JNJ7777120 inhibits the effects of histamine in various cell systems expressing the H_4R (see e.g., Thurmond et al., 2008; Leurs et al., 2009).

Molecular Pharmacology Fast Forward. Published on January 25, 2011 as DOI: 10.1124/mol.111.071266
This article has not been copyedited and formatted. The final version may differ from this version.

Although JNJ7777120 has a relatively short plasma half-life and limited bioavailability, rendering animal experiments requiring continuous exposure to the compound technically difficult (Thurmond et al., 2004), researchers in the H_4R field readily embraced JNJ7777120 because of its availability and high H_4R-selectivity (Jablonowski et al., 2003; Venable et al., 2005). Table 1 summarizes pharmacological data for JNJ7777120 in various *in vitro* test systems. The implementation of JNJ7777120 as "standard" H_4R antagonist was also facilitated by the fact that the first known "standard" H_4R antagonist, thioperamide (Fig. 1), is not selective for the H_4R but it is also a potent H_3R antagonist, rendering it potentially difficult to discriminate H_3R- and H_4R effects *in vivo*. More precisely, thioperamide is a dual H_3R/H_4R inverse agonist, i.e. according to the two-state model of 7TM receptor activation, the compound stabilizes the inactive R state, resulting in a reduction of basal G-protein activation promoted by the agonist-free H_3R and H_4R (Schneider et al., 2009; Schnell et al., 2010). Numerous H_4R ligands structurally related to JNJ7777120 have been synthesized (Venable et al., 2005), but with some exceptions (Schneider et al., 2010), these compounds have not yet been characterized in depth pharmacologically.

Activation of β-arrestin by JNJ7777120: A twisted story. Based on this background, in this issue of *Molecular Pharmacology*, Rosethorne and Charlton (2011) provide important insights into the pharmacological properties of the hH_4R in general and into the properties of JNJ7777120 in particular. Using the U2OS osteosarcoma cell expression system, the authors show that, as expected (Schneider et al., 2009), the endogenous and full agonist histamine stimulates [^{35}S]GTPγS binding in cell membranes, whereas the inverse agonist thioperamide effectively reduces [^{35}S]GTPγS binding, reflecting constitutive activity of hH_4R. With respect to [^{35}S]GTPγS binding, JNJ 7777120 exhibits, to a variable degree, partial inverse agonist or neutral antagonist properties at hH_4R (Schneider et al., 2009, 2010; Rosethorne and Charlton, 2011) (Fig. 2A, Table 1). In addition to stimulation of [^{35}S]GTPγS binding, histamine also

Molecular Pharmacology Fast Forward. Published on January 25, 2011 as DOI: 10.1124/mol.111.071266
This article has not been copyedited and formatted. The final version may differ from this version.

stimulates binding of β-arrestin to hH_4R. Traditionally, β-arrestin recruitment to 7TM receptors has been linked to receptor-uncoupling from G-proteins and desensitization (Rajagopal et al., 2010; Luttrell and Gesty-Palmer, 2010). However, more recently, it has become clear that β-arrestin can also serve as signal-transducing protein, stimulating G-protein-independent signal transduction pathways such as ERK (Rajagopal et al., 2010; Luttrell and Gesty-Palmer, 2010). For this reason, GPCRs should actually be more correctly referred to as 7TM receptors, giving credit to the universal heptahelical structure of these proteins instead of their signal transduction pathways that are not necessarily G-protein-mediated.

So far, the data of Rosethorne and Charlton (2011) fit into established paradigms, but the headache starts with their finding that JNJ7777120 behaves as a partial agonist with respect to β-arrestin binding to hH_4R (Fig. 2A, Table 1). This effect was observed at various H_4R expression levels, ruling out the possibility that excess H_4R molecules, referred to as receptor reserve, could account for the unexpected effects. Moreover, the effect of JNJ7777120 appears to be G_i-protein-independent as is suggested by the lacking influence of the ADP-ribosyltransferase, PTX, on β-arrestin binding (but see Discussion below regarding the G-protein aspect). The authors provide evidence that the effect of JNJ7777120 on β-arrestin recruitment is mediated by the H_4R and not through another receptor. Specifically, thioperamide which is without stimulatory effect in this assay by itself, blocks the effects of JNJ7777120 on β-arrestin recruitment competitively, and the pA_2 values of thioperamide for blockade of the JNJ7777120 response and the response of the H_4R agonist clobenpropit are very similar. Unfortunately, the pA_2 for the endogenous H_4R ligand histamine was not reported. This is not trivial since the apparent affinity of thioperamide may be ligand-dependent.

Molecular Pharmacology Fast Forward. Published on January 25, 2011 as DOI: 10.1124/mol.111.071266
This article has not been copyedited and formatted. The final version may differ from this version.

Most striking is the finding that β-arrestin binding to hH_4R is not a dead-end. Particularly, JNJ7777120 induces very effective and prolonged ERK activation, whereas histamine induces only transient ERK activation. These time courses are typical for arrestin-dependent and G-protein-dependent signal transduction, respectively (Luttrell and Gesty-Palmer, 2010), but the two pathways were not dissected with PTX in the study of Rosethorne and Charlton (2011) (see Discussion below). In any case, these data show that JNJ7777120 is capable of stabilizing a conformation in hH_4R that induces β-arrestin recruitment and stimulates an important down-stream signalling pathway, at least in an osteosarcoma cell line. Thus, JNJ7777120 cannot be considered as the "standard" H_4R antagonist anymore, but depending on the parameter assessed, JNJ7777120 may also act as agonist. Noteworthy, with respect to β-arrestin recruitment, JNJ7777120 acts only as partial agonist, but with respect to ERK activation, JNJ7777120 is actually a full agonist. However, the stimulatory effects of JNJ7777120 on ERK activation was only reported for an exceedingly high ligand concentration (100 μM), a concentration that is almost 10,000-fold higher than the EC_{50} for β-arrestin recruitment (Table 1). Thus, one cannot exclude the possibility that in addition to β-arrestin, other signal transduction pathways are involved in JNJ7777120-induced ERK activation. Evidently, it will now be very important to study in great detail the effect of JNJ7777120 on ERK activation in more commonly used and well-characterized expression systems such as HEK293 cells and in cells endogenously expressing hH_4R. Eosinophils are a well-established native cell system for studying hH_4R functions (Table 1). It is likely that the effects of JNJ7777120 strongly depend on the endogenous complement of signal transduction proteins in the cells harbouring hH_4R.

Additional evidence for JNJ7777120 and structurally related compounds being more than just H_4R "antagonists" comes from a recent analysis of a series of 25 indole-, benzimidazole- and thienopyrrole compounds at the recombinant hH_4R expressed in Sf9 insect ells (Schneider et al., 2010). In this system, for a subset of compounds including

Molecular Pharmacology Fast Forward. Published on January 25, 2011 as DOI: 10.1124/mol.111.071266
This article has not been copyedited and formatted. The final version may differ from this version.

JNJ7777120 we observed quite substantial differences between K_i values in [^{3}H]histamine competition binding studies and EC_{50} values for inverse agonistic activity in the GTPase assays (Schneider et al., 2010) (Table 1). Although the data on JNJ7777120 compiled in Table 1 have to be compared with caution because they were obtained in different cell types and because different parameters were determined, it is evident that the apparent affinities/potencies of JNJ7777120 can vary considerably among the various studies, a property that is not commonly observed for classic receptor antagonists. For example, in the studies of Ling et al. (2004) and Barnard et al. (2008), the IC_{50} values for JNJ7777120 on various functional parameters in human eosinophils endogenously expressing hH_4R differ up to 50-fold (Table 1). These differences cannot be explained by the relatively small differences in the stimulatory histamine concentration used. Such data on divergent ligand affinities/potencies regarding various parameters support the notion that JNJ7777120 and related compounds stabilize functionally distinct hH_4R conformations.

While the data from Rosethorne and Charlton (2011) are certainly unexpected for the H_4R community and raise many questions of how JNJ7777120 effects *in vitro* and particularly *in vivo* should be interpreted, in a broader conceptual context, the findings are actually not that surprising. It is just that the H_4R now joins the growing family of 7TM receptors showing *functional selectivity* or *biased signalling*. This concept states that any given ligand stabilizes a unique conformation in a particular 7TM receptor that is then capable of activating a unique pattern of G-protein-dependent and -independent signal transduction pathways (Galandrin et al., 2007; Rajagopal et al., 2010). β-Adrenergic receptor antagonists are a very prominent and well-studied class of ligands for which functional selectivity has been documented (Galandrin et al., 2007).

Even more twists with JNJ7777120, extending to thioperamide. But the JNJ7777120 story has some additional unexpected twists. First, we have recently studied the effects of

Molecular Pharmacology Fast Forward. Published on January 25, 2011 as DOI: 10.1124/mol.111.071266
This article has not been copyedited and formatted. The final version may differ from this version.

JNJ7777120 at recombinant hH_4R, mH_4R and cH_4R expressed in Sf9 cells, using the steady-state high-affinity GTPase assay as parameter (Schnell et al., 2011). It is important to characterize the effects of JNJ7777120 at those H_4R species orthologs because mouse, rat and dog are important laboratory animal species for assessing the pathophysiological role of the H_4R (Liu et al., 2001; Dunford et al., 2006). Most strikingly, at mH_4R, rH_4R and cH_4R expressed in S9 cells, JNJ7777120 exhibits strong partial agonism with respect to activation of G_i-proteins (Fig. 2B). However, a comparison of the efficacies must consider the constitutive activity which is very high in the case of the hH_4R and very low for the mH_4R, rH_4R and cH_4R (Schnell et al., 2011). Thus, JNJ7777120 may lead to a similar equilibrium of active and inactive states for all H_4R orthologs which appears as inverse agonism at hH_4R due to the shift of the basal equilibrium towards the active state (R*), and as partial agonism at mH_4R, rH_4R and cH_4R because of the shift of the basal equilibrium towards the inactive (R) state. The effect of JNJ7777120 on β-arrestin activation has not yet been studied with these H_4R species orthologs.

And then there is still another twist extending to thioperamide. To his end, in all studies including the study of Rosethorne and Charlton (2011), there has been consensus that thioperamide acts as H_4R inverse agonist (Leurs et al., 2009; Schneider et al., 2009). However, at cH_4R, thioperamide clearly exhibits partial agonistic efficacy, and at mH_4R and rH_4R, thioperamide is a neutral antagonist with respect to G_i-protein activation (Schnell et al., 2011). Thus, depending on the species studied, thioperamide can stabilize either only inactive or both inactive and active H_4R conformations, and the functional behavior of this compound is clearly different from the behavior of JNJ7777120 (Figs. 2B and 2C). It is also possible that the controversial effects of H_1R antagonists observed at the H_4R (Hough, 2001; Nguyen et al., 2001; Deml et al., 2009) are due to functional selectivity. At least, the H_1R antagonist data are reminiscent of what has been observed for JNJ7777120 (Hough, 2001; Deml et al., 2009) (Table 1), and a careful analysis of this problem is warranted.

Molecular Pharmacology Fast Forward. Published on January 25, 2011 as DOI: 10.1124/mol.111.071266
This article has not been copyedited and formatted. The final version may differ from this version.

What is the molecular basis for the divergent effects of JNJ7777120 on various signalling pathways promoted by hH_4R? Also a twisted story. Recently, we have developed a model of the interaction of JNJ7777120 and related compounds with hH_4R (Schneider et al., 2010). We suggested that JNJ7777120 prevents the indole ring of $Trp^{6.48}$, the key element of the proposed 7TM receptor-activating toggle switch, from changing the inactive vertical position into the horizontal position (Schneider et al., 2010). However, JNJ7777120 stabilizes both inactive and active hH_4R states (see above and Fig. 2). Therefore, the compound was docked into models of the inactive hH_4R (Fig. 3A) and of the active hH_4R (Fig. 3B). The positively charged amine moiety of JNJ7777120 interacts electrostatically with the highly conserved $Asp^{3.32}$ in both states. In the inactive hH_4R, the indole moiety of JNJ7777120 adopts a nearly vertical position with respect to the longitudinal axis of the receptor, placed between TM III and TM VI. This conformation stabilizes the indole ring of $Trp^{6.48}$ in vertical position, too (Fig. 3A). These assumptions correspond to published data of related compounds (Schneider et al., 2010). For the active state of the hH_4R, the docking studies suggest an alternative binding mode of JNJ7777120 where the indole moiety is stacked between the aromatic side chains of $Trp^{6.48}$ and $Tyr^{6.51}$. This interaction stabilizes the indole ring of $Trp^{6.48}$, discussed to be involved in the rotamer toggle switch during receptor activation, in a more horizontal position (Fig. 3B). Based on the present models, no differences in amino acids directly interacting with JNJ7777120 in the binding pocket of inactive and active hH_4R are obvious. Unfortunately, no conclusions can be drawn from the models about the molecular mechanism by which JNJ7777120 induces and stabilizes a β-arrestin-binding conformation of the hH_4R, but in the following, we will propose a mechanism based on the available literature.

Non-visual arrestins 2 and 3 preferentially bind to active phosphorylated 7TM receptors but, e.g. in the case of β_2 adrenergic and M_2 muscarinergic receptors, also to phosphorylated inactive states (~ two-fold lower binding, Gurevich and Gurevich, 2006). However, unphosphorylated receptor states must be active to bind arrestins with sufficient

Molecular Pharmacology Fast Forward. Published on January 25, 2011 as DOI: 10.1124/mol.111.071266
This article has not been copyedited and formatted. The final version may differ from this version.

affinity. Thus, recruitment of β-arrestin to an inactive (not G-protein coupled) receptor state requires phosphorylation of multiple serine and threonine residues in the intracellular loops and the C-terminal tail, 7TM receptor regions that all have been shown to contain phosphorylation sites relevant for arrestin binding (Gurevich and Gurevich, 2006). 7TM receptors are mainly phosphorylated by GRKs. Among them, GRKs 5 and 6 are independent of translocation by Gβγ-subunits and exclusively responsible for ERK1/2 activation by arrestins (Kim et al., 2005; Ren et al., 2005).

Since the JNJ7777120-mediated recruitment of β-arrestin supposedly does not depend on G-proteins (Rosethorne and Charlton, 2011) (see Discussion below), JNJ7777120 may stabilize a specific hH_4R state which does not activate G_i-proteins but which in the first place facilitates phosphorylation of newly exposed serine and threonine residues by Gβγ-independent GRKs. Some of the phosphorylated serine/threonine sites or clusters may then interact with the lysine- and arginine-rich polar core of β-arrestin. Specific phosphorylation patterns differentially orient β-arrestin on the receptor and stabilize different active arrestin conformations, leading to structurally and functionally distinct arrestin-receptor complexes (Gurevich and Gurevich, 2006). To enable high-affinity hH_4R-binding and activation of such a functionally distinct β-arrestin state resulting in prolonged ERK activation (Rosethorne and Charlton, 2011), JNJ7777120 should therefore expose the proper pattern of hH_4R phosphorylation sites by conformational changes in the intracellular loops and the C-terminal tail. Possibly, these conformational changes also enable interactions with additional arrestin regions, contributing to the activation of the complex *via* induced fit.

And even more twists: Are the effects of JNJ7777120 on β-arrestin recruitment really G-protein-independent? The conclusion that JNJ7777120 induces β-arrestin recruitment in a G-protein-independent manner is based on the findings that the ligand does

Molecular Pharmacology Fast Forward. Published on January 25, 2011 as DOI: 10.1124/mol.111.071266
This article has not been copyedited and formatted. The final version may differ from this version.

not stimulate [^{35}S]GTPγS binding and the lacking effect of PTX on β-arrestin recruitment (Rosethorne and Charlton, 2011). PTX, *via* ADP-ribosylation of G_i-protein α-subunits, uncouples 7TM-receptors from G_i-proteins and is a most valuable tool for unmasking G_i-protein-dependent pathways (Ui and Katada, 1990). While inhibitory effects of PTX on receptor-mediated signalling can be readily interpreted, specifically if the proper controls with the B-oligomer (and not only the carrier solvent) are performed, the interpretation of negative PTX data is more complicated. In particular, it is difficult to completely ADP-ribosylate all G_i-protein α-subunits in cells because G_i-proteins are so abundant (Ui and Katada, 1990). For this reason, PTX effects are often only incomplete. Accordingly, one cannot exclude the possibility that a fraction of the available G_i-proteins in the osteosarcoma cell expression system used by Rosethorne and Charlton (2011) was still functional, and evidently, those non-ADP-ribosylated G_i-proteins could participate in signalling, including β-arrestin recruitment and ERK activation (Walters et al., 2009). At the membrane level, activation of G_i-protein may be too small to be detected, but the β-arrestin assay may be sufficiently sensitive to detect G_i-protein activation.

Rosethorne and Charlton (2011) incubated the cells for 20-hours with 200 ng/ml PTX, but even a 24-hour incubation with PTX at a concentration of 1 μg/ml may be insufficient to functionally eliminate all G_i-proteins from receptor-coupling (Ui and Katada, 1990). It is possible to assess the effectiveness of PTX-catalyzed ADP-ribosylation by treating membranes from PTX-treated cells with pre-activated PTX and [^{32}P]NAD and then performing SDS polyacrylamide electrophoresis with subsequent quantitative autoradiography. Effective ADP-ribosylation of G_i-proteins in intact cells results in poor incorporation of [^{32}P]ADP-ribose in the subsequent membrane incubation with activated PTX. Unfortunately, this important control experiment was not performed in the study of Rosethorne and Charlton (2011).

Molecular Pharmacology Fast Forward. Published on January 25, 2011 as DOI: 10.1124/mol.111.071266
This article has not been copyedited and formatted. The final version may differ from this version.

Moreover, the specific activity of PTX from various commercial suppliers can be quite different and vary considerably from batch to batch, so that it is always essential to include a positive control experiment to document functionality of PTX. Unfortunately, the source of PTX is not mentioned in the study by Rosethorne and Charlton (2011), and the authors also did not present a positive control experiment showing that PTX actually functioned properly. Such a positive control could have been provided by demonstrating a lack of stimulatory effect of histamine on [^{35}S]GTPγS binding in membranes from PTX-treated cells and a decrease in basal [^{35}S]GTPγS binding, reflecting uncoupling of constitutively active receptors from G_i-proteins (Seifert and Wenzel-Seifert, 2003).

Considering the constitutive activity of the hH_4R, the function of JNJ7777120 as weak partial inverse agonist or even neutral antagonist on [^{35}S]GTPγS binding in the study of Rosethorne and Charlton (2011) implies that at least a small part of JNJ7777120-bound hH_4R molecules stays in an active state further activating G_i-proteins. Insofar, a fraction of remaining functional G_i-proteins would not necessarily change β-arrestin recruitment after PTX incubation and may also lead to the congruent concentration-response curves of JNJ7777120-mediated β-arrestin binding with and without PTX (Rosethorne and Charlton, 2011).

Alternatively, Rosethorne and Charlton (2011) could have studied the effects of PTX on histamine- and JNJ7777120-induced ERK activation. Based on the time course, the effects of histamine would be expected to be PTX-sensitive, whereas the effects of JNJ7777120 would be predicted to be PTX-insensitive (Luttrell and Gesty-Palmer, 2010). However, no such control experiments were performed. PTX-sensitive arrestin recruitment has been reported for G_i-coupled receptors (Walters et al., 2009). Intriguingly, the effect of histamine on β-arrestin recruitment in the osteosarcoma cell line is apparently PTX-insensitive, too (Rosethorne and Charlton, 2011). This result is not necessarily in contrast with the possibility

Molecular Pharmacology Fast Forward. Published on January 25, 2011 as DOI: 10.1124/mol.111.071266
This article has not been copyedited and formatted. The final version may differ from this version.

that, in the presence of PTX, histamine recruits β-arrestin in a G_i-protein-independent manner different from a Gβγ-dependent manner without PTX, since β-arrestin *binding curves* may be similar in both cases. But this possibility would imply that the *time course of ERK activation* by histamine changes after PTX treatment (Luttrell and Gesty-Palmer, 2010; Rosethorne and Charlton, 2011), a consequence which must be checked to exclude insufficient PTX function in the study of Rosethorne and Charlton (2011).

Moreover, one cannot dismiss the possibility that JNJ7777120 stabilizes an hH_4R conformation that enables the receptor to interact even with ADP-ribosylated G_i-protein α-subunits or to preferentially interact with PTX-insensitive G-proteins. Because of slow guanine nucleotide exchange, it can be very difficult or impossible to detect activation of PTX-insensitive G-proteins in the [^{35}S]GTPγS binding assay (Wenzel-Seifert and Seifert, 2000), but activation of PTX-insensitive G-proteins can be assessed more readily with the G-protein photoaffinity labelling/immunoprecipitation technique (Laugwitz et al., 1996). However, such experiments were not conducted in the study of Rosethorne and Charlton (2011). These limitations regarding the G-protein aspect of the study of Rosethorne and Charlton (2011) should be addressed in future studies but do not question the fundamental issue of paradoxical effects of JNJ7777120 at the hH_4R level. We simply have to be cautious with the conclusion that JNJ7777120 recruits β-arrestin without any involvement of active G proteins.

Are H_4R agonists an alternative to JNJ7777120 and thioperamide? So, if pharmacological effects of JNJ7777120 and thioperamide cannot be anymore taken for granted as antagonist actions, what about the use of agonists as experimental tools for assessing the pathophysiological role of H_4R? We have recently reviewed the hH_4R agonist literature (Igel et al., 2010). Unfortunately, the situation with agonists is not easier than with H_4R "antagonists". For example, 5(4)-methylhistamine, originally described as H_2R agonist

Molecular Pharmacology Fast Forward. Published on January 25, 2011 as DOI: 10.1124/mol.111.071266
This article has not been copyedited and formatted. The final version may differ from this version.

(Black et al., 1972), displays selectivity for recombinant hH_4R relative to the other H_xRs (Lim et al., 2005). However, when applied *in vivo*, one does not know what the actual concentration of the ligand in a particular organ is, so that effects on H_xRs other than the H_4R, specifically the H_2R, cannot be excluded. The anti-inflammatory effects of 5(4)-methylhistamine and JNJ7777120 in a mouse asthma model could be interpreted as agonistic effects of the ligands on the H_4R (Morgan et al., 2007; Neumann et al., 2010), but the effects of 5(4)-methylhistamine could also be mediated *via* activation of H_2R, exhibiting an established anti-inflammatory role (Hill et al., 1997).

Recently, we described the cyanoguanidine UR-PI376 as a potent and selective hH_4R agonist (Igel et al., 2009a), but for studies in mouse, rat and dog, the compound is not useful because of low potency and efficacy (Schnell et al., 2011). The N^G-acylated imidazolylpropylguanidine H_4R agonist UR-PI294 (Igel et al., 2009b) should also only be used with caution, considering the mechanistically still unexplained and very unusual "superagonism" at recombinant rH_4R, measuring G_i-protein-catalyzed GTP hydrolysis as parameter (Schnell et al., 2011).

Conclusions and future studies. Even at the very beginning, the H_4R pharmacology caused headache as exemplified by contradictory and still unresolved H_1R antagonist effects (Hough, 2001). This initial phase was followed by a relatively headache-free phase in which these intriguing H_1R antagonist effects were put aside and JNJ7777120 rapidly advanced to the status of "standard" H_4R antagonist. And now we have headache, again, because multiple ligands encompassing classic H_4R "antagonists" such as JNJ7777120 and thioperamide, and even agonists show paradoxical, unexpected, unexplained and complex effects in various systems (Table 1 and Figs. 2 and 3).

The recent data by Rosethorne and Charlton (2011) and other groups have important implications for future research in the H_4R field. First, considering the lack of a standard H_4R

Molecular Pharmacology Fast Forward. Published on January 25, 2011 as DOI: 10.1124/mol.111.071266
This article has not been copyedited and formatted. The final version may differ from this version.

antagonist, it is necessary to study multiple structurally diverse compounds at multiple H_4R orthologs, assessing multiple G-protein-dependent and -independent parameters, both in agonist, antagonist and inverse agonist modes. There is no way to avoid these painful experimental approaches since even among structurally very similar compounds, unexpected pharmacological differences may be uncovered (Schneider et al., 2010). Second, the above-mentioned pharmacological studies have to be accompanied by mechanistic studies aiming at the elucidation of the structural basis for the functional diversity, both with respect to receptors and ligands. This is also not an easy task in view of the fact that the H_4R species orthologs are structurally very different from each other (Lim et al., 2008, 2010; Schnell et al., 2011). In fact, combinations of amino acids and entire receptor regions may account for the different pharmacological properties of H_4R orthologs. Third, crystal structures of the H_4R would be most useful, but again, structures with multiple ligands and multiple interacting proteins such as G-proteins and arrestins would be required. From all these considerations, it becomes clear that the challenges in the H_4R field are formidable, and presently, it is not clear whether a "standard" H_4R antagonist will ever be identified. But perhaps another strategy will help us out of the dilemma in a relatively short period of time: It is possible that other groups have observed paradoxical effects of H_4R ligands in general and JNJ7777120 in particular as well but have elected not to publish the data so far because they "don't fit" to *current*, or more correctly, as is outlined in this article, *past* paradigms. Open and *unbiased* documentation of H_4R ligand effects in recombinant and native systems will help us understand *biased* H_4R signalling and the still poorly understood pathophysiological function of the H_4R.

Acknowledgements

We would like to thanks Drs. P. Igel and D. Schnell for long-standing collaboration on the H_4R project and Mrs. A. Stanke for design of Figure 2.

Molecular Pharmacology Fast Forward. Published on January 25, 2011 as DOI: 10.1124/mol.111.071266
This article has not been copyedited and formatted. The final version may differ from this version.

Authorship contributions

Performed data analysis: Seifert, Schneider, Dove, Brunskole, Neumann, Strasser, Buschauer

Wrote or contributed to the writing of the manuscript: Seifert, Schneider, Dove, Brunskole, Neumann, Strasser, Buschauer

Other (conceptual design of the article): Seifert, Buschauer

Other (development of receptor models): Dove, Strasser

Molecular Pharmacology Fast Forward. Published on January 25, 2011 as DOI: 10.1124/mol.111.071266
This article has not been copyedited and formatted. The final version may differ from this version.

References

Barnard R, Barnard A, Salmon G, Liu W, Sreckovic S (2008) Histamine-induced actin polymerization in human eosinophils: an imaging approach for histamine H_4 receptor. *Cytometry A* 73:299-304.

Black JW, Duncan WA, Durant CJ, Ganellin CR, Parsons EM (1972) Definition and antagonism at histamine H_2-receptors. *Nature* 236:385-390.

Deml KF, Beermann S, Neumann D, Strasser A, Seifert R (2009) Interactions of histamine H_1-receptor agonists and antagonists with the human histamine H_4-receptor. *Mol Pharmacol* 76:1019-1030.

Dunford PJ, O'Donnell N, Riley JP, Williams KN, Karlsson L, Thurmond RL (2006) The histamine H_4 receptor mediates allergic airway inflammation by regulating the activation of CD^{4+} T cells. *J Immunol* 176:7062-7070.

Galandrin S, Oligny-Longpré G, Bouvier M (2007) The evasive nature of drug efficacy: implications for drug discovery. *Trends Pharmacol Sci* 28:423-430.

Gurevich VV, Gurevich EV (2006) The structural basis of arrestin-mediated regulation of G-protein-coupled receptors. *Pharmacol Ther* 110**:**465–502.

Hill SJ, Ganellin CR, Timmerman H, Schwartz JC, Shankley NP, Young JM, Schunack W, Levi R, and Haas HL (1997) International Union of Pharmacology. XIII. Classification of histamine receptors. *Pharmacol Rev* 49:253-278.

Hough LB (2001) Genomics meets histamine receptors: new subtypes, new receptors. *Mol Pharmacol* 59:415-419.

Jablonowski JA, Grice CA, Chai W, Dvorak CA, Venable JD, Kwok AK, Ly KS, Wei J, Baker SM, Desai PJ, Jiang W, Wilson SJ, Thurmond RL, Karlsson L, Edwards JP, Lovenberg TW, Carruthers NI (2003) The first potent and selective non-imidazole human histamine H_4 receptor antagonists. *J Med Chem* 46:3957-3960.

Jiang W, Lim HD, Zhang M, Desai P, Dai H, Colling PM, Leurs R, Thurmond RL (2008) Cloning and pharmacological characterization of the dog histamine H_4 receptor. *Eur J Pharmacol* 592:26-32.

Igel P, Geyer R, Straßer A, Dove S, Seifert R and Buschauer A (2009a) Synthesis and structure-activity relationships of cyanoguanidine-type and structurally related histamine H_4 receptor agonists. *J Med Chem* 52:6297-6313.

Igel P, Schneider E, Schnell D, Elz S, Seifert R and Buschauer A (2009b) N^G-Acylated imidazolylpropylguanidines as potent histamine H_4 receptor agonists: selectivity by variation of the N^G-substituent. *J Med Chem* 52:2623-2627.

Igel P, Dove S, Buschauer A (2010) Histamine H_4 receptor agonists. *Bioorg Med Chem Lett* 20:7191-7199.

Kim J, Ahn S, Ren XR, Whalen EJ, Reiter E, Wei H, Lefkowitz RJ (2005) Functional antagonism of different G protein-coupled receptor kinases for betaarrestin-mediated angiotensin II receptor signaling. *Proc Natl Acad Sci USA* 102:1442–1447.

Laugwitz KL, Allgeier A, Offermanns S, Spicher K, Van Sande J, Dumont JE, Schultz G (1996) The human thyrotropin receptor, a heptahelical receptor capable of stimulating members of all four G protein families. *Proc Natl Acad Sci USA* 93:116-120.

Leurs R, Chazot PL, Shenton FC, Lim HD, de Esch IJ (2009) Molecular and biochemical pharmacology of the histamine H_4 receptor. *Br J Pharmacol* 157:14-23.

Lim HD, van Rijn RM, Ling P, Bakker RA, Thurmond RL, Leurs R (2005) Evaluation of histamine H_1-, H_2-, and H_3-receptor ligands at the human histamine H_4 receptor: identification of 4-methylhistamine as the first potent and selective H_4 receptor agonist. *J Pharmacol Exp Ther* 314:1310-1321.

Lim HD, Jongejan A, Bakker RA, Haaksma E, de Esch IJ, Leurs R (2008) Phenylalanine 169 in the second extracellular loop of the human histamine H_4 receptor is responsible for

the difference in agonist binding between human and mouse H_4 receptors. *J Pharmacol Exp Ther* 327:88-96.

Lim HD, de Graaf C, Jiang W, Sadek P, McGovern PM, Istyastono EP, Bakker RA, de Esch IJ, Thurmond RL, Leurs R (2010) Molecular determinants of ligand binding to H_4R species variants. *Mol Pharmacol* 77:734-743.

Ling P, Ngo K, Nguyen S, Thurmond RL, Edwards JP, Karlsson L , Fung-Leung WP (2004) Histamine H_4 receptor mediates eosinophil chemotaxis with cell shape change and adhesion molecule upregulation. *Br J Pharmacol* 142:161-171.

Liu C, Wilson SJ, Kuei C, Lovenberg TW (2001) Comparison of human, mouse, rat, and guinea pig histamine H_4 receptors reveals substantial pharmacological species variation. *J Pharmacol Exp Ther* 299:121-130.

Luttrell LM, Gesty-Palmer D (2010) Beyond desensitization: Physiological relevance of arrestin-mediated signaling. *Pharmacol Rev* 62:305-330.

Meng J, Ma X, Li M, Jia M, Luo X (2008) Histamine H_4 receptors regulate ACTH release in AtT-20 cells. *Eur J Pharmacol* 587:336-338.

Morgan RK, McAllister B, Cross L, Green DS, Kornfeld H, Center DM, Cruikshank WW (2007) Histamine 4 receptor activation induces recruitment of FoxP3+ T cells and inhibits allergic asthma in a murine asthma model. *J Immunol* 178:8081-8089.

Neumann D, Beermann S, and Seifert R (2010) Does the histamine H_4 receptor have a pro- or anti-inflammatory role in murine bronchial asthma? *Pharmacology* 85:217-223.

Nguyen T, Shapiro DA, George SR, Setola V, Lee DK, Cheng R, Rauser L, Lee SP, Lynch KR, Roth BL, O'Dowd BF (2001) Discovery of a novel member of the histamine receptor family. *Mol Pharmacol* 59:427-433.

Rajagopal S, Rajagopal K, Lefkowitz RJ (2010) Teaching old receptors new tricks: biasing seven-transmembrane receptors. *Nat Rev Drug Disc* 9:373-386.

Ren XR, Reiter E, Ahn S, Kim J, Chen W, Lefkowitz RJ (2005) Different G protein-coupled receptor kinases govern G protein and beta-arrestin-mediated signaling of V2 vasopressin receptor. *Proc Natl Acad Sci USA.* 102:1448–1453.

Rosethorne EM, Charlton SJ (2011) Agonist-biased signalling at the histamine H_4 receptor. JNJ7777120 recruits β-arrestin without activating G proteins. *Mol Pharmacol* (in press).

Schneider EH, Schnell D, Papa D, and Seifert R (2009) High constitutive activity and a G-protein-independent high-affinity state of the human histamine H_4-receptor. *Biochemistry* 48:1424-1438.

Schneider EH, Strasser A, Thurmond RL, Seifert R (2010) Structural requirements for inverse agonism and neutral antagonism of indole-, benzimidazole-, and thienopyrrole-derived histamine H_4 receptor ligands. *J Pharmacol Exp Ther* 334:513-521.

Schnell D, Burleigh K, Trick J, Seifert R (2010) No evidence for functional selectivity of proxyfan at the human histamine H_3 receptor coupled to defined G_i/G_o protein heterotrimers. *J Pharmacol Exp Ther* 332:996-1005.

Schnell D, Brunskole I, Ladova K, Schneider EH, Igel P, Dove S, Buschauer A, Seifert R (2011) Expression and functional properties of canine, rat and murine histamine H_4-receptors in Sf9 insect cells. *Naunyn-Schmiedeberg's Arch Pharmacol* (in press)

Seifert R, Wenzel-Seifert K (2003) The human formyl peptide receptor as model system for constitutively active G-protein-coupled receptors. *Life Sci* 73:2263-2280.

Thurmond RL, Desai PJ, Dunford PJ, Fung-Leung WP, Hofstra CL, Jiang W, Nguyen S, Riley JP, Sun S, Williams KN, Edwards JP, Karlsson L (2004) A potent and selective histamine H_4 receptor antagonist with anti-inflammatory properties. *J Pharmacol Exp Ther* 309:404-413.

Thurmond RL, Gelfand EW, Dunford PJ (2008) The role of histamine H_1 and H_4 receptors in allergic inflammation: the search for new antihistamines. *Nat Rev Drug Discov* 7:41-53.

Ui M, Katada T (1990) Bacterial toxins as probe for receptor-G_i coupling. *Adv Second Messenger Phosphoprotein Res* 24:63-69.

Venable JD, Cai H, Chai W, Dvorak CA, Grice CA, Jablonowski JA, Shah CR, Kwok AK, Ly KS, Pio B, Wei J, Desai PJ, Jiang W, Nguyen S, Ling P, Wilson SJ, Dunford PJ, Thurmond RL, Lovenberg TW, Karlsson L, Carrthers NI, Edwards JP (2005) Preparation and biological evaluation of indole, benzimidazole, and thienopyrrole piperazine carboxamides: potent human H_4 antagonists. *J Med Chem* 48:8289-8298.

Walters RW, Shukla AK, Kovacs JJ, Violin JD, DeWire SM, Lam CM, Chen JR, Muehlbauer MJ, Whalen EJ, Lefkowitz RJ (2009) Beta-arrestin 1 mediates nicotinic acid-induced flushing, but not its antilipolytic effect, in mice. *J Clin Invest* 119:1312-1321.

Wenzel-Seifert K, Seifert R (2000) Molecular analysis of β_2-adrenoceptor coupling to G_s-, G_i-, and G_q-proteins. *Mol Pharmacol* 58:954-966.

Molecular Pharmacology Fast Forward. Published on January 25, 2011 as DOI: 10.1124/mol.111.071266
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes

This work was supported by grants of the Deutsche Forschungsgemeinschaft [GRK 760, GRK 1441, SFB 587, STR 1125/1-1] and the European Union [COST program BM0806 (H_4R network)].

Correspondence address:

Dr. Roland Seifert

Institute of Pharmacology

Medical School of Hannover

Carl-Neuberg-Str. 1

D-30625 Hannover, Germany

Phone: +49-511-532-2805

Fax: +49-511-532-4081

Email: seifert.roland@mh-hannover.de

Molecular Pharmacology Fast Forward. Published on January 25, 2011 as DOI: 10.1124/mol.111.071266
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 1. Structures of selected H_4R ligands analyzed in the present study. According to conventional definition, histamine, 5(4)-methylhistamine, UR-PI294 and UR-PI376 are H_4R agonists, whereas thioperamide and JNJ7777120 are H_4R antagonists. The classification of the compounds depends substantially on the specific system analyzed, and most strikingly, even the "standard" antagonists JNJ7777120 and thioperamide can exhibit agonistic effects at H_4R, reflecting *functional selectivity* or *biased signalling*.

Figure 2. Functional selectivity of JNJ7777120 and thioperamide. A, at hH_4R histamine is a full agonist with respect to G_i- and β-arrestin activation. JNJ7777120 is a partial inverse agonist with respect to G_i-protein activation and a partial agonist with respect to β-arrestin activation. **B**, at mH_4R, rH_4R and cH_4R, HA is a full agonist with respect to G_i activation in Sf9 insect cell membranes, while JNJ7777120 is a partial agonist at these H_4R orthologs. The effects on β-arrestin activation have not yet been studied. **C**, Considering the effects of thioperamide on G_i-protein activation catalyzed by various H_4R species orthologs, effects may range from "full" inverse agonism (even thioperamide is actually not a full inverse hH_4R agonist (Schneider et al., 2009)) over neutral antagonism to weak partial agonism.

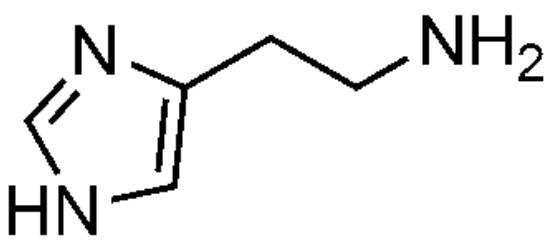
Figure 3. Model of the interaction of JNJ7777120 with inactive and active hH_4R. Homology models of the inactive and active hH_4R were generated as described (Schneider et al., 2010). For modelling of the inactive hH_4R, the crystal structure of the β_2 adrenergic receptor 2RH1.pdb was used as template, whereas for modelling of the active hH_4R, the crystal structure of active opsin 3DQB.pdb was applied (http://www.pdb.org). JNJ7777120 was docked into both models, using SYBYL 7.3 (Tripos, St. Louis, MO). **A**, JNJ7777120 docked into the inactive hH_4R. **B**, JNJ7777120 docked into the active hH_4R.

Table 1: Characterization of JNJ7777120 in different *in vitro* test systems

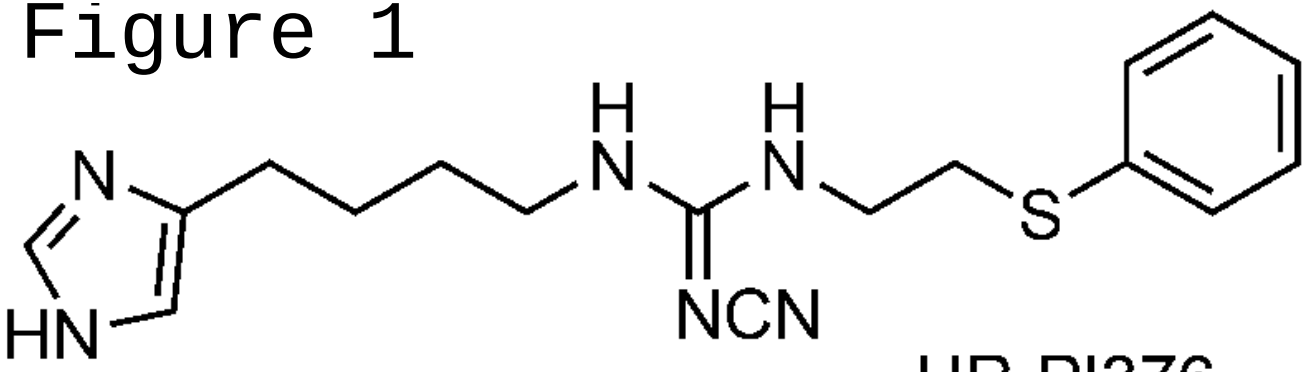
Expression system	Assays	Important findings concerning JNJ7777120	Reference
Human SK-N-MC cells	Radioligand binding cAMP-CRE gene reporter assay	Human H_4R, [^{3}H]histamine competition binding: K_i = 4.1 nM, K_d = 4 nM and pA_2 = 7.9 nM cAMP assay with human, mouse, rat H_4R: equipotent **antagonistic** potency 1000-fold selectivity relative to H_1R, H_2R and H_3R no cross-reactivity with 50 other targets	(Jablonowski et al., 2003) (Thurmond et al., 2004)
Mouse bone marrow-derived mast cells	*in vitro* mast cell chemotaxis assay	**Antagonist**: inhibition of chemotaxis in mast cells, induced by 10 µM of histamine: IC_{50} = 40 nM	
Endogenous (human eosinophils)	Flow cytometry, in vitro chemotaxis assays	**Antagonist:** inhibition of eosinophil shape change, induced by 1 µM of histamine: IC_{50} = 300 nM inhibition of chemotaxis, induced by 1 µM of histamine: IC_{50} = 86 nM	(Ling et al., 2004)
	Fluorescence imaging	**Antagonist:** inhibition of actin polymerization, induced by 300 nM of histamine: IC_{50} = 6 nM	(Barnard et al., 2008)
Sf9 insect cells	Radioligand binding, steady-state GTPase assay	**Partial inverse agonist** at human H_4R: EC_{50} = 37.7 ± 8.5 nM; α = -0.31 (related to the efficacy of thioperamide)	(Schneider et al., 2009)
		Partial agonist at mouse (EC_{50} = 186 nM; α = 0.61), canine (EC_{50} = 155 nM; α = 0.66) and rat H_4R (EC_{50} = 316 nM; α = 0.51) in the GTPase assay (G_i-protein activation)	(Schnell et al., 2011)
		Differences between K_i value in [^{3}H]histamine competition binding (18.6 nM) and EC_{50} value with respect to inverse agonistic activity in the GTPase assay (77.6 nM); for other compounds structurally related to JNJ7777120, even larger differences were found	(Schneider et al., 2010)
Human HEK 293 / HEK 293T cells	Radioligand binding ([^{3}H]histamine competition)	Substantial affinity differences of JNJ7777120 at various species isoforms: human (K_i = 5 nM), monkey (K_i = 32 nM), pig (K_i = 501 nM), dog (K_i = 79 nM), mouse (K_i = 4 nM), rat (K_i = 4 nM) and guinea pig H_4R (K_i = 1 µM)	(Lim et al., 2010)
	SRE-luciferase reporter gene assay (cH_4R + $G\alpha q_i$)	**Antagonist** at canine H_4R: rightward shift of the histamine concentration-response-curve, no pA_2 reported	(Jiang et al., 2008)
COS-7 cells	Radioligand binding	[^{3}H]histamine competition binding assay: K_i: 50 nM	
Human U2OS cells	GTPγS binding assay, β-arrestin recruitment assay, ERK phosphorylation	**Weak partial inverse agonist** in [^{35}S]GTPγS binding assay (EC_{50} = 79 nM and α = -0.05, Table 2). However, in Fig. 1, JNJ7777120 appears to be a neutral antagonist with respect to [^{35}S]GTPγS binding **Partial agonist:** increase in recruitment of β-arrestin in a supposedly G-protein independent (PTX-insensitive) manner (EC_{50} = 12.5 nM and α = 0.64); effective and prolonged ERK activation at a very high ligand concentration (100 µM!)	(Rosethorne and Charlton, 2011)
Mouse pituitary tumor AtT-20 cells	ACTH release (enzyme-linked immunosorbent assay)	**Antagonist:** inhibition of ACTH secretion, induced by histamine (10 nM) or R-α-methylhistamine (100 nM): IC_{50} = 360 nM or 230 nM, respectively	(Meng et al., 2008)

JNJ7777120 is the best-studied selective H_4R ligand. The table summarizes the data of important studies characterizing the compound in *in vitro* test systems. Information on the expression system, the parameter (functional assay or radioligand binding)) measured and pharmacological parameters is provided. α Designates the efficacy of the ligand. To facilitate comparison of the data from various studies, K_i, IC_{50} and EC_{50} values are all provided in molar units. For functional inhibition experiments, the concentration of the stimulus histamine is provided as well.

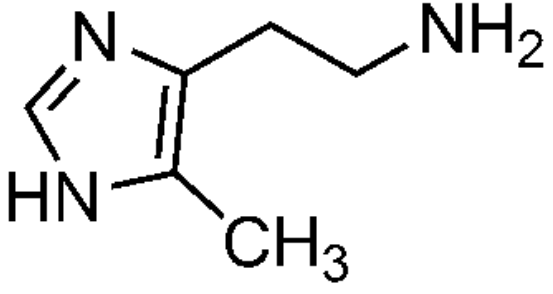
Figure 1



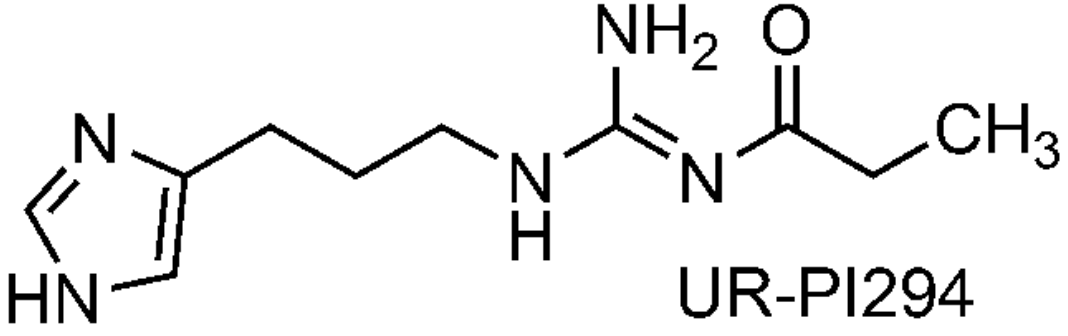
Histamine



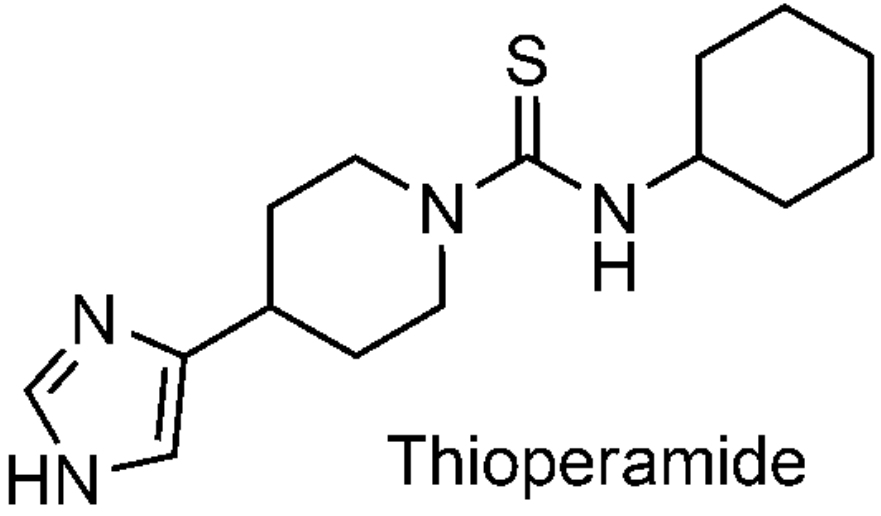
UR-PI376



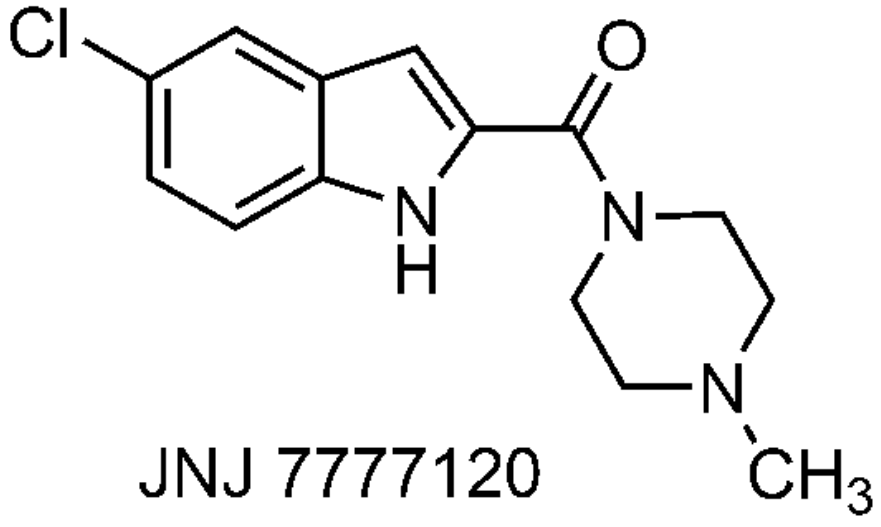
5(4)-Methylhistamine



UR-PI294



Thioperamide



JNJ 7777120

Figure 2

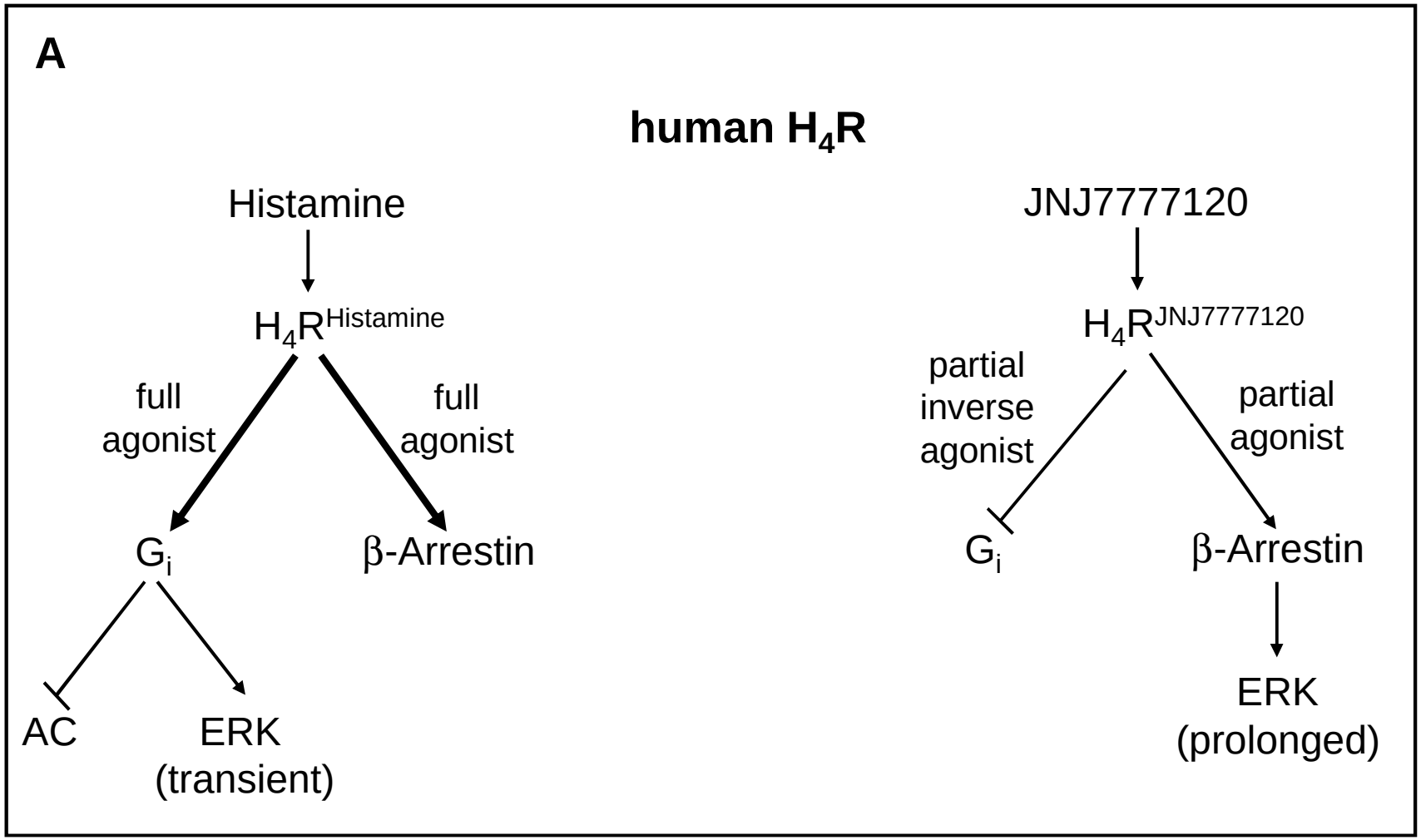


Figure 2

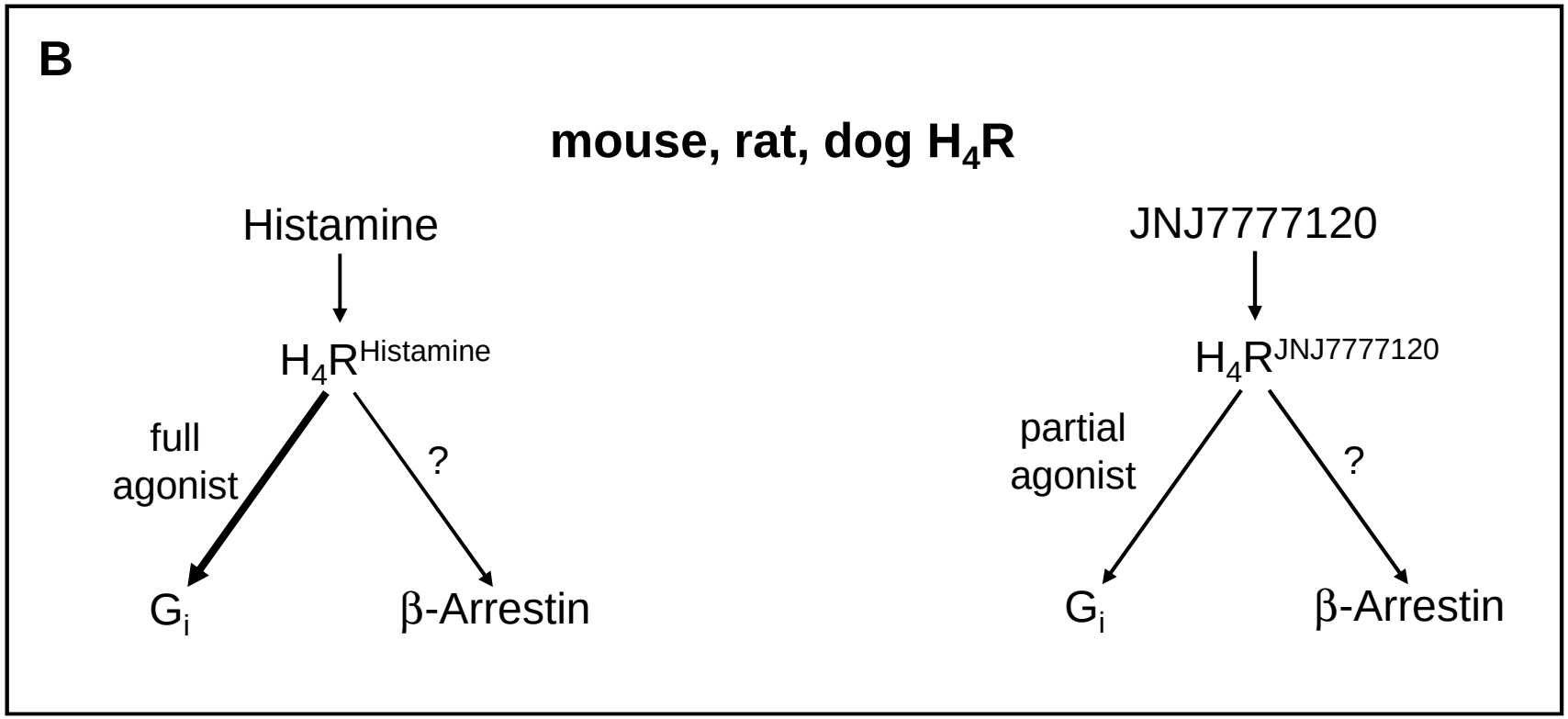


Figure 2

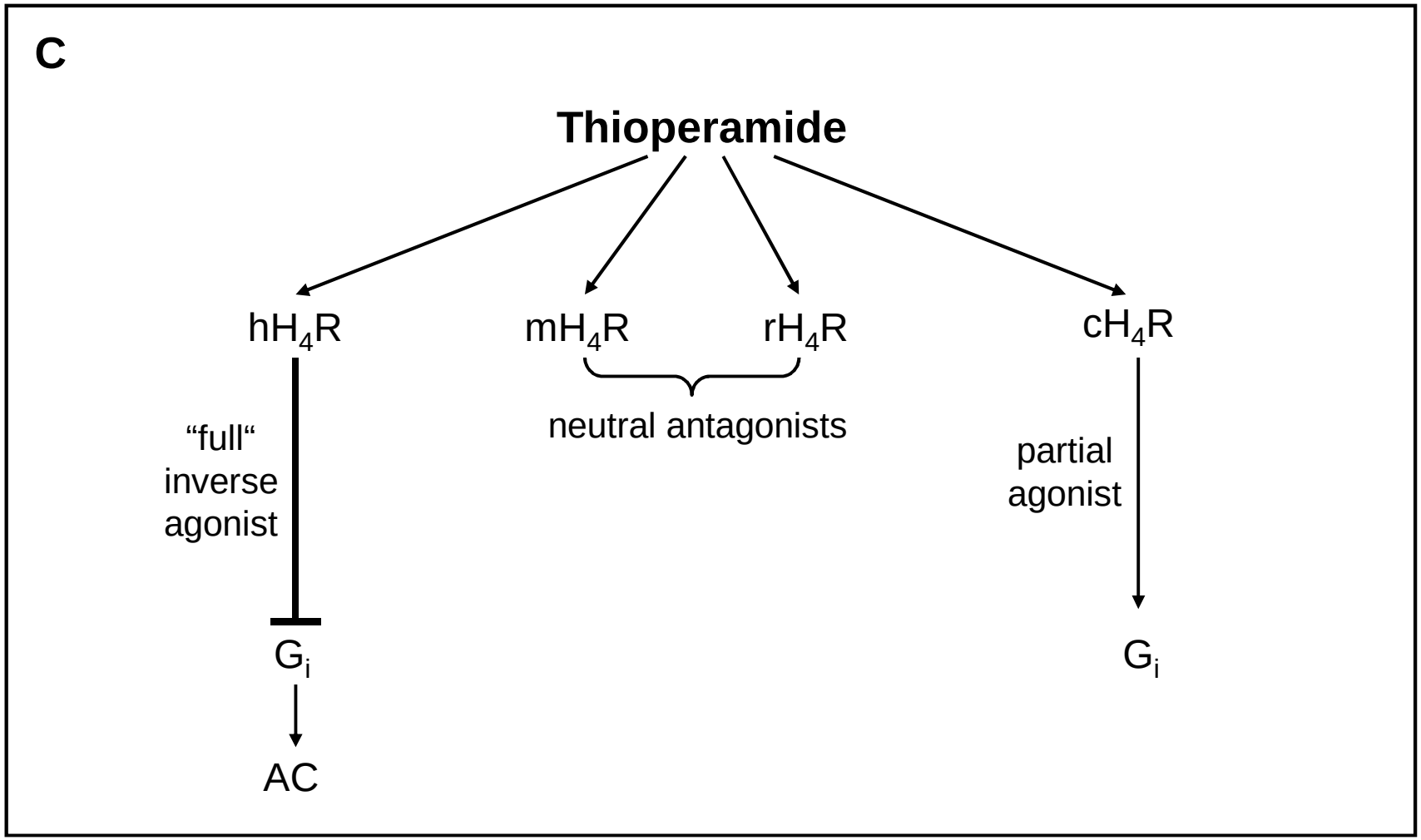
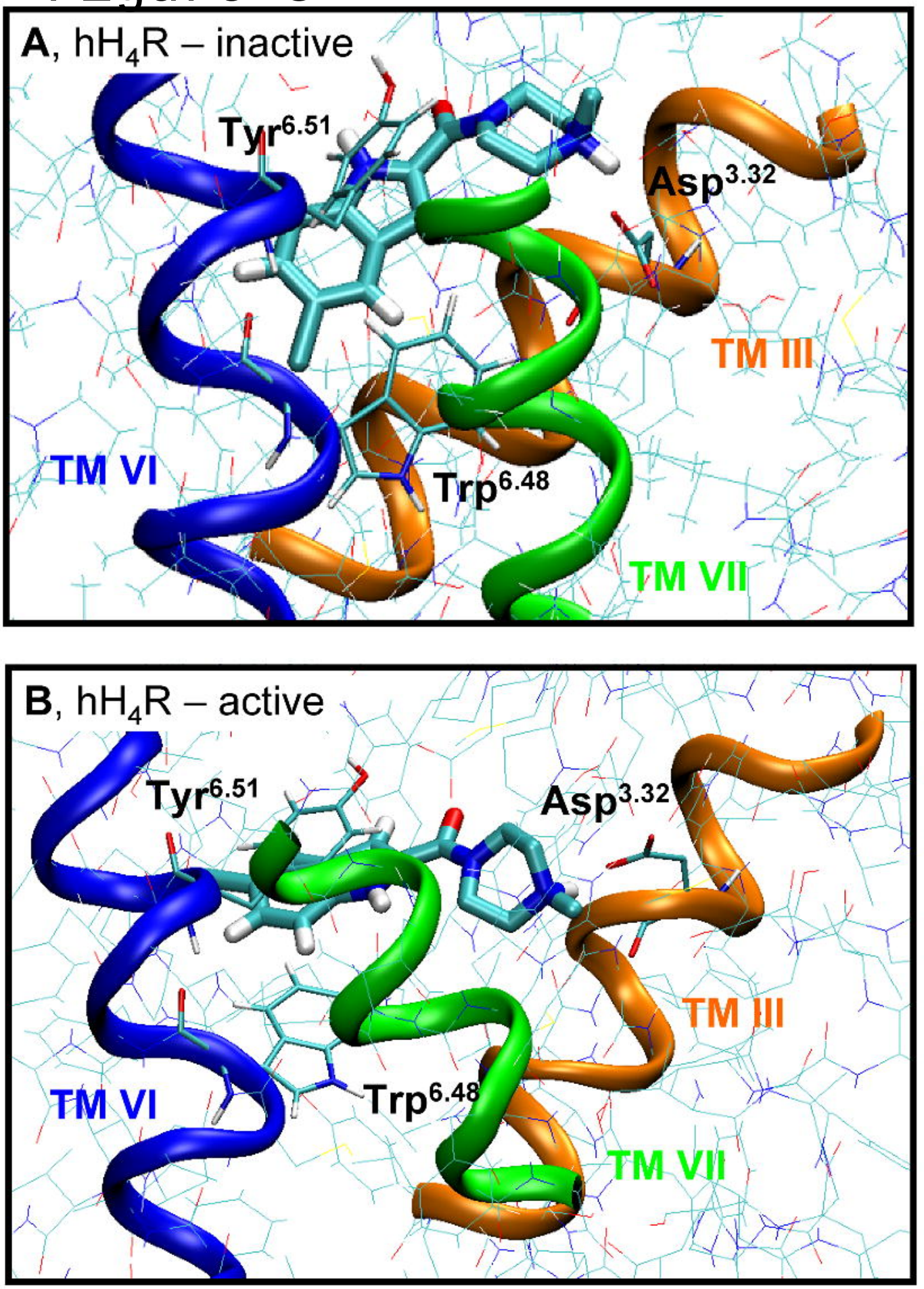


Figure 3


A, hH4R – inactive
Tyr6.51
Asp3.32
TM III
TM VI
Trp6.48
TM VII
B, hH4R – active
Tyr6.51
Asp3.32
TM III
TM VI
Trp6.48
TM VII