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MOL #68395

Title: Agonist-biased signalling at the histamine H_4 receptor: JNJ7777120 recruits beta-arrestin without activating G proteins

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Running title: JNJ7777120 is a biased agonist at the histamine H_4 receptor

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Abstract - 248 words

Introduction - 584 words

Discussion - 991 words

Abbreviations: [^{35}S]-GTPγS, guanosine 5'-*O*-(3-[^{35}S]-thio)triphosphate; BSA, bovine serum albumin; HBSS, Hanks' balanced salt solution; H_4, histamine H_4; JNJ7777120, 1-[(5-Chloro-1H-indol-2-yl) carbonyl]-4-methyl-piperazine; 2-PED, 2-pyridylethylamine dihydrochloride; U2OS; PathHunter™ U2OS β-arrestin:EA; U2OS-H_4; PathHunter™ U2OS β-arrestin:EA-H_4:ProLink

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Abstract

The $G_{i/o}$-coupled histamine H_4 receptor is highly expressed in haemopoietic cells and is a promising new target for the treatment of chronic inflammatory diseases. JNJ7777120 has been described as a selective antagonist at the H_4 receptor and is widely used to characterise the physiological role of the H_4 receptor. We have investigated the pharmacological properties of JNJ7777120 using two distinct downstream signalling readouts, G protein activation and β-arrestin recruitment. The H_4 receptor agonists histamine and clobenpropit, but not JNJ7777120, were both able to induce [^{35}S]-GTPγS binding in membranes prepared from U2OS-H_4 cells. Thioperamide, a dual H_3/H_4 receptor antagonist, and JNJ7777120 were both able to inhibit the [^{35}S]-GTPγS binding induced by clobenpropit. Agonists and antagonists specific for other members of the histamine receptor family had no effect in this assay format. Histamine and clobenpropit increased β-arrestin recruitment to the H_4 receptor in a concentration-dependent manner. This β-arrestin recruitment could be inhibited by pre-incubation with thioperamide. Surprisingly, pre-incubation with the H_4-selective antagonist JNJ7777120 did not antagonise, but rather potentiated the response to histamine. JNJ7777120 treatment alone resulted in an increase in β-arrestin recruitment, which again could be inhibited by pre-incubation with thioperamide. Schild analysis demonstrated competitive antagonism between thioperamide and both clobenpropit and JNJ7777120. Histamine and clobenpropit had comparable potencies for both [^{35}S]-GTPγS binding β-arrestin recruitment, suggesting little difference in the levels of receptor reserve between the two assays. In conclusion, we have demonstrated that JNJ7777120 recruits β-arrestin to the H_4 receptor, independent of G protein activation.

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Introduction

Histamine has many diverse biological roles and exerts its effects via 4 distinct receptor sub-types: H_1, H_2, H_3 and H_4 receptors. The most recent addition to the family, the H_4 receptor shares a relatively higher degree of homology with the H_3 receptor than either of the H_1 or H_2 receptors (Nakamura et al., 2000; Oda et al., 2000). Unlike the H_1 receptor ($G_{q/11}$) and H_2 receptor (G_s), the H_3 and H_4 receptors couple to $G_{i/o}$, and their activation results in the inhibition of adenylyl cyclase. The expression of the H_4 receptor appears to be limited to the haematopoietic cells including mast cells (Hofstra et al., 2003), eosinophils (Liu et al., 2001) and T cells (Gutzmer et al., 2009), and as such a role for this receptor in inflammatory responses has been suggested (for review see: Zhang et al., 2007).

Initial investigation into the pharmacology and physiological roles of the H_4 receptor were complicated by the lack of specific ligands at this receptor. The H_4 receptor was found to cross-react with known histamine receptor ligands, most notably those that were previously identified as selective for the H_3 receptor. The H_3 receptor antagonists clobenpropit and clozapine have subsequently been shown to have partial agonist activity at the H_4 receptor (Liu et al., 2001), whereas thioperamide has now been identified as a dual $H_{3/4}$ receptor antagonist (Gbahou et al., 2006). Many studies investigating the roles of the H_4 receptor have been performed using a combination of these compounds and knock-out mice. The identification of JNJ7777120 as a potent and highly selective antagonist at the H_4 receptor marked the advent of more direct investigation into the roles of the H_4 receptor. JNJ7777120 binds to the H_4 receptor with high affinity, K_i equal to 4 nM, and at least 1000-fold selectivity over the H_1 or H_3 receptors. The antagonist properties of this compound have also been described against histamine in a cAMP-mediated reporter gene assay with a resulting pA_2 of 8.1 (Thurmond et al., 2004).

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JNJ7777120 has been used extensively to elucidate the roles of the H_4 receptor in a variety of allergic and inflammatory processes including chemotaxis of eosinophils (Ling et al., 2004) and mast cells (Thurmond et al., 2004), as well as allergic rhinitis (Takahashi et al., 2009) and allergic airway inflammation (Dunford et al., 2006). Taken together these data demonstrate that JNJ7777120 has anti-inflammatory properties, and suggests that H_4 receptor antagonists may constitute a new class of anti-inflammatory drugs.

The H_4 receptor has previously been shown to couple to multiple signalling pathways, dependent upon cell background. In recombinant systems activation of $G_{i/o}$ proteins results in a decrease in adenylyl cyclase activity and hence reduction in levels of cAMP within the cell (Nakamura et al., 2000; Oda et al., 2000). However, in some cell-types, such as mouse mast cells, the endogenous H_4 receptor has been shown to couple to Ca^{2+} mobilization in a pertussis toxin-sensitive manner, but not to cAMP (Hofstra et al., 2003). Furthermore, there is growing evidence that where multiple pathways are activated by the same receptor, certain agonists are able to preferentially activate one pathway over another, displaying collateral efficacy (Kenakin, 2005). This pathway specificity is an important consideration, particularly where ligands are used to characterise the pharmacology and physiology of novel receptors.

In this study we investigate the ability of several ligands to initiate diverse signalling pathways from the H_4 receptor, comparing G protein-dependent signalling with β-arrestin recruitment that can occur independently of G protein activation (Lefkowitz and Shenoy, 2005;Wei et al., 2003).

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Materials & Methods:

Histamine receptor agonists / antagonists used (Table 1):

Histamine (2-(4-Imidazolyl) ethylamine), JNJ7777120 (1-[(5-Chloro-1H-indol-2-yl) carbonyl]-4-methyl-piperazine), triprolidine ((E)-2-[3-(1-Pyrrolidinyl)-1-*p*-tolylpropenyl] pyridine hydrochloride), mepyramine (N-(4-Methoxyphenyl) methyl-N′,N′-dimethyl-N-(2-pyridinyl)-1,2-ethanediamine maleate salt), ranitidine hydrochloride (*N*-(2-[(5-(dimethylaminomethyl)furan- 2-yl)methylthio]ethyl)- *N*-methyl- 2-nitroethene- 1,1-diamine), clobenpropit dihydrobromide ([(4-Chlorophenyl) methyl]-3-(1H-imidazol-4-yl) propyl ester carbamimidothioic acid dihydrobromide), thioperamide maleate salt (N-Cyclohexyl-4-(1H-imidazol-4-yl)-1-piperidinecarbothioamide maleate salt) were all purchased from Sigma Ltd., (Poole, UK); 2-PED (2-pyridylethylamine dihydrochloride) was purchased from Tocris Cookson Ltd; (Bristol, UK).

Cell Culture

PathHunter™ U2OS β-arrestin:EA (U2OS) cells (DiscoveRx) were maintained in MEM medium containing L-glutamine supplemented with foetal bovine serum (10 % v/v), penicillin (100 $iu.mL^{-1}$), streptomycin (100 $\mu g.mL^{-1}$) and hygromycin (250 $\mu g.mL^{-1}$) at 37 °C, 5 % CO_2. For experiments, cells were harvested using trypsin/EDTA and seeded in medium composed as above.

H_4 receptor transfection

The PathHunter™ β-arrestin assay (DiscoveRx) uses enzyme fragment complementation to measure recruitment of β-arrestin to a GPCR after activation. U2OS cells were transfected with

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the H_4:ProLink receptor using Fugene6 according to manufacturer's instructions. They were grown under antibiotic selection (250 μg.mL^{-1} hygromycin; 500 ug.mL^{-1} geneticin) to create a "stable pool" of transfected cells. This pool was subsequently single-cell sorted and clones selected that expressed the H_4 receptor at various levels (from here on known as U2OS-H_4 cells). Unless otherwise indicated, the same clonal cell-line was used for all experiments.

β-arrestin recruitment assay

U2OS or U2OS-H_4 cells were seeded overnight in white, clear bottomed 384-well ViewPlates (PerkinElmer, UK) at 10,000 cells/well in 20 μl MEM medium, supplemented as above, and incubated at 37 °C, 5 % CO_2 overnight to achieve a confluent monolayer. Spent media was removed and replaced with 15 μL of assay buffer (HBSS supplemented with 20 mM HEPES, 0.1 % bovine serum albumin). Cells were then stimulated with agonist or vehicle (5 μL) for 2h at room temperature. All incubations were performed in HBSS supplemented with 20 mM HEPES and 0.1 % bovine serum albumin. For antagonist experiments, cells were pre-incubated with antagonist (5 μL) for 15 min prior to agonist addition. 25 μL Flash detection reagent was added and luminescence read on the LeadSeeker (GE Healthcare, UK) after a 1 min incubation.

Membrane preparation

Membranes were prepared from U2OS or U2OS-H_4 cell lines. Cells were grown to approximately 90 % confluence, removed from culture flask using a lifting buffer (10 mM HEPES, 0.9% w v^{-1} NaCl, 0.2% w v^{-1} EDTA pH 7.4) and cell scraper, pelleted by centrifugation (1500 rpm, 10 min) and resuspended in wash buffer 1 (10 mM HEPES, 10 mM EDTA, pH 7.4). The

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cell suspension was homogenised with an Ultra-Turrax disperser (5× 10 s bursts). The resultant homogenate was ultra-centrifuged at 48 000 xg for 30 min at 4 °C using a Beckman Avanti J-251 ultracentrifuge, supernatant discarded and pellet resuspended. This was repeated to wash, and final pellet resuspended in wash buffer 2 (10 mM HEPES, 0.1 mM EDTA, pH 7.4 at a concentration of 3–5 mg ml^{-1}, as determined by the method of (Bradford, 1976) using BSA as a standard. Aliquots were flash frozen and maintained at -80 °C until required.

[^{35}S]-GTPγS binding assay

[^{35}S]-GTPγS binding was measured using scintillation proximity assay (SPA). All experiments were run at room temperature (~21ºC) in the following buffer (20 mM HEPES, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM EDTA (pH 7.4) and 0.01 % (w/v) BSA) with 10 µg.mL^{-1} saponin added. U2OS and U2OS0-H_4 membranes (5 µg) were incubated with 3 µM GDP, 0.5 mg/well WGA SPA beads and a range of histamine receptor agonist and antagonists for 30 minutes. This initial pre-incubation was performed to allow the agonist and antagonist to equilibrate prior to the addition of [^{35}S]-GTPγS (0.3 nM), which was followed by a further 60 minute incubation. The assay plates were centrifuged prior to detection of [^{35}S]-GTPγS binding using single-photon counting (30 sec/well read) on a Topcount scintillation counter (PerkinElmer Life & Analytical Sciences, UK).

[^{3}H]-histamine binding assay

[^{3}H]-histamine binding assays were performed in 96-deep-well plates in a final volume of 500 µL, using a range of concentrations of [^{3}H]-histamine (~1 – 100 nM) to construct saturation

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binding curves as described by (Dowling and Charlton, 2006). Membranes derived from U2OS and U2OS-H_4 cells (15 μg mL^{-1}) were incubated in assay binding buffer (50 mM Tris-HCl) at 25 °C, with gentle agitation for 60 min. Non-specific binding was defined using 10 μM cold histamine. Following incubation period, bound and free [^{3}H]-histamine were separated by rapid vacuum filtration using a FilterMate Cell Harvester (PerkinElmer Life & Analytical Sciences, UK) onto 96-well GF/C filter plates pre-coated with 0.3 % PEI. Plates were rapidly washed 3x with ice-cold assay binding buffer. After drying (>4 h), 40 μL of Microscint-20 (PerkinElmer Life & Analytical Sciences, UK) was added to each well and radioactivity quantified using single-photon counting (60 sec/well read) on a TopCount microplate scintillation reader (PerkinElmer Life & Analytical Sciences, UK).

Western Blot

U2OS-H_4 cells were seeded overnight in clear 12-well plates at 150,000 cells/well in 2 mL MEM medium, supplemented as above, and incubated at 37 °C, 5 % CO_2 overnight to achieve a confluent monolayer. On the day of the experiment, cells were washed in assay buffer (HBSS supplemented with 20 mM HEPES, 0.1 % bovine serum albumin) and rested in 450 μL assay buffer at 37 °C for 15 minutes. Cells were then stimulated with agonist or vehicle (50 μL) for between 0 and 60 min at 37 °C. Assay buffer was aspirated, and cells lysed with ice-cold 1x Laemelli Buffer containing 1 mM DTT, scraped and harvested. Samples were sonicated for 15-20 sec, boiled for 5 min and spun for 2 min at 14000 g, 4 °C. Samples were separated on SDS-PAGE at 200V (constant) for 60 min in MOPS running buffer containing anti-oxidant. Proteins were then transferred from the gel onto nitrocellulase membrane at 30 V (constant) for 60 min.

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Membranes were blocked in TBS-T (50 mM Tris-base, 150 mM NaCl (pH 7.6), 0.1 % (v/v) Tween-20) containing 5 % non-fat dry milk and 1 % BSA overnight, at 4 °C with gentle agitation. Membranes were washed briefly and incubated with primary antibody (anti-phospho-ERK, 1:5000 in TBS-T + 0.1 % BSA) for 2 h at room temperature with gentle agitation. Following 3x 5 min wash in TBS-T, membranes were incubated with 2° antibody (IRDye 800CW donkey anti-rabbit IgG, 1:15000) for 1 hr at room temperature, with gentle agitation. Membranes were washed 3x 10 min in TBS-T and flourescence detected using the Li-cor Odyssey.

Quantification and data analysis

Graphs were fitted to data using GraphPad Prism (Version 4.0) and results are expressed as the mean ± standard error of mean from at least three separate experiments, unless otherwise stated. Statistical analysis performed using GraphPad Prism (Version 4.0). Concentration-response data were fitted using a four-parameter logistic equation.

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Results

Stimulation of [^{35}S]-GTPγS binding

To investigate G protein-dependent activities at the H_4 receptor we first determined whether histamine was capable of increasing levels of [^{35}S]-GTPγS accumulation in membranes prepared from U2OS cells which are reported to endogenously express H_1R (Verdonk *et al.,* 2006). In this assay, cells are incubated with a [^{35}S]-labelled non-hydrolysable form of GTP ([^{35}S]-GTPγS) that will accumulate following agonist activation of the receptor, and give a measurement of G protein activation in the cell.

In membranes prepared from U2OS cells, we were unable to detect any agonistic activity with either histamine or the histamine H_1 agonist 2-PED in (Fig. 1a). To measure G protein-dependent activities at the H_4R we then monitored histamine-mediated [^{35}S]-GTPγS binding in membranes prepared from the U2OS-H_4 cells (stably expressing the H_4:ProLink construct). The K_d for histamine in our cell-line was found to be 10.5 nM (table 3), which correlates well with that reported previously for a HEK-H_4 cell-line (15.3 nM) (Morse et al., 2001). This suggests that the addition of the ProLink to the receptor does not interfere with agonist binding to the receptor.

Histamine and clobenpropit, but not JNJ7777120, were both able to induce [^{35}S]-GTPγS binding in membranes prepared from U2OS-H_4 cells, with pEC_{50} values of 7.8 ± 0.2 and 7.1 ± 0.2, respectively. Additionally, we demonstrated that this assay was capable of detecting constitutive activity, as thioperamide treatment resulted in a reduction in the basal levels of [^{35}S]-GTPγS binding (Fig. 1b, Table 2). In contrast, triprolidine had no effect on levels of either basal or histamine stimulated [^{35}S]-GTPγS binding in the U2OS-H_4 cells (data not shown).

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Thioperamide and JNJ777120 were also both able to inhibit the H_4 receptor-mediated [^{35}S]-GTPγS binding induced by clobenpropit (Fig. 1c) with pIC_{50} values of 6.3 ± 0.04 and 6.7 ± 0.19, respectively. Again, we saw a decrease in [^{35}S]-GTPγS binding below basal levels with thioperamide demonstrating that this compound is an inverse agonist in this assay format.

Recruitment of β-arrestin to the H_4 receptor

Following receptor activation and phosphorylation, β-arrestins are recruited to the active receptor to initiate signal termination via endocytosis of the receptor and uncoupling of the receptor from G protein (Lohse et al., 1990; Pitcher et al., 1992). In addition to this role it has also been demonstrated that the association of β-arrestin is involved in G-protein independent signalling, such as ERK1/2 activation (Shenoy et al., 2006; Dewire et al., 2007). The PathHunter™ β-arrestin assay (DiscoveRx) uses β-galactosidase enzyme fragment complementation to measure recruitment of β-arrestin to a GPCR after activation (for details see: (Dewire et al., 2007; Olson and Eglen, 2007).

The parental U2OS cell-line was tested in this assay format to confirm that the assay was specific for coupling between the transfected β-arrestin:EA and GPCR:ProLink tags. No β-arrestin recruitment could be detected in cells where the β-arrestin:EA, but no ProLink-tagged receptor, was present (Figure 2a).

After transfection and clonal selection of the H_4:ProLink receptor, a range of histamine receptor agonists were tested in the β-arrestin assay to determine their ability to recruit β-arrestin to the H_4 receptor. Histamine and clobenpropit, but not the H_1 receptor selective 2-PED, were able to induce β-arrestin recruitment to the H_4 receptor (Figure 2a). Histamine- and clobenpropit-

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mediated β-arrestin recruitment occurred in a concentration-dependent manner (figure 2b), with pEC_{50} values of 7.3 ± 0.1 and 7.1 ± 0.1, respectively. EC_{80} concentrations of agonists were used to demonstrate that the β-arrestin recruitment observed could be inhibited by pre-incubation with thioperamide (Figure 2c). In contrast, pre-incubation with JNJ7777120 did not antagonise the histamine response and moderately potentiated the response to a sub-maximal concentration of clobenprobit where the system was not already saturated (Figure 2d). JNJ7777120 treatment alone resulted in a concentration-dependent increase in β-arrestin recruitment (pEC_{50} of 7.6 ± 0.1; Figure 2e), which could be inhibited with pre-treatment of the cells with thioperamide (pIC_{50} of 6.4 ± 0.2) but not the H_1-selective antagonist triprolidine. Thioperamide was not able to decrease β-arrestin recruitment below basal levels, demonstrating that this compound is a neutral antagonist in this assay format (figure 2f). It has previously been demonstrated that the complementation between the ProLink and EA tags is reversible making it possible to detect inverse agonist activity in this assay system (Mcguinness et al., 2009). This would imply that although we do not observe constitutive recruitment of β-arrestin to the H_4 receptor in this cell-line, the assay system itself is capable of measuring inverse agonism.

To further ensure the JNJ7777120 agonist activity was due to a specific interaction with the H_4 receptor, Schild plots for the antagonist thioperamide were compared against JNJ7777120 and clobenpropit concentration-response curves. Thioperamide produced parallel rightward shifts of both clobenpropit and JNJ7777120 concentration-response curves, yielding pA_2 values of 6.95 ± 0.76 and 6.95 ± 0.62, and Schild slopes of 0.99 and 0.97, respectively (Figure 3). This suggests thioperamide is a competitive antagonist against both JNJ7777120 and clobenpropit.

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To determine if the level of receptor expression was responsible for the agonistic properties of JNJ7777120 in the β-arrestin recruitment assay, a range of clonal cell lines were selected that expressed different levels of receptor, assessed using [^{3}H]-histamine saturation binding (Table 3). These cell lines were then treated with the full agonist histamine and JNJ7777120 to determine levels of β-arrestin recruitment (Figure 4). The maximal response observed in each cell line increased linearly with receptor expression (Figure 5), but there was no change in the EC_{50} for either histamine or JNJ7777120 (Table 3; $P > 0.1$ by one-way ANOVA), suggesting little receptor reserve in this system.

β-arrestin recruitment does not require active G protein to be recruited to the receptor, but rather serves to uncouple the G protein from the activated receptor and hence initiate signal termination (Lohse et al., 1990; Pitcher et al., 1992). To determine whether the β-arrestin recruitment observed in this study was dependent upon activation of G proteins, cells were treated with agonists after prior incubation with pertussis toxin (PTx) to inactivate $G_{i/o}$ proteins. Pre-treatment for 20 h with 200 ng.mL^{-1}. PTx had no effect on histamine- or JNJ7777120-mediated β-arrestin recruitment (Figure 6).

Measurement of ERK phosphorylation

One of the downstream consequences of β-arrestin recruitment is the scaffolding of the mitogen-activated protein kinase (MAPK) complex which results in extracellular signal-regulated kinase (ERK) phosphorylation and activation (Shenoy et al., 2006; Dewire et al., 2007). To determine if the JNJ7777120-mediated recruitment of β-arrestin translated into a functional ERK response, ERK phosphorylation was monitored by Western Blot after treatment of cells with maximal (100

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μM) concentrations of JNJ7777120 and histamine for between 0 and 60 min. Treatment with both agonists resulted in ERK phosphorylation in a time-dependent manner (Figure 7). The magnitude of ERK phosphorylation was similar for both compounds but the time courses were very different. Histamine induced a transient phosphorylation that peaked at 5 min and returned to basal levels within 30 min. In contrast, JNJ7777120 did not stimulate any appreciable ERK phosphorylation over the first 10 min, but then generated a much more prolonged pERK response that peaked at 30 min and was still elevated after 60 min.

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Discussion

The H_4 receptor has been shown to be largely expressed in haemopoietic cells and possess the ability to modulate eosinophil migration and selective recruitment of mast cells leading to amplification of histamine-mediated immune responses and eventually to chronic inflammation. The discovery of this role in inflammation has led to the proposal that H_4 receptor antagonists may possess anti-inflammatory properties.

JNJ7777120 has been described previously as a novel antagonist at the H_4 receptor with K_i of 4 nM. It has been characterised in cAMP-CRE reporter assays, where it acts as a competitive antagonist, producing parallel rightward shift of histamine concentration-response curves with increasing concentrations. JNJ7777120 was shown to have a pA_2 equal to 8.1, and demonstrated good selectivity with little or no affinity for over 50 other targets (Thurmond et al., 2004). In addition to studies in recombinant cell systems, JNJ7777120 has been shown to exhibit functional antagonism of Ca^{2+} mobilization and chemotaxis to histamine in mast cells *in vitro* and *in vivo* (Jablonowski et al., 2003; Thurmond et al., 2004). We have demonstrated that JNJ7777120 is a biased agonist at the H_4 receptor, acting as a neutral antagonist for [^{35}S]-GTPγS binding and a partial agonist for β-arrestin recruitment.

The phenomenon of agonist-biased signalling has been described for a number of different systems (for reviews see: Rajagopal et al., 2010; Kenakin and Miller, 2010), both *in vitro* (Berg et al., 1998; Gay et al., 2004; Cordeaux et al., 2001; Lane et al., 2007; Cordeaux et al., 2000) and *in vivo* in the case of the nicotinic acid receptor (GPR109a) (Semple et al., 2008). This has been attributed to the existence of multiple active conformations, with each agonist able to stabilise a subtly different state that may preferentially couple to one pathway over another (Bokoch et al., 2010; Kenakin, 2001). It is, however, very important to rule out other potential explanations for

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the observation of biased signalling before speculating that different receptor conformations may be responsible.

First, and most importantly, it is critical to ensure that differences in agonist efficacy are not responsible. For example, in a system with poor coupling efficiency a low efficacy agonist may appear to be a neutral antagonist. If, however, the receptor reserve is increased, a low efficacy agonist may become a full agonist in that particular system (Baker et al., 2002; Kenakin 2009). For this reason it is important to consider receptor reserve in each pathway examined.

We observed that clobenpropit and histamine display similar potency and efficacy in both the [^{35}S]-GTPγS binding assay and β-arrestin recruitment assay, indicating there is little difference in the efficiency of coupling of the two different signalling pathways (Table 2). If JNJ7777120 were truly a partial agonist that failed to score in the [^{35}S]-GTPγS assay, we would also expect it to appear inactive in the similarly coupled β-arrestin recruitment assay. In addition, previous work utilising the highly amplified cAMP-CRE transcription factor assay has demonstrated that JNJ7777120 is a neutral antagonist, in an assay system that is capable of measuring inverse agonism (Lim et al., 2005).

To further test whether the high level of maximal JNJ7777120 agonist responses in the β-arrestin recruitment assay were an artefact of over-expressing the H_4 receptor in the U2OS cells, 4 different clonal cell-lines were tested in a radioligand saturation binding assay and the β-arrestin recruitment assay. In all of the clones tested there was a linear correlation between receptor expression and maximal activation by both histamine and JNJ7777120, but no shift in the pEC_{50} of either compound. The absence of receptor reserve is probably a result of the fixed one to one stoichiometry between receptor and β-arrestin in this assay system, as has recently been

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suggested by Waterfield and colleagues (2009). It is therefore unlikely that the agonist activity of JNJ7777120 in the β-arrestin recruitment assay is an artefact of receptor over-expression in these cells. Although it may not be indicative of endogenous receptor:β-arrestin interactions, this lack of reserve might be expected for this type of assay system.

It is important to also ensure that the agonists are acting at a single receptor. If the ligand is not fully selective it may activate other pathways via a different receptor, apparently displaying biased signalling. We have taken several steps to prove that JNJ7777120 is not acting as an agonist at a different receptor to recruit β-arrestin. Firstly, we tested a range of ligands with different histamine receptor selectivity to show the signalling we observed was solely via the H_4 receptor. Secondly, the assay employed to measure β-arrestin recruitment utilises enzyme complementation, so only recruitment of β-arrestin to the tagged receptor will result in reconstitution of the enzyme activity. This means the signal is very specific for the H_4 receptor. Finally, we performed Schild plots with the H_4 receptor antagonist thioperamide to demonstrate that the resulting pA2 against JNJ7777120 was the same as that against clobenpropit, strongly suggesting they are acting as the same receptor site.

It has recently been shown that differences in dissociation rates between ligands can also lead to the misinterpretation of agonist biased signalling, particularly if one of the assay readouts is rapid whilst the other is slower (Vauquelin and Charlton, 2010). Whilst the lack of kinetic data on these compounds means this explanation can not be completely excluded, the long nature of both assay readouts means that hemi-equilibrium is unlikely to be an issue in this study.

Having ruled out many of the common artefacts that may complicate the interpretation of biased signalling, we conclude that JNJ7777120 is likely to stabilise an alternative active conformation

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of the H_4R that is able to initiate β-arrestin recruitment but not G protein activation. By examining ERK phosphorylation in these cells downstream of JNJ7777120 we have also been able to demonstrate that this recruitment of β-arrestin translates into a functional response. The timecourse of JNJ7777120 activation of ERK was delayed compared to that of histamine, with a peak response being observed between 20 and 30 minutes, rather than the rapid and transient response observed with histamine. This observation is consistent with previous reports that show G protein-mediated ERK phosphorylation is fast and short-lived, while β-arrestin-dependent pERK responses are slower and more prolonged (Ahn et al., 2004).

JNJ7777120 has been used extensively *in vitro* and *in vivo* to elucidate H_4 receptor biology. We have demonstrated that although JNJ7777120 is an antagonist with regards to G protein-dependent signalling, it acts as an agonist in a non-G protein dependent manner to recruit β-arrestin to the receptor, as demonstrated by the lack of sensitivity to pertussis toxin treatment. In addition to its classical role in desensitization, β-arrestin has been shown to be involved in the scaffolding and regulation of G protein-independent signalling pathways such as ERK1/2, JNK and c-Raf-1 (Lefkowitz and Shenoy, 2005). For this reason, the agonist properties of JNJ7777120 could potentially complicate interpretation of experimental data, especially from *in vivo* work, and lead to misleading conclusions regarding the role of the H_4R. This work has demonstrated that the properties of JNJ7777120 are dependent upon the system studied and highlights the need to consider more than one down-stream signalling pathway when evaluating compounds for efficacy at GPCRs.

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Reference List

Ahn S, Shenoy SK, Wei H, Lefkowitz RJ (2004) Differential kinetic and spatial patterns of β-arrestin and G protein-mediated ERK activation by the angiotensin II receptor. *J Biol Chem* **279**:35518-35525

Baker JG, Hall IP and Hill SJ (2002) Pharmacological Characterization of CGP 12177 at the Human Beta(2)-Adrenoceptor. *British Journal of Pharmacology* **137**:400-408

Berg KA, Maayani S, Goldfarb J, Scaramellini C, Leff P and Clarke WP (1998) Effector Pathway-Dependent Relative Efficacy at Serotonin Type 2A and 2C Receptors: Evidence for Agonist-Directed Trafficking of Receptor Stimulus. *Mol Pharmacol* **54**:94-104

Bokoch MP, Zou Y, Rasmussen SGF, Liu CW, Nygaard R, Rosenbaum DM, Fung JJ, Choi HJ, Thian FS, Kobilka TS, Puglisi JD, Weis WI, Pardo L, Prosser RS, Mueller L and Kobilka BK (2010) Ligand-Specific Regulation of the Extracellular Surface of a G-Protein-Coupled Receptor. *Nature* **463**:108-112

Bradford MM (1976) Rapid and Sensitive Method for Quantitation of Microgram Quantities of Protein Utilizing Principle of Protein-Dye Binding. *Analytical Biochemistry* **72**:248-254

Cordeaux Y, Briddon SJ, Megson AE, McDonnell J, Dickenson JM and Hill SJ (2000) Influence of Receptor Number on Functional Responses Elicited by Agonists Acting at the Human Adenosine A(1) Receptor: Evidence for Signaling Pathway-Dependent Changes in Agonist Potency and Relative Intrinsic Activity. *Mol Pharmacol* **58**:1075-1084

Cordeaux Y, Nickolls SA, Flood LA, Graber SG and Strange PG (2001) Agonist Regulation of D2 Dopamine Receptor/G Protein Interaction. *J Biol Chem* **276**:28667-28675

Dewire SM, Ahn S, Lefkowitz RJ and Shenoy SK (2007) Beta-Arrestins and Cell Signaling. *Annual Review of Physiology* **69**:483-510

Dowling MR and Charlton SJ (2006) Quantifying the Association and Dissociation Rates of Unlabelled Antagonists at the Muscarinic M-3 Receptor. *British Journal of Pharmacology* **148**:927-937

Dunford PJ, O'Donnell N, Riley JP, Williams KN, Karlsson L and Thurmond RL (2006) The Histamine H4 Receptor Mediates Allergic Airway Inflammation by Regulating the Activation of CD4+ T Cells. *J Immunol* **176**:7062-7070

Gay EA, Urban JD, Nichols DE, Oxford GS and Mailman RB (2004) Functional Selectivity of D2 Receptor Ligands in a Chinese Hamster Ovary HD2L Cell Line: Evidence for Induction of Ligand-Specific Receptor States. *Mol Pharmacol* **66**:97-105

Gbahou F, Vincent L, Humbert-Claude M, Tardivel-Lacombe J, Chabret C and Arrang JM (2006) Compared Pharmacology of Human Histamine H-3 and H-4 Receptors: Structure-Activity Relationships of Histamine Derivatives. *British Journal of Pharmacology* **147**:744-754

Gutzmer R, Mommert S, Gschwandtner M, Zwingmann K, Stark H and Werfel T (2009) The Histamine H4 Receptor Is Functionally Expressed on TH2 Cells. *J Allergy Clin Immunol* **123**:619-625

Hofstra CL, Desai PJ, Thurmond RL and Fung-Leung WP (2003) Histamine H-4 Receptor Mediates Chemotaxis and Calcium Mobilization of Mast Cells. *J Pharmacol Exp Ther* **305**:1212-1221

Jablonowski JA, Grice CA, Chai WY, Dvorak CA, Venable JD, Kwok AK, Ly KS, Wei JM, Baker SM, Dsesai PJ, Jiang W, Wilson SJ, Thurmond RL, Karlsson L, Edwards JP,

Lovenberg TW and Carruthers NI (2003) The First Potent and Selective Non-Imidazole Human Histamine H-4 Receptor Antagonists. *J Med Chem* **46**:3957-3960

Kenakin T (2001) Inverse, Protean, and Ligand-Selective Agonism: Matters of Receptor Conformation. *Faseb Journal* **15**:598-611

Kenakin T (2005) New Concepts in Drug Discovery: Collateral Efficacy and Permissive Antagonism. *Nat Rev Drug Discov* **4**:919-927

Kenakin T (2009) System effects on agonist response: full and partial agonists, in *A Pharmacology Primer* 3rd ed. pp 26-9, Elsevier Academic Press, London, UK

Kenakin T and Miller LJ (2010) Seven Transmembrane Receptors As Shapeshifting Proteins: The Impact of Allosteric Modulation and Functional Selectivity on New Drug Discovery. *Pharmacol Rev* **62**:265-304

Lane JR, Powney B, Wise A, Rees S and Milligan G (2007) Protean Agonism at the Dopamine D2 Receptor: (S)-3-(3-Hydroxyphenyl)-N-Propylpiperidine Is an Agonist for Activation of Go1 but an Antagonist/Inverse Agonist for Gi1,Gi2, and Gi3. *Mol Pharmacol* **71**:1349-1359

Lefkowitz RJ and Shenoy SK (2005) Transduction of Receptor Signals by {Beta}-Arrestins. *Science* **308**:512-517

Lim HD, van Rijn RM, Ling P, Bakker RA, Thurmond RL and Leurs R (2005) Evaluation of Histamine H1-, H2-, and H3-Receptor Ligands at the Human Histamine H4 Receptor: Identification of 4-Methylhistamine As the First Potent and Selective H4 Receptor Agonist. *J Pharmacol Exp Ther* **314**:1310-1321

Ling P, Ngo K, Nguyen S, Thurmond RL, Edwards JP, Karlsson L and Fung-Leung WP (2004) Histamine H-4 Receptor Mediates Eosinophil Chemotaxis With Cell Shape Change and Adhesion Molecule Upregulation. *British Journal of Pharmacology* **142**:161-171

Liu C, Ma XJ, Jiang X, Wilson SJ, Hofstra CL, Blevitt J, Pyati J, Li X, Chai W, Carruthers N and Lovenberg TW (2001) Cloning and Pharmacological Characterization of a Fourth Histamine Receptor (H4) Expressed in Bone Marrow. *Mol Pharmacol* **59**:420-426

Lohse MJ, Benovic J L, Codina J, Cargon M G and Lefkowitz R J (1990) Beta-Arrestin: A Protein That Regulates Beta-Adrenergic Receptor Function. *Science* **248**:1547-1550

Mcguinness D, Malikzay A, Visconti R, Lin K, Bayne M, Monsma F and Lunn C A (2009) Characterizing Cannabinoid CB2 Receptor Ligands Using DiscoveRx PathHunter™ β-Arrestin Assay. *J Biomol Screen* **14**:49-58

Morse KL, Behan J, Laz TM, West RE, Greenfeder SA, Anthes JC, Umland S, Wan YT, Hipkin R W, Gonsiorek W, Shin N, Gustafson EL, Qiao XD, Wang SK, Hedrick JA, Greene J, Bayne M and Monsma FJ (2001) Cloning and Characterization of a Novel Human Histamine Receptor. *J Pharmacol Exp Ther* **296**:1058-1066

Nakamura T, Itadani H, Hidaka Y, Ohta M and Tanaka K (2000) Molecular Cloning and Characterization of a New Human Histamine Receptor, HH4R. *Biochem Biophys Res Commun* **279**:615-620

Oda T, Morikawa N, Saito Y, Masuho Y and Matsumoto S i (2000) Molecular Cloning and Characterization of a Novel Type of Histamine Receptor Preferentially Expressed in Leukocytes. *J Biol Chem* **275**:36781-36786

Olson KR and Eglen RM (2007) Beta Galactosidase Complementation: A Cell-Based Luminescent Assay Platform for Drug Discovery. *Assay Drug Dev Technol* **5**:137-144

Pitcher J, Lohse MJ, Codina J, Caron MG and Lefkowitz RJ (1992) Desensitization of the Isolated .Beta.2-Adrenergic Receptor by Beta-Adrenergic Receptor Kinase, cAMP-Dependent Protein Kinase, and Protein Kinase C Occurs Via Distinct Molecular Mechanisms. *Biochemistry* **31**:3193-3197

Rajagopal S, Rajagopal K and Lefkowitz RJ (2010) Teaching Old Receptors New Tricks: Biasing Seven-Transmembrane Receptors. *Nat Rev Drug Discov* **9**:373-386

Semple G, Skinner PJ, Gharbaoui T, Shin YJ, Jung JK, Cherrier MC, Webb PJ, Tamura SY, Boatman PD, Sage CR, Schrader TO, Chen R, Colletti SL, Tata JR, Waters MG, Cheng K, Taggart AK, Cai TQ, Carballo-Jane E, Behan DP, Connolly DT and Richman JG (2008) 3-(1H-Tetrazol-5-Yl)-1,4,5,6-Tetrahydro-Cyclopentapyrazole (MK-0354): A Partial Agonist of the Nicotinic Acid Receptor, G-Protein Coupled Receptor 109a, With Antilipolytic but No Vasodilatory Activity in Mice. *J Med Chem* 51:5101-5108

Shenoy SK, Drake MT, Nelson CD, Houtz DA, Xiao K, Madabushi S, Reiter E, Premont RT, Lichtarge O and Lefkowitz RJ (2006) Beta-Arrestin-Dependent, G Protein-Independent ERK1/2 Activation by the Beta2 Adrenergic Receptor. *J Biol Chem* **281**:1261-1273

Takahashi Y, Kagawa Y, Izawa K, Ono R, Akagi M and Kamei C (2009) Effect of Histamine H4 Receptor Antagonist on Allergic Rhinitis in Mice. *International Immunopharmacology* **9**:734-738

Thurmond RL, Desai PJ, Dunford PJ, Fung-Leung WP, Hofstra CL, Jiang W, Nguyen S, Riley J P, Sun S, Williams KN, Edwards JP and Karlsson L (2004) A Potent and Selective

Histamine H4 Receptor Antagonist With Anti-Inflammatory Properties. *J Pharmacol Exp Ther* **309**:404-413

Vauquelin and Charlton (2010) Long-lasting target binding and rebinding as mechanisms to prolong *in vivo* drug action. *British Journal of Pharmacology* doi:

10.1111/j.1476-5381.2010.00936.x

Verdonk E, Johnson K, McGuinness R, Leung G, Chen Y, Tang HR, Michelotti JM, and Liu VF (2006) Cellular Dielectric Spectroscopy: A Label-Free Comprehensive Platform for Functional Evaluation of Endogenous Receptors. *Assay Drug De. Technol* **4**(5):609-619

Waterfield A, Kinloch R, Williams R, Young T, Nickolls S (2009) The DiscoveRx PathHunter β-arrestin assay; A measure of agonist affinity? ***p****A2online* **3(2):**001P

Wei HJ, Ahn S, Shenoy SK, Karnik SS, Hunyady L, Luttrell LM and Lefkowitz RJ (2003) Independent Beta-Arrestin 2 and G Protein-Mediated Pathways for Angiotensin II Activation of Extracellular Signal-Regulated Kinases 1 and 2. *Proceedings of the National Academy of Sciences of the United States of America* **100**:10782-10787

Zhang M, Thurmond RL and Dunford PJ (2007) The Histamine H4 Receptor: A Novel Modulator of Inflammatory and Immune Disorders. *Pharmacology & Therapeutics* **113**:594-606

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Figure Legends.

Figure 1 – Receptor-mediated [^{35}S]-GTPγS binding in membranes prepared from naïve U2OS cells (A), or U2OS-H_4 cells (B&C). (A) Concentration-dependent increases in [^{35}S]-GTPγS binding were assessed after a 1 h stimulation with the indicated concentrations of histamine, 2-PED or triprolidine plus a single concentration of histamine (100 μM). Concentration-dependent increases in [^{35}S]-GTPγS binding were assessed after a 1 h stimulation with the indicated concentrations of (B) histamine, clobenpropit, thioperamide or JNJ7777120, or (C) JNJ7777120 and thioperamide plus a single concentration of clobenpropit (300 nM). For each individual experiment, data have been normalized to the [^{35}S]-GTPγS binding detected after assay buffer addition alone, and are expressed as means ± S.E.M. for at least 3 independent experiments.

Figure 2 – Receptor-mediated β-arrestin recruitment in U2OS and U2OS-H_4 cells. (A) Treatment of U2OS or U2OS-H_4 cells with maximal concentration (100 μM) of each agonist for 2 h. (B) Concentration-dependent increases in β-arrestin recruitment were assessed after a 2 h stimulation with the indicated concentrations of histamine or clobenpropit. Levels of β-arrestin recruitment were monitored after a 15 min pre-incubation with indicated concentrations of (C) thioperamide followed by a 2 h incubation with a single, EC_{80} concentration of either histamine (300 nM) or clobenpropit (1 μM), or (D) β-arrestin recruitment was assessed after a 2 h stimulation with the indicated concentrations of histamine, or pre-incubation for 30 min with indicated concentrations of JNJ7777120 followed by a 2 h incubation with a single concentration of either histamine (maxima;: 10 μM) or clobenpropit (sub-maximal: 300 nM). (E) Concentration-dependent increases in β-arrestin recruitment were assessed after a 2 h stimulation

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with the indicated concentrations of JNJ7777120. (F) Levels of β-arrestin recruitment were monitored after a 15 min pre-incubation with indicated concentrations of thioperamide or triprolidine, followed by a 2 h incubation with a single, EC_{80} concentration of JNJ7777120 (100 nM). For each individual experiment, data have been normalized to the β-arrestin recruitment detected after assay buffer addition alone, and are expressed as means ± S.E.M. for at least 3 independent experiments.

Figure 3 – Levels of β-arrestin recruitment were monitored after a 15 minute pre-incubation with indicated concentrations of thioperamide, followed by a 2 h incubation with concentration-response curves to (A) JNJ7777120 or (B) clobenpropit. (C) Data were used to calculate dose ratios and Schild analysis performed using GraphPad Prism. For each individual experiment, data have been normalized to the β-arrestin recruitment detected after assay buffer addition alone, and are expressed as means ± S.E.M. for at least 3 independent experiments.

Figure 4 – Receptor-mediated β-arrestin recruitment in U2OS cells expressing different levels of the H_4:ProLink receptor. Concentration-dependent increases in β-arrestin recruitment were assessed in 4 separate clones of U2OS-H_4 cells after a 2 h stimulation with the indicated concentrations of histamine (A) or JNJ7777120 (B). For each individual experiment, data have been normalized to the β-arrestin recruitment detected after assay buffer addition alone, and are expressed as means ± S.E.M. for at least 3 independent experiments.

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Figure 5 – Correlation between H_4 receptor expression (in $pmol.mg^{-1}$) and maximal histamine-induced recruitment of β-arrestin (as a percentage of that observed in the highest expressing cell line) in 4 different clonal U2OS-H_4 cell lines.

Figure 6 – Receptor-mediated β-arrestin recruitment in U2OS-H_4 cells after pertussis toxin treatment. Concentration-dependent increases in β-arrestin recruitment were assessed after a 2 h stimulation with the indicated concentrations of histamine or JNJ7777120, 20 h after treatment with pertussis toxin (200 $ng.mL^{-1}$). For each individual experiment, data have been normalized to the β-arrestin recruitment detected after assay buffer addition alone, and are expressed as means ± S.E.M. for at least 3 independent experiments.

Figure 7 – Receptor-mediated ERK phosphorylation. Time-dependent increases in the levels of phosphorylated ERK were assessed by Western blot after treatment of U2OS-H_4 cells with a maximal (100 μM) concentration of JNJ7777120 or histamine. For each individual experiment, data have been normalized to the ERK phosphorylation detected after addition of vehicle alone, and are expressed as means ± S.E.M. for at least 3 independent experiments.

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Table 1 Compounds used in this study

Compound	Receptor sub-type	Mode of action
Histamine	all	Agonist
2-PED	H_1	Agonist
Triprolidine	H_1	Selective antagonist
Mepyramine	H_1	Selective antagonist
Ranitidine	H_2	Selective antagonist
Clobenpropit	H_3	Selective antagonist
	H_4	Reduced efficacy agonist
Thioperamide	H_3	Antagonist
	H_4	Inverse agonist
JNJ7777120	H_4	Selective antagonist

Histamine receptor agonists and antagonists used, with receptor sub-type selectivity.

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Table 2 Efficacy and potency of agonists in different assay formats

	GTPγS		β-arrestin	
	pEC50	% efficacy	pEC50	% efficacy
Histamine	6.2 ± 0.2	100	7.3 ±0.1	100
Clobenpropit	7.4 ± 0.2	84.1 ± 9.7	7.1 ± 0.1	86.2 ± 3.9
JNJ7777120	7.1 ± 0.2	-5.45 ± 2.8	7.9 ± 0.2	64.3 ± 5.1
JNJ + 1 μM clob	6.9 ± 0.2	-5.95 ± 1.9		
JNJ + 300 nM clob			7.4 ± 0.1	68 ± 12.9
JNJ + 10 μM HA			5.6 ± 0.0	119 ± 0.0
Thioperamide	8.0 ± 0.3	-47.3 ± 4.1	NE	NE
Thio + 100 nM HA			8.0 ± 0.02	-6.7 ± 3.3
Thio + 100 nM JNJ			6.4 ± 0.1	-6.7 ± 2.4
Thio + 300 nM clob	6.33 ± 0.1	-21.5 ± 1.1	7.6 ± 0.2	-9.5 ± 1.3

NE, no effect

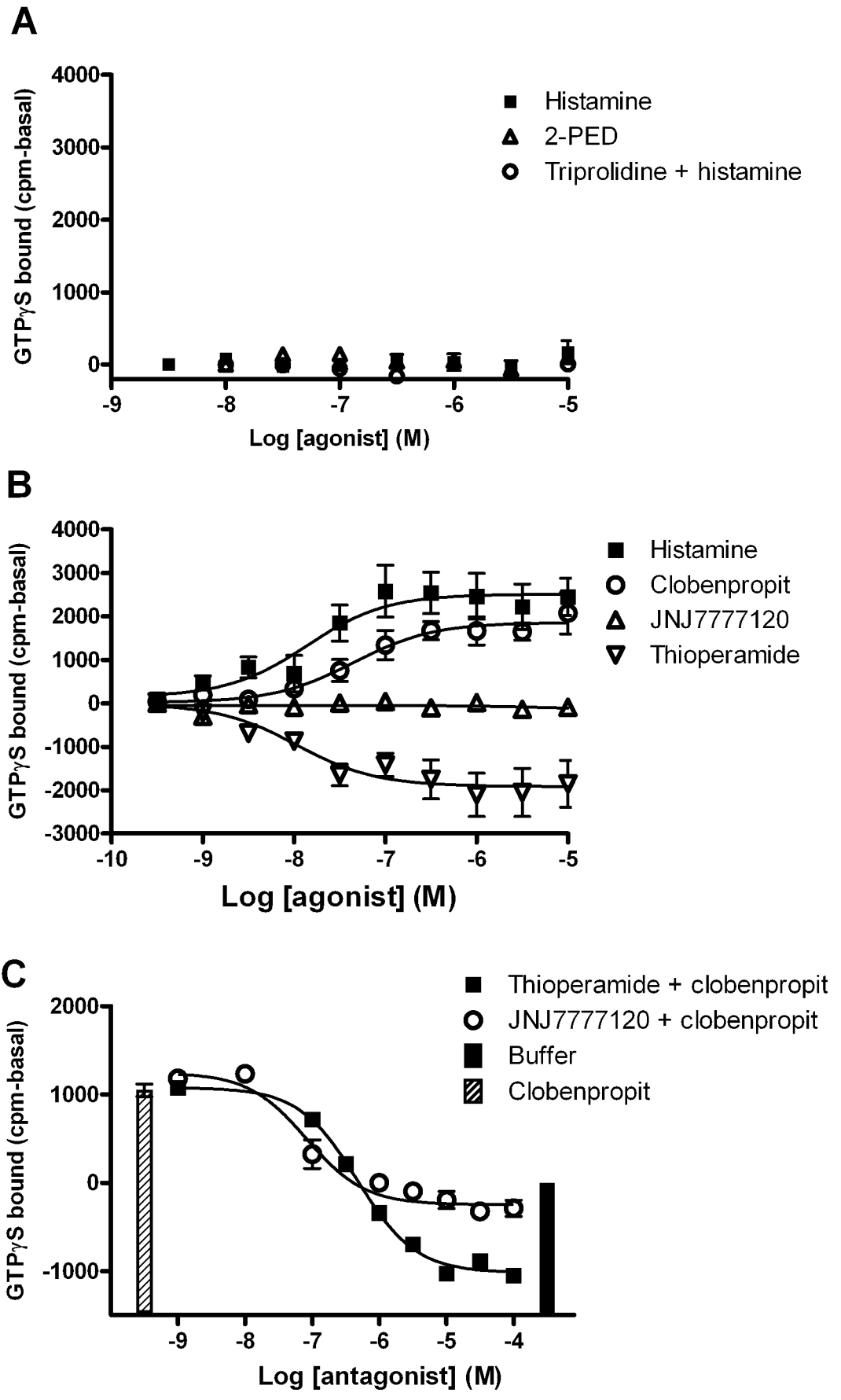
Histamine, JNJ7777120, clobenpropit and thioperamide efficacy and potency in different assay formats using the U2OS-H_4 cell-line. The efficacy of each agonist is expressed as a % of the maximal response to histamine. Data are shown as means ± S.E.M. for at least 3 independent experiments.

Table 3 H_4 receptor expression and agonist efficacy and potency in cell-lines expressing different levels of the H_4 receptor.

	[^{3}H]-histamine		Histamine		JNJ7777120	
	B_{max} (pmol.mg^{-1})	K_d (nM)	pEC_{50}	% efficacy	pEC_{50}	% efficacy
U2OS-H_4_ER1J	2.2 ± 0.2	10.5 ± 2.3	7.5 ± 0.07	75.7	7.9 ± 0.10	100 (66.0)
U2OS-H_4_ER1L	1.3 ± 0.1	11.6 ± 3.7	7.7 ± 0.10	42.9	8.1 ± 0.10	57.2 (75.9)
U2OS-H_4_ER1B	1.1 ± 0.2	10.8 ± 3.4	7.5 ± 0.05	27.8	8.0 ± 0.05	37.2 (64.8)
U2OS-H_4_ER1K	0.3 ± 0.1	22.2 ± 13.0	7.7 ± 0.08	3.1	7.9 ± 0.26	4.2 (97.0)

[^{3}H]-histamine binding was used to determine the level of H_4 receptor expression in a number of U2OS-H_4 cell-lines. These cell-lines were then used to characterize the efficacy and potency of histamine and JNJ7777120. Efficacy of each agonist is expressed as a % of the maximal response to that ligand observed in the highest expressing clone (ER1J). Additionally, the % efficacy for JNJ7777120 compared to the histamine response in that clone is shown in parentheses. Data are shown as means ± S.E.M. for 3 independent experiments.

Figure 1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-8
-7
-6
-5
Log [agonist] (M)
Histamine
2-PED
Triprolidine + histamine
B
4000
3000
2000
1000
0
-1000
-2000
-3000
GTPγS bound (cpm-basal)
-10
-9
-8
-7
-6
-5
Log [agonist] (M)
Histamine
Clobenpropit
JNJ7777120
Thioperamide
C
2000
1000
0
-1000
GTPγS bound (cpm-basal)
-9
-8
-7
-6
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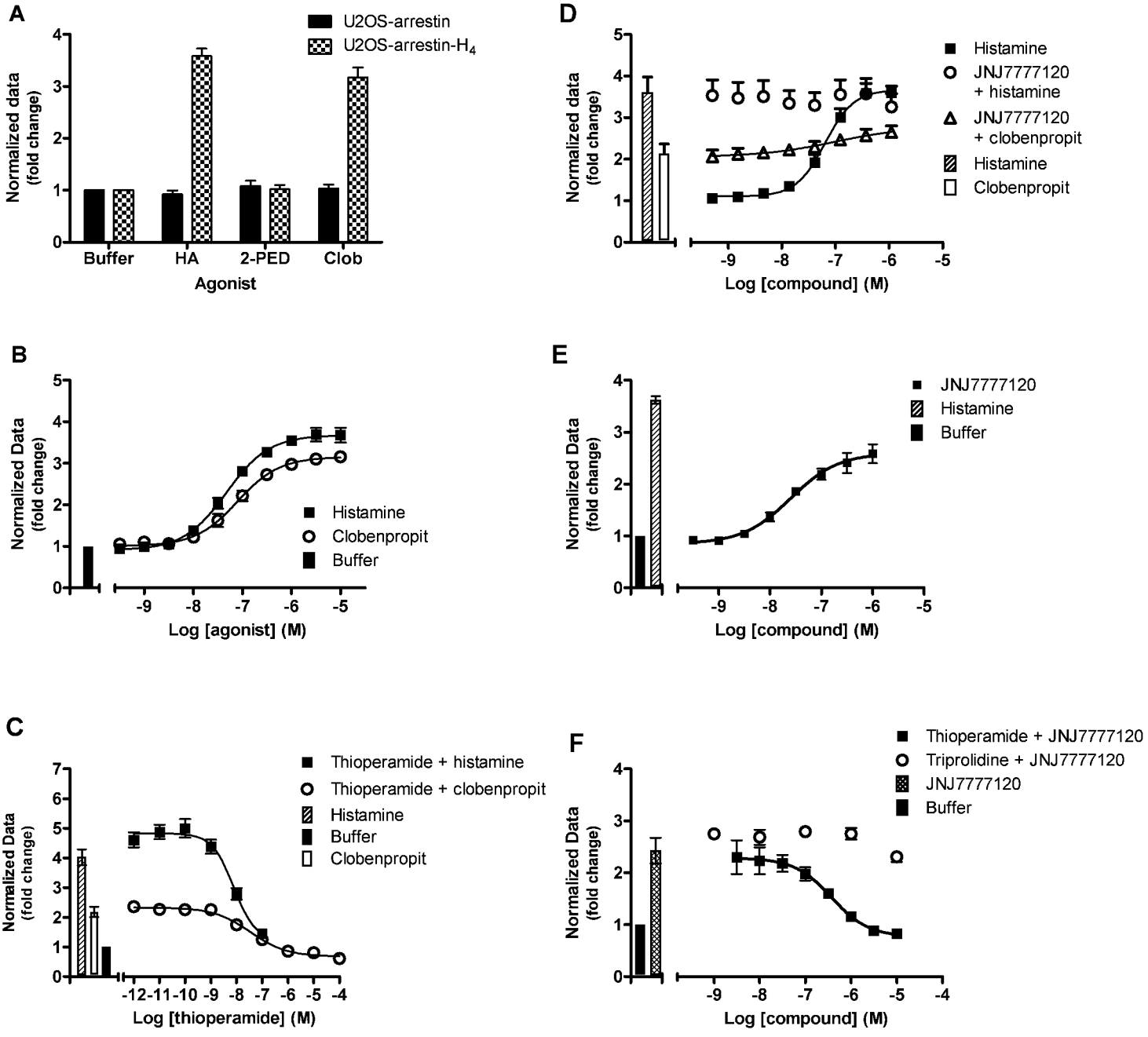
JNJ7777120 + clobenpropit
Buffer
Clobenpropit

Figure 2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2-PED
Clob
Agonist
B
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Histamine
Clobenpropit
Buffer
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C
Thioperamide + histamine
Thioperamide + clobenpropit
Histamine
Buffer
Clobenpropit
Log [thioperamide] (M)
D
Histamine
JNJ7777120 + histamine
JNJ7777120 + clobenpropit
Histamine
Clobenpropit
Log [compound] (M)
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JNJ7777120
Histamine
Buffer
Log [compound] (M)
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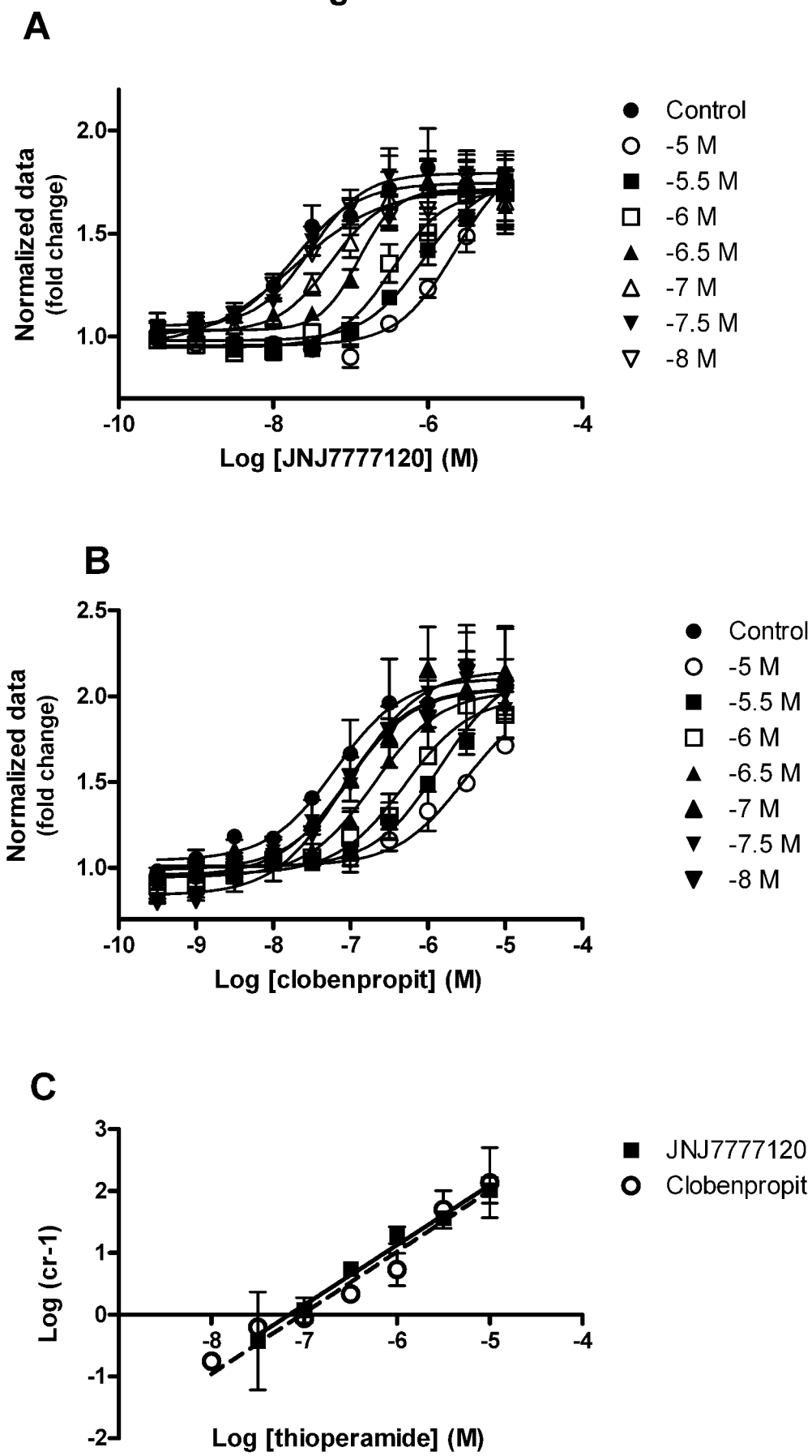
Triprolidine + JNJ7777120
JNJ7777120
Buffer
Log [compound] (M)

Figure 3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Normalized data
(fold change)
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1.0
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Log [JNJ7777120] (M)
Control
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-5.5 M
-6 M
-6.5 M
-7 M
-7.5 M
-8 M
B
Normalized data
(fold change)
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2.0
1.5
1.0
-10
-9
-8
-7
-6
-5
-4
Log [clobenpropit] (M)
Control
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-5.5 M
-6 M
-6.5 M
-7 M
-7.5 M
-8 M
C
Log (cr-1)
3
2
1
0
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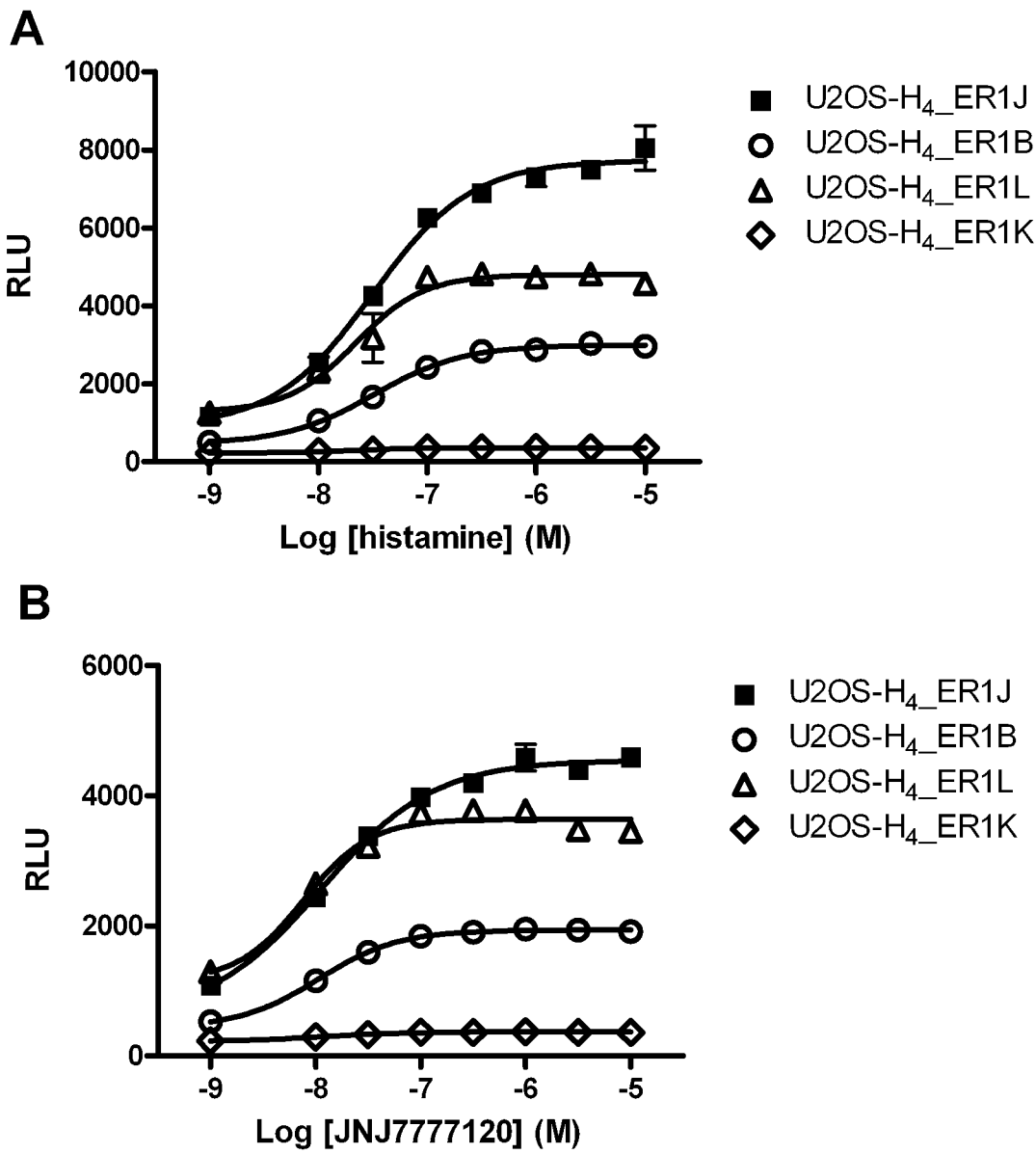
Clobenpropit

Figure 4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RLU
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-8
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-6
-5
Log [histamine] (M)
U2OS-H4_ER1J
U2OS-H4_ER1B
U2OS-H4_ER1L
U2OS-H4_ER1K
B
6000
4000
2000
0
RLU
-9
-8
-7
-6
-5
Log [JNJ7777120] (M)
U2OS-H4_ER1J
U2OS-H4_ER1B
U2OS-H4_ER1L
U2OS-H4_ER1K

Figure 5

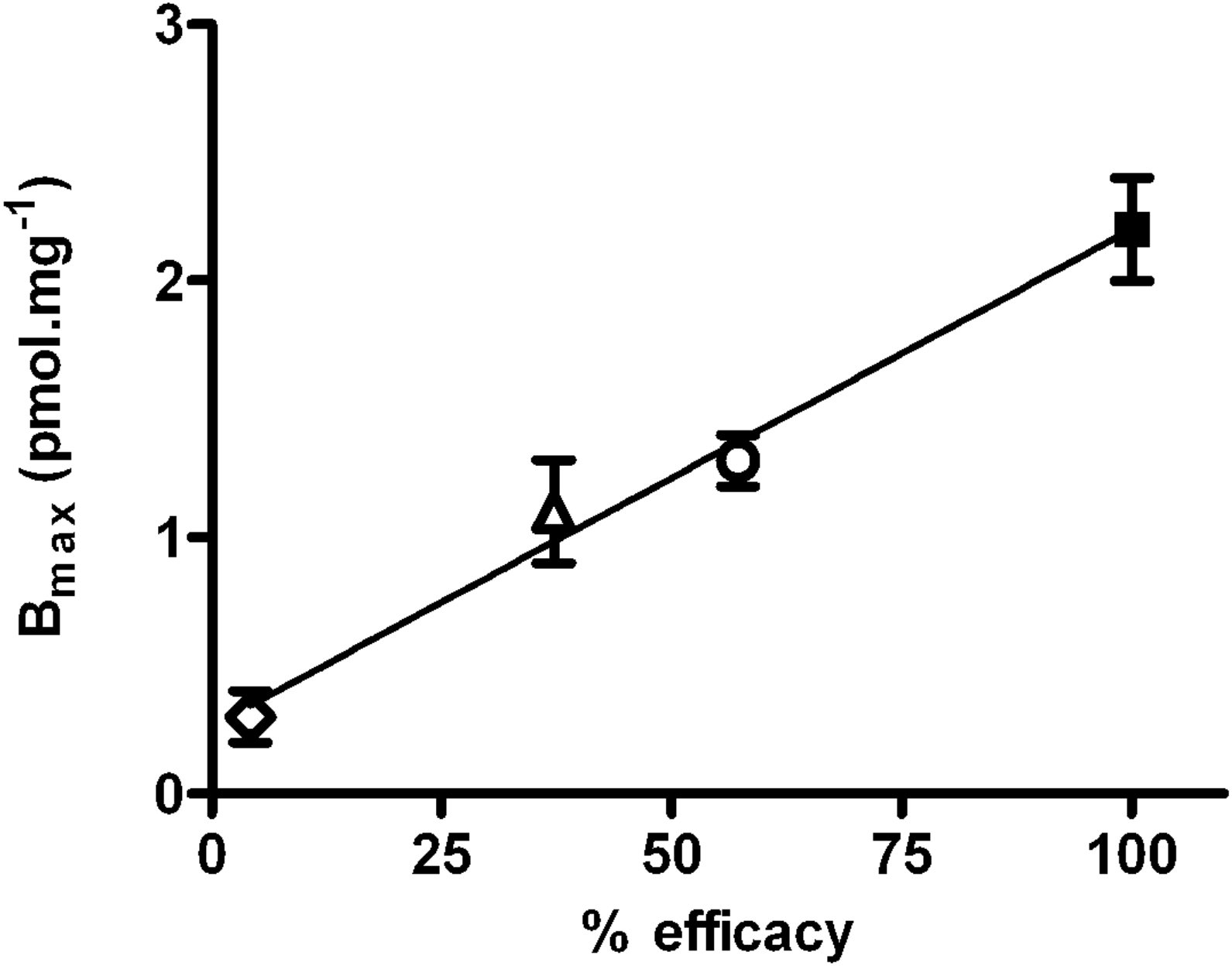
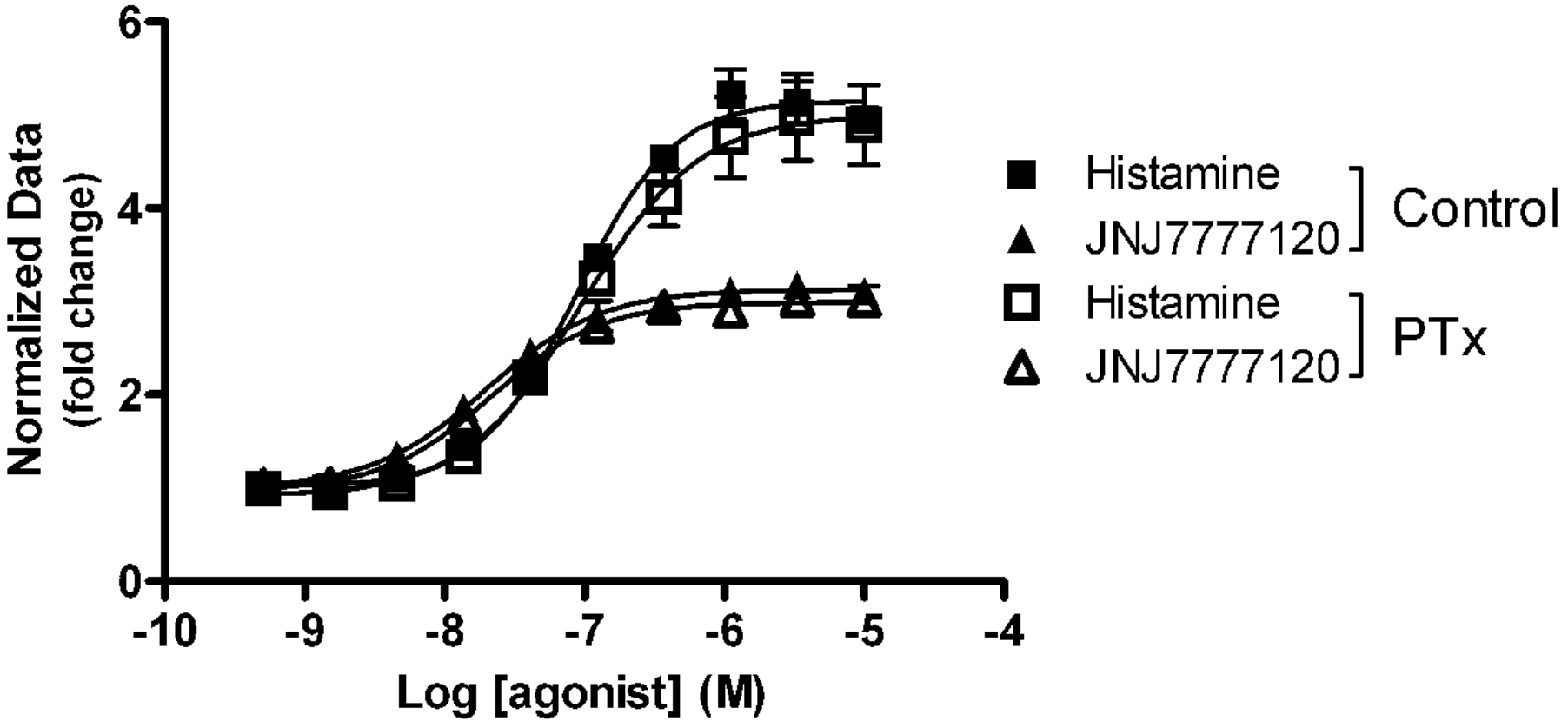


Figure 6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-10
-9
-8
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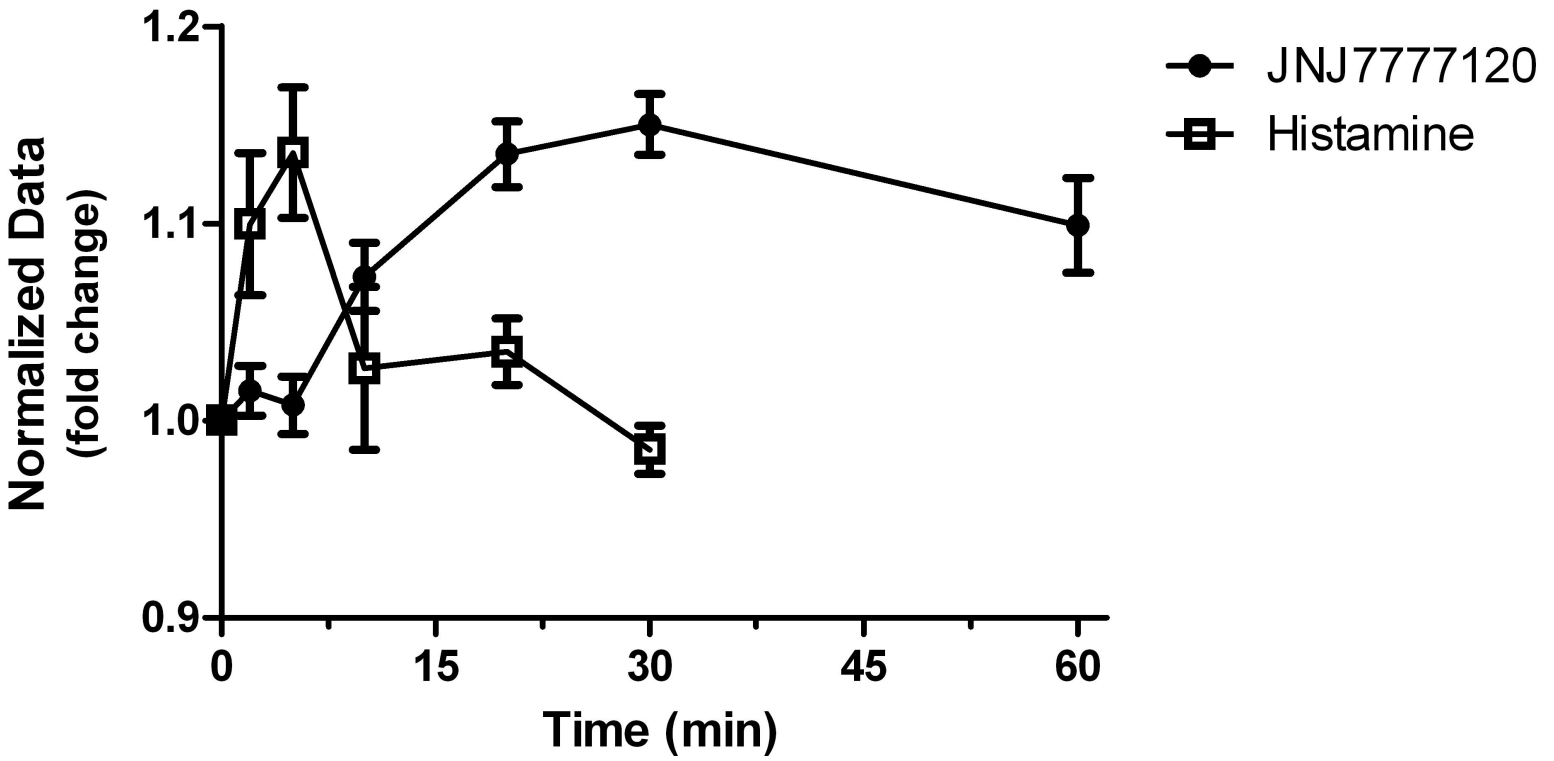
Log [agonist] (M)
Histamine
JNJ7777120
Control
Histamine
JNJ7777120
PTx

Figure 7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Normalized Data
(fold change)
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15
30
45
60
Time (min)
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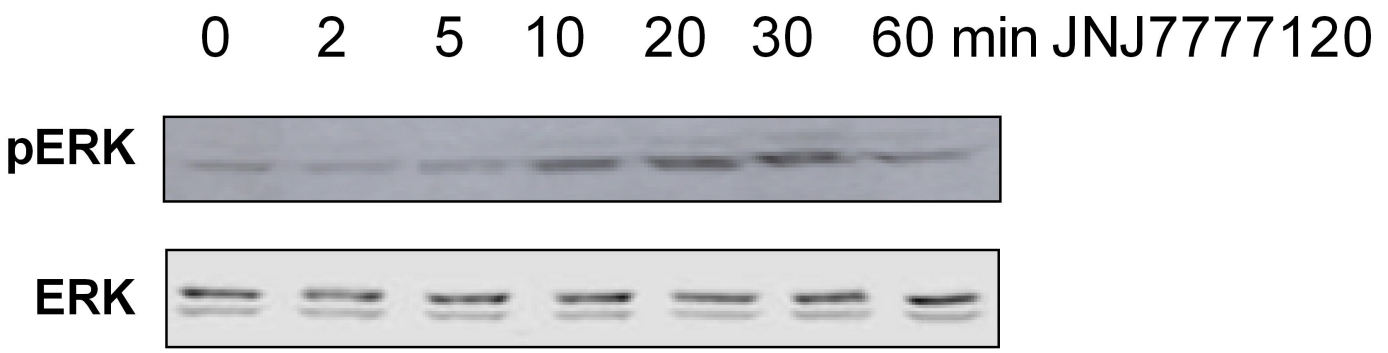
Histamine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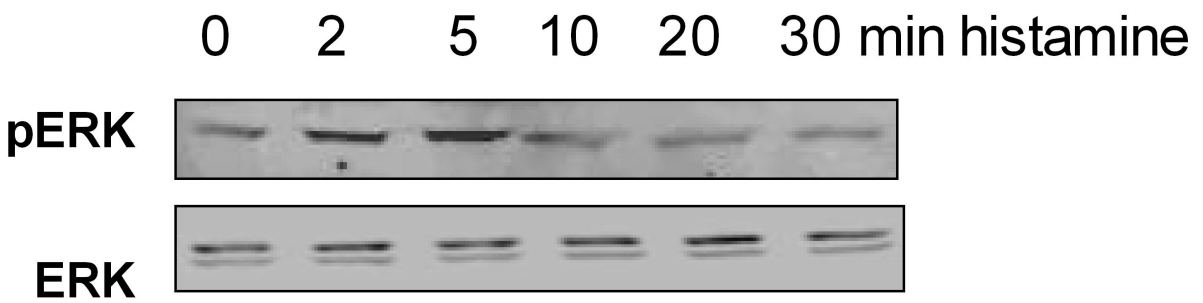
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