Structural requirements for inverse agonism and neutral antagonism of indole-benzimidazole- and thienopyrrole-derived histamine H₄R ligands

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Running title: Inverse Agonism at the Histamine H₄ Receptor

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Number of pages: 27
Number of Tables: 3
Number of Figures: 4
Number of References: 39
Number of words in the Abstract: 240
Number of words in the Introduction: 690
Number of words in the Discussion: 1499

Abbreviations used:

FLAG, octapeptide epitope for the labelling of proteins; GPCR, G-protein coupled receptor;
HA, histamine; hH₄R, human histamine H₄ receptor; Kᵦ, binding affinity of an antagonist/inverse agonist calculated from functional assays; Kᵦ, binding affinity of an antagonist/inverse agonist calculated from ligand binding studies; THIO, thioperamide (N-Cyclohexyl-4-(imidazol-4-yl)-1-piperidinecarbothioamide);
Abstract

The human histamine H₄ receptor (hH₄R), co-expressed with Gαᵢ₂ and Gβ₁γ₂ in Sf9 insect cells, is highly constitutively active and thioperamide (THIO) is one of the most efficacious hH₄R inverse agonists. High constitutive hH₄R activity may have pathophysiological implications in which case inverse agonists may behave differently than neutral antagonists. To learn more about the structural requirements for hH₄R inverse agonism, we investigated 25 compounds (indole-, benzimidazole- and thienopyrrole-derivatives) structurally related to the standard antagonist JNJ-7777120 (1-[(5-Chloro-1H-indol-2-yl)carbonyl]-4-methyl-piperazine). We characterized the compounds in radioligand binding assays using [³H]histamine ([³H]HA) and in steady-state GTPase assays in the presence (antagonist mode) and absence (agonist mode) of the agonist HA, yielding the following results: i) Twenty-two compounds were inverse agonists (efficacy: 15 - 25 % of the THIO-effect) and only 3 compounds (12 %) showed neutral antagonism. Thus, inverse agonism is far more common than neutral antagonism. ii) The inverse agonistic efficacy of the R5-monosubstituted indole-derived compounds increased with the volume of R5. R5 may interact with Trp₆.₄₈ of the rotamer-toggle switch and stabilize the inactive receptor conformation. iii) A subset of compounds showed large differences between the Kᵢ value from [³H]HA competition binding and the EC₅₀ value from steady-state GTPase assays, whereas the Kᵦ values in general were closer to the Kᵢ values. Thus, the two-state model should be extended to a model comprising a constitutively active hH₄R state, which can be discriminated by inverse agonists from a structurally distinct HA-stabilized active state.

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Introduction

The concept of inverse agonism is derived from the two-state model of receptor activation (Seifert and Wenzel-Seifert, 2002). According to this model, GPCRs exist in an equilibrium of an active G-protein-coupling conformation (R*) and an uncoupled inactive state (R). R* promotes GDP/GTP exchange at the G$\alpha$ subunit and shows a higher affinity for agonists than R. Thus, agonists activate the receptor by stabilizing the R* state. Neutral antagonists bind to R and R* states with the same affinity without altering the equilibrium. Some receptor molecules spontaneously adopt the R* state and promote G-protein signalling in the absence of agonists, which is referred to as constitutive activity. Inverse agonists are ligands that bind preferentially to the R state and reduce the percentage of spontaneously active receptors and, thereby, reduce constitutive activity. Similarly, inverse agonism can be explained by the extended ternary complex model, which takes into account that both free and ligand-occupied receptors can interact with G-proteins (Samama et al., 1994).

In the last two decades, constitutive receptor activity and inverse agonism of ligands have been reported for a large number of wild-type GPCRs, e.g. for the $\delta$-opioid receptor (Costa and Herz, 1989), the human formyl peptide receptor FPR-26 (Wenzel-Seifert et al., 1998), the cannabinoid CB$_1$ receptor (Bouaboula et al., 1997) and the histamine receptors H$_1$R (Leurs et al., 2002), H$_2$R (Alewijse et al., 1998), H$_3$R (Wieland et al., 2001) and H$_4$R (Liu et al., 2001; Schneider et al., 2009). Constitutive activity is not only a common property of wild-type GPCRs, but also cause of several diseases (Seifert and Wenzel-Seifert, 2002). Even cancerous cell transformation may be caused by a long-term elevation of second messengers, resulting from constitutive activity of overexpressed receptors (Seifert and Wenzel-Seifert, 2002; Kenakin, 2004). Thus, it is obvious that the targeted development of inverse agonistic drugs will gain more and more importance. However, for most receptors only very little is known about the structural requirements governing inverse agonism and neutral antagonism. Although first efforts were made to establish structure-activity relationships for inverse
agonists at GPCRs (Soudijn et al., 2005), most drug development programs are still not designed to systematically investigate this issue.

As recently reported by our laboratory (Schneider et al., 2009), the human hH₄R is highly constitutively active, when co-expressed with Gα₁₂ and Gβ₁γ₂ in Sf9 insect cells using the baculovirus expression system. In steady-state GTPase and [³⁵S]GTPγS binding assays THIO was found to be one of the most efficacious inverse agonists at the hH₄R. The benzimidazole derivative JNJ-7777120, which behaves as a neutral hH₄R antagonist in reporter gene assays (Lim et al., 2005), showed a partial inverse agonistic effect in our system, reaching ~ 30 % of the THIO efficacy (Schneider et al., 2009; Schneider and Seifert, 2009).

In contrast to the hH₃R, which plays an important role in neurological and psychiatric diseases, the hH₄R is an interesting drug target for the therapy of inflammation, allergy and autoimmune disorders (Tiligada et al., 2009). Specifically, the hH₄R is involved in the pathogenesis of pruritus and asthma (Thurmond et al., 2008; Neumann et al., 2010). In the past years, a large number of structurally diverse compounds targeting the hH₄R was developed with an increasing focus on the aminopyrimidine scaffold (Smits et al., 2009; Sander et al., 2009). If hH₄R-related diseases are caused or promoted by extraordinarily high constitutive hH₄R activity, inverse agonists at the hH₄R would be an interesting therapeutic option. However, if inverse agonists caused receptor up-regulation resulting in rebound effects after discontinuation of the therapy, neutral hH₄R antagonists would be preferred.

Therefore, we started a project to investigate structure-activity relationships for inverse agonism and neutral antagonism at the hH₄R. Here we report on the investigation of a series of 25 indole-, benzimidazole- and thienopyrrole derivatives that were previously described as hH₄R ligands (Venable et al., 2005) and are structurally related to the reference compound JNJ-7777120. We characterized the compounds using Sf9 cell membranes co-expressing the hH₄R with Gα₁₂ and Gβ₁γ₂. We performed steady-state GTPase assays and
high-affinity radioligand binding assays with $[^3H]HA$. Structure-activity relationships were established by molecular modelling and by correlating the efficacy values with different molecular descriptors such as log P and molar volume.

**Methods**

**Materials.** The baculoviruses encoding the N-terminally FLAG- and C-terminally hexahistidine-tagged hH4R were prepared as described recently (Schneider et al., 2009).

Recombinant baculovirus for the unmodified versions of the G$\beta_1$$\gamma_2$ subunits was a kind gift of Dr. P. Gierschik (Dept. of Pharmacology and Toxicology, University of Ulm, Germany). Baculovirus encoding G$\alpha_i$ was donated by Dr. A. G. Gilman (Department of Pharmacology, University of Southwestern Medical Center, Dallas, TX). The antibody selective for G$\alpha_{i1/2}$ was purchased from Calbiochem and the M1 anti-FLAG antibody was obtained from Sigma (St. Louis, MO, USA). The series of H4R antagonists investigated in this paper were synthesized as previously described (Venable, 2005) and were provided by Johnson & Johnson Pharmaceutical Research and Development (San Diego, CA). Stock solutions (10 mM) were prepared in Me$_2$SO. The dilution series was prepared in 10 % (v/v) DMSO. Because of low solubility, for compounds (9) and (10) 50 % (v/v) DMSO were used for preparation of the 1 mM solution. THIO was obtained from Tocris (Avonmouth, Bristol, UK) and the dilution series was prepared in 10 % DMSO. The final concentration of DMSO in the sample was 5 % for compounds (9) and (10) and 1 % for the other compounds. $[^3H]HA$ (specific activity 14.2 - 18.1 Ci/mmol) was obtained from Perkin Elmer (Boston, MA). [$^{32}$P]GTP was prepared in our laboratory using GDP and [$^{32}$P] (orthophosphoric acid, 150 mCi/ml, obtained from Perkin Elmer) according to a previously described enzymatic labelling procedure. All other reagents were of the highest purity available from standard suppliers. Radioactive samples were counted in a PerkinElmer Tricarb 2800TR liquid scintillation analyzer.
Generation of recombinant baculoviruses, cell culture and membrane preparation. Sf9 cells were cultured in 250 or 500 ml disposable Erlenmeyer flasks at 28°C under rotation at 150 r.p.m in SF 900 II medium (Invitrogen, Carlsbad, CA) supplemented with 5% (v/v) fetal calf serum (Biochrom, Berlin, Germany) and 0.1 mg/ml gentamicin (Cambrex Bio Science, Walkersville, MD, USA). Cells were maintained at a density of 0.5 – 6.0 x 10^6 cells/ml. Recombinant baculoviruses were generated in Sf9 cells using the BaculoGOLD transfection kit (BDPharmingen, San Diego, CA) according to the manufacturer’s instructions. After initial transfection, high-titer virus stocks were generated by two sequential virus amplifications. The supernatant fluid from the second amplification was stored under light protection at 4°C and used as routine virus stock for membrane preparations.

Infection of the cells with baculoviruses encoding hH4R, Gαi2 and Gβ1γ2 was performed as previously described (Preuss et al., 2007). Sf9 membranes were prepared as described (Gether et al., 1995), using 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 10 µg/ml benzamidine and 10 µg/ml leupeptin as protease inhibitors. Membranes were suspended in binding buffer (12.5 mM MgCl2, 1 mM EDTA and 75 mM Tris/HCl, pH 7.4). All membrane preparations were stored at -80°C until use.

[^3H]HA binding experiments. Prior to the experiments, membranes were sedimented by a 10 min centrifugation at 4°C and 13,000 r.p.m. and resuspended in binding buffer (12.5 mM MgCl2, 1 mM EDTA and 75 mM Tris/HCl, pH 7.4). For competition binding experiments 10 nM of [^3H]HA and 4-6 appropriate concentrations between 1 nM and 100 µM of the test compound were used. Non-specific binding was determined in the presence of THIO (10 µM). Incubations were performed for 60 min at 25°C and shaking at 250 rpm. Bound radioligand was separated from free radioligand by filtration through GF/C filters pretreated
with 0.3% (m/v) polyethyleneimine and washed three times with 2 ml of ice-cold binding buffer (4°C). Filter-bound radioactivity was determined by liquid scintillation counting.

**Steady-state GTPase assay.** Steady-state GTPase assays were essentially performed as previously described (Schneider et al., 2009). The samples did not contain NaCl, and the reaction temperature was 25°C. Inverse agonist pEC$_{50}$ values were determined in the absence of HA, whereas pK$_{b}$ values were calculated from pIC$_{50}$ values determined in the presence of agonist (HA, 100 nM). For all assays 4-6 appropriate concentrations between 1 nM and 100 µM of the test compound were used. To quantify efficacies, THIO (10 µM) was used as reference for the maximum inverse agonistic effect.

**Molecular modelling.** Based on the crystal structure of the human β$_2$ adrenergic receptor a homology model of the inactive hH$_4$R was generated as described (Deml et al., 2009). Glu$^{5.46}$ was modelled in its protonated state, as proposed (Jongejan et al., 2008). The unsubstituted compound (compound 2) was docked manually into the binding-pocket of hH$_4$R. Subsequently, molecular dynamic simulations were performed, as described previously (Strasser et al., 2008), including the natural surrounding of the receptor, like lipid bilayer and water. For all molecular dynamic simulations, the software Gromacs (http://www.gromacs.org) was used.

**Determination of descriptors for QSAR.** In order to search for dependency of efficacy, several ligand specific descriptors, like logP, polar surface area, molar refractivity, molar volume, refraction index and polarizability were calculated (ACD/PhysChem, Release 11.0, Toronto, Ontario, Canada).
**Miscellaneous.** Protein concentrations were determined with the Bio-Rad DC protein assay kit. Membrane proteins were separated on SDS polyacrylamide gels containing 10 or 12% (mass/vol.) acrylamide. Proteins were transferred onto Trans-Blot nitrocellulose membranes (Bio-Rad, Hercules, CA) and reacted with M1 anti-FLAG (1:1000) or anti-Gα1/2 (1:1000). Protein bands were visualized by enhanced chemiluminescence (Pierce Chemical, Rockford, IL) using goat anti-mouse IgG (Sigma, 1:2000) or donkey anti-rabbit IgG (GE Healthcare, Little Chalfont, Buckinghamshire, UK, 1:10000) both coupled to horseradish peroxidase.

Radioligand competition experiments and steady-state GTPase assays were analyzed by non-linear regression with the Prism 5.01 software (GraphPad, San Diego, CA, USA). For every compound the curves of all assays were merged in one file and a global fit over all data was performed. All values are given as means ± SEM. Significance was defined as p < 0.05 (confidence interval 95%). In case of multiple t-tests (comparisons of pEC50 with pK_i or pK_b) the Bonferroni correction was applied (44 t-tests), resulting in a new significance level of p < 0.001.

**Results**

**Radioligand Binding.** The compounds were characterized in radioligand competition binding assays using [3H]HA (10 nM) and membranes from Sf9 cells co-expressing the hH4R with Gαi2 and Gβ1γ2. As shown in Fig. 1A, there is a good correlation (p < 0.0001, r² = 0.88, slope: 1.12 ± 0.09) between the pK_i values determined in the Sf9 cell system and the previously reported results (Venable et al., 2005), indicating that the rank order of affinity within the compound series was retained in our experimental system. However, the pK_i values determined in Sf9 cell membranes were in average by 0.68 log units lower than the literature data.

Our results show (Table 1) that in the series of piperazine-methylated and R5-mono-substituted indole derivatives the pK_i values are influenced by the R5 residues in the following...
rank order (compound numbers in brackets): Cl (4) > Br (5) ~ F (3) > CH₃ (6) ~ NH₂ (8) ~ H (2) >> OCH₃ (7) ~ CF₃ (9). Thus, halogen substitution in position R₅ increases binding affinity, whereas an OCH₃- or a CF₃- residue in this position considerably reduces the pKi value. Introduction of an additional F- in position R₇ of compound 3 yields the structure 10 with slightly (0.28 log units) reduced affinity (Table 1). R₅-/R₇-disubstitution with Cl- (11) or CH₃- (12) did not significantly alter the binding affinity, compared to the corresponding R₅-monosubstituted compounds 4 and 6. This indicates that substituents in R₇ are well tolerated without major reduction of affinity (Table 1). In fact, R₇-monosubstitution with NH₂ (15) and CH₃- (16) is even beneficial and increases the binding affinity by ~ 0.5 log units, compared to the totally unsubstituted compound 2 (table 1).

We also investigated different positions of a Br- residue in the ring system and found that the pKi value correlates with the position of Br- in the rank order R₅ (5) >> R₆ (13) ~ R₇ (14), with ~ 1 log unit affinity difference between Br- in R₅ and R₆ (Table 1). This shows again that halogen substitution in position R₅ is favoured and leads to compounds with high binding affinity. Compound 4 – the experimentally most widely used H₄R antagonist JNJ-7777120 (Jablonowski et al., 2003; Thurmond et al., 2004) – shows the highest affinity in the investigated series and is substituted with Cl- in position R₅.

When the indole scaffold is replaced by a benzimidazol structure (Table 2, upper part), in most cases the affinity is reduced by at least 0.3 log units (cpds. 17, 18, 20, 21 in Table 2 vs. 2, 3, 6, 16 in Table 1). Only when a CF₃-substituent is located in position R₅, a change from the indole- (9) to the benzimidazole (19) structure leads to an increase of the pKi by ~ 0.4 log units. Replacement of the phenyl moiety of the indole scaffold by the bioisosteric thiophene ring yielded the thienopyrrole series shown in Table 2 (lower part). When completely unsubstituted, this change of scaffold led to a reduction of the pKi value by ~0.5 log units (indole derivative 2 in Table 1 vs. thienopyrrole derivative 23 in Table 2).
Compound 25, which is substituted with Cl- in position R4 and CH3 in R5 shows an affinity comparable to the (R5-) Cl-substituted compound 4 (JNJ-7777120).

**Steady-state GTPase assays: efficacies.** We also investigated the functional properties of the compounds in steady-state GTPase assays. As shown in Tables 1 and 2, the majority of compounds exhibited inverse agonism with efficacies between 15 and 62 % of the effect of the hH4R inverse agonist thioperamide (THIO). Interestingly, we also found three neutral antagonists (cpds. 9, 14 and 19). Fig. 2 shows examples for compounds with effects between neutral antagonism and 60-70 % of the THIO effect. As suggested by the structures of 9 (indole derivative) and 19 (benzimidazole derivative), a CF3- residue in position R5 favours neutral antagonism. Compound 14, which has a Br- substituent located in position R7, is also a neutral antagonist. Replacement of Br in R7 by either -H (2), -NH2 (15) or -CH3 (16) results in inverse agonists with efficacies of -0.40 (2), -0.37 (15) or -0.29 (16). This indicates that neutral antagonism requires an electronegative, electron-withdrawing substituent in position R7.

Relatively high inverse agonistic efficacies (range between -0.45 and -0.65) were found for compounds 1, 3, 10, 11, 17, 21 and 22. Interestingly, compound 1 is not methylated at the piperazine ring. Although the piperazine methyl substituent increases affinity in radioligand binding (cpd. 1 vs. 2 in table 1), it is obviously not necessary for a large inverse agonistic effect. Within the series of R5-monosubstituted indole derivatives (cpd. 2-9, Table 1), the efficacy depends on the R5-substituent in the following rank order: CF3 (9) < OCH3 (7) < Cl (4) < CH3 (6) < Br (5) < H (2) < NH2 (8) < F (3). When in the structures of 3 (R5 = F), 4 (R5 = Cl) or 6 (R5 = CH3) a second -F (10), -Cl (11) or CH3 (12) substituent is introduced in position R7 this does only slightly change inverse agonistic efficacy by 0.10 – 0.20 units. The Br- substituent can be moved from R5 (5) to R6 (13) without a significant
change in efficacy (Table 1). However, as already mentioned, -Br in position R7 leads to a complete loss of inverse agonistic effect, yielding a neutral antagonist.

In the benzimidazole series, for compounds 18, 19 and 20 (Table 2), the efficacies were not significantly different from those of the corresponding indole compounds 3, 6 and 9 (Table 1). Only in case of the completely unsubstituted and the (R7-) CH3-substituted compounds did a change from the indole structure (2 and 16 in Table 1) to the benzimidazole scaffold (17 and 21 in Table 2) increase inverse agonistic efficacy by 0.15 – 0.20. The compounds of the thienopyrrole series show only moderate to low inverse agonism. The thienopyrroles 24 and 25 and the indole derivative 12 represent the three inverse agonists with the lowest efficacy in the series, thus, representing almost neutral antagonists. Interestingly, all these compounds have a CH3-substituent located in similar regions of the molecule (R7 in the indole series or R5 of the thienopyrrole scaffold).

**Steady-state GTPase assays: pEC50 and pKb values.** The pEC50 values of the inverse agonists were determined in the absence of HA (“inverse agonist mode”). The pKb values were calculated from pIC50 values (Cheng-Prusoff equation), determined in the presence of 100 nM of HA (“antagonist mode”). As shown in Fig. 1B, we obtained a clear correlation (p < 0.001, r2 = 0.62) between pEC50 values from steady-state GTPase assays and radioligand binding pKb values. Interestingly, tables 1 and 2 indicate that most of the compounds show tendency towards increased pKb and pKb values compared to the corresponding pEC50 values. This is also demonstrated in Fig. 1B and 1D by the reduced slope of the regression lines and by the fact that the majority of data points are located left of the diagonal line. This trend reached significance (p < 0.001, t-test with Bonferroni correction for the total number of t-tests) for the compounds 3 and 11 (pKb >> pEC50, filled circles in Fig. 1B) as well as for the compounds 1, 3, 7, 10, 11 and 17 (pKb >> pEC50, filled circles in Fig. 1D). The pKb- or pKb-
values that are significantly higher than the corresponding pEC\textsubscript{50} values are also highlighted in tables 1 and 2.

The correlation between pEC\textsubscript{50} values and pK\textsubscript{b} values was significant, but widely scattered (Fig. 1D, p < 0.001, r\textsuperscript{2} = 0.36). The best correlation was found for the pK\textsubscript{b} values from antagonist mode GTPase assays, compared to the pK\textsubscript{i} values from radioligand competition binding (Fig. 1C, p < 0.0001, r\textsuperscript{2} = 0.66).

**Binding mode of compound (2).** The molecular dynamics revealed a stable interaction between compound (2) and the receptor (Fig. 3, A). The positively charged terminal amino moiety of the piperazine establishes an electrostatic interaction with the highly conserved Asp\textsuperscript{3.32}. A bivalent interaction is established between the side chain of the non charged Glu\textsuperscript{5.46} and the carbonyl moiety and the NH of the indole moiety, respectively. The indole moieties of the ligand and of Trp\textsuperscript{6.48} establish a hydrophobic, aromatic interaction. Besides, this interaction stabilizes Trp\textsuperscript{6.48} in its vertical conformation, which is proposed to be an essential part of the so-called rotamer toggle-switch (Shi et al., 2002) and which is considered typical for the inactive state of a biogenic amine receptor. Thus, the binding mode of (2) described here, differs significantly to that from the related compound (4), which was already described (Jojart et al., 2008). These significant differences in binding mode with regard to the structurally highly related compounds 2 and 4 may be explained by different hH\textsubscript{4}R models. The model of Jojart et al (2008) is based on the crystal structure of bovine rhodopsin, whereas the hH\textsubscript{4}R model used within this study is based on the crystal structure of h\beta\textsubscript{2}R. Another reason for the different findings may be the differences in simulation and docking protocol. A comparison of the backbone of TMVII of the compound (2)-hH\textsubscript{4}R-complex with the backbone of TMVII of the ligand-free hH\textsubscript{4}R revealed a slight shift of TMVII away from TMVI (Fig. 3, B). The indole moiety of the ligand switches into the pocket neighboured to Trp\textsuperscript{6.48} and thus induces a slight movement of Trp\textsuperscript{6.48} towards TMVII. As a consequence,
TMVII is slightly shifted away from TMVI. Furthermore, the simulations revealed two small pockets in R5 and R7 position of the indole derivatives (Fig. 3, C). These pockets can be filled with additional substituents in the corresponding positions. Docking of (11) into the binding-pocket reveals, that both chlorine atoms optimally fit into these additional pockets (Fig. 3, D). Thus, the interactions between ligand and receptor are not disturbed. Further docking studies with compound (7) (data not shown) revealed, that the space-filling methoxy moiety does not fit into the additional pocket.

Since the benzimidazole derivatives can exist in two tautomeric states, both tautomers of derivative (20) were docked into the binding pocket of hH4R. The first tautomer (methyl moiety in R5 position) fits well into the binding pocket with its additional methyl moiety, and no loss in interaction is observed (Fig. 3, E). In contrast, the second tautomer (methyl moiety in R6 position) does not fit into the binding pocket, because in position of the methyl moiety, there is no additional space left. Further simulations showed (data not shown), that the tautomer two (methyl moiety in R6 position) shifts away from TMVI. As a consequence, the interaction with Glu\(^{5.46}\) is lost. Besides that it is possible that the indole moiety rotates about 180°. In this conformation, there is sufficient space for the additional methyl group, but again, due to the rotation of the indole, the interaction between the NH of the indole and Glu\(^{5.46}\) is lost again.

**Dependency of the efficacy of compounds (2) – (9) on molar volume.** In order to describe the efficacy of the indole-derivatives with differences in substitution pattern in R5, we calculated the descriptors logP, polar surface area, molar refractivity, molar volume (van-der-Waals volume of one mol), refraction index and polarizability. The calculations showed that the efficacy is significantly dependent on the molar volume of the ligand, but not on the other properties, mentioned above. A correlation of the molar volume with the efficacy for compounds (2) – (9) led to a linear relationship (Fig. 4) described by the following equation:

\[
\text{efficacy} = -3.036 \pm 0.591 + 0.0129 \pm 0.00279 \ V \ [\text{cm}^3]
\]
with the statistical parameters: \( n = 8, r^2 = 0.782, F = 21.56, \) standard error of estimate = 0.085, \( p < 0.005. \) Thus, the efficacy of the compounds (2) – (9), with variations in the substitution pattern in R5 position is strongly dependent on volume of the substituent. All other properties did not show any influence onto efficacy. However, the efficacy of compounds (10) – (16) with different substitution patterns in R7 do neither correlate with the molar volume, nor with any of the other properties, mentioned above.

Discussion

The extraordinarily high and Na\(^+\)-insensitive constitutive activity of the hH\(_4\)R (Schneider et al., 2009) suggests that inverse agonists and neutral antagonists may behave differently in clinical conditions. If high constitutive receptor activity leads to pathological condition, then inverse hH\(_4\)R agonists may be more effective than neutral antagonists. On the other hand, neutral antagonists could be advantageous, because they may induce less up-regulation of binding sites, compared to inverse agonists.

Therefore, it is very important for the development of hH\(_4\)R antagonistic drugs that the structural requirements for neutral antagonism and inverse agonism are elucidated, enabling the synthesis of tailored compounds with the intended quality of action. To learn more about structure-activity relationships for hH\(_4\)R neutral antagonists and inverse agonists we investigated a series of 25 hH\(_4\)R antagonists previously described as H\(_4\)R ligands (Venable et al., 2005). The compounds were characterized in the recently reported Sf9 insect cell-based test system, co-expressing hH\(_4\)R with G\(\alpha_2\) and G\(\beta\gamma_2\) (Schneider et al., 2009). The expression level of G\(\alpha_2\) in Sf9 cells is more than 100-fold higher than the hH\(_4\)R expression level (Schneider et al., 2009). Thus, signal transduction of hH\(_4\)R is not hampered by a limited G\(\alpha_2\) availability. It has to be emphasized that we determined inverse-agonistic activity at the point of GDP/GTP exchange, which is very proximal in the signal transduction cascade. Functional assays at more distal points like adenylyl cyclase- (AC) or reporter gene assays often suffer
from non-linear signal transduction, e.g. due to limited availability of distal signalling components. Such effects can bias determination of correct inverse agonistic activities.

Radioligand competition assays with [3H]HA revealed a good correlation of our data with the previously reported results (Venable et al., 2005). Interestingly, among the 25 compounds investigated in our study, 3 compounds (12%) were neutral antagonists, suggesting that neutral antagonism is rather exceptional. This is confirmed by a previously reported survey of 105 articles describing 380 antagonists acting on 73 GPCRs. Only 15% of these compounds acted as neutral antagonists (Kenakin, 2004). The similar percentage of inverse agonists potentially indicates a general mechanism of regulation of constitutive activity that most GPCRs may have in common. Interestingly, the neutral antagonists identified in our studies all show very low affinities with pKᵣ values around 6.50 (cpd. 14) or lower. (cpds. 9 and 19) We do not have an explanation for these data. There is no indication in the literature that neutral antagonism goes along with low affinity. In fact, some low-affinity H₄R ligands are actually highly efficacious inverse agonists (Deml et al., 2009).

All inverse agonists investigated in our study exhibited lower efficacies than the reference compound THIO, confirming that THIO is one of the most effective inverse hH₄R agonists. However, as recently reported, THIO does not exert the maximum possible inverse agonistic effect (Schneider et al., 2009), and a quinazoline derivative with higher inverse agonistic efficacy than THIO was described (Smits et al., 2008). This compound is closely related to the structures investigated in our study, suggesting that small structural changes can largely affect inverse agonistic efficacy. This was also observed within our compound series. For instance, introduction of -Br in position R₇ of the unsubstituted indole derivative 2 (efficacy = -0.40) eliminates inverse agonism and produces the neutral antagonist 14. A similar sensitivity of inverse agonistic efficacy for subtle structural changes was previously described e.g. for α₁AR antagonists (Rossier et al., 1999) or 5-HT₁B R antagonists (Gaster et al., 1998).
Unexpectedly, we found a subset of compounds (filled circles in Figs. 1B and 1D) with pEC₅₀ values significantly lower than the pKᵢ and/or pKᵦ values. Such discrepancies are not unusual for inverse agonists (Table 3). The pKᵦ values in our compound series were much closer to the pKᵢ values than the pEC₅₀ values. Since pKᵦ and pKᵢ values were determined in the presence of HA, but pEC₅₀ values in its absence, it is possible that some of the inverse agonists discriminate between the constitutively active receptor state and the active state stabilized by histamine. However, this phenomenon seems to be complex, since the pKᵢ/pEC₅₀ difference was only observed in some hH₄R inverse agonists. Also the cimetidine/famotidine pair in Table 3 (Alewijnse et al., 1998) confirms that even in the same system the EC₅₀/Kᵢ ratio may show large variability. The literature examples shown in Table 3 for the cannabinoid CB₂R (Ross et al., 1999) and the dopamine D₂R (Roberts et al., 2004) show that also systems with good correlation between EC₅₀ and Kᵢ values exist. Interestingly, the difference in EC₅₀/Kᵢ ratios obtained with the neutral D₂R antagonist [³H]spiperone and the D₂R agonist [³H]NPA suggest that the quality of action of the radioligand may result in different Kᵢ values. Our results and the literature examples in Table 3 show that the two-state model of receptor activation, which describes only one active and one inactive receptor state, is not sufficient to describe the pharmacological properties of inverse agonists. Hence, the model must be extended to include different active states, dependent on whether the receptor is agonist-activated or constitutively active. Other data confirm that the two-state model of receptor activation has to be expanded into a more complex model. For example, the replacement of GTP by xanthosine 5’-triphosphate switches the effect of ICI 118551 (erythro-DL-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol) at the β₂AR-Gₛα₅ fusion protein expressed in Sf9 cells in the cAMP assay from inverse agonism to partial agonism (Seifert et al., 1999). Furthermore, certain β₂AR agonists act as partial agonists or as inverse agonists depending on whether the functional assay is performed in intact cells or in cell membranes (Chidiac et al., 1994).
Molecular dynamic simulations revealed a stable binding mode for (2), different from the already published binding mode of the related compound (4) (Jojart et al., 2008). However, the binding mode, described in this work, is similar to the recently published binding mode of clozapine at hH4R (Jongejan et al., 2008). The indole or the benzimidazole moiety of the compounds described here are embedded in a small pocket, parallel to the indole moiety of Trp\textsuperscript{6.48} in its inactive, more vertical conformation. A conformational change, the rotamer toggle switch of the highly conserved amino acids Trp\textsuperscript{6.48} and Phe\textsuperscript{6.52} is necessary for GPCR activation (Crocker et al., 2006; Strasser et al., 2008). Molecular dynamic simulations revealed that the hydrophobic interaction between the benzimidazole or indole moiety of the compounds stabilizes the inactive conformation of the Trp\textsuperscript{6.48}. Due to this interaction between the ligand and Trp\textsuperscript{6.48}, Trp\textsuperscript{6.48} cannot switch into its active, vertical conformation. Thus, the inverse agonism or antagonism of the analyzed compounds can be explained.

The exchange of the hydrogen in R5 and/or R7 position in the indole-derivatives into more space filling substituents, like chlorine, as given in compounds (4) and (11) leads to an increase in affinity, due to an increase in contact area between ligand and receptor. If the substituent is too large, like in compound (7), a decrease in affinity is observed. However, since no significant dependence between affinity and molar volume of compounds (2) – (9) could be observed, further and as yet unknown descriptors may be relevant. In contrast, the efficacy of compounds (2) – (9) is significant dependent from molar volume. With decreasing molar volume, corresponding to a decreasing volume of substituent in R5 position, a significant decrease in efficacy is observed (Fig. 4). Thus, small substituents in R5 position shift the equilibrium between inactive and active conformation towards the inactive state.

It should be noted that our study comprised a relatively small number of compounds. Important substitution patterns (e.g. benzimidazoles derivatives substituted in analogy to the indole derivatives) were, most unfortunately and despite substantial efforts, not available to us. Therefore, our study does not allow more detailed conclusions about the interactions at the
binding site. Binding modes of structurally completely different H₄R inverse agonists, e.g. of thioperamide or some 2,4-diaminopirimidines (Sander et al., 2009), are not predictable from our results. Additionally, establishing analogies between the binding mode of the JNJ-compounds and other compounds, would lead to an extensive modelling study, which is beyond the scope of this paper.

Nevertheless, to the best of our knowledge, our study is one of the first that systematically investigate structure-activity-relationships of inverse GPCR-agonists, using structurally closely related compounds. Our experiments showed that explaining the impact of structural alterations on the pharmacological properties of inverse agonists is much more difficult than expected, since structure-activity relationships could only be established for substituents in position R5 of the indole-derived compounds. Seemingly, the sole determination of pK₉ and pKᵢ values is not sufficient to establish structure-activity-relationships for inverse agonists. Similarly to previously reported results for 2,4-diaminopyrimidine-derived hH₄R ligands (Sander et al., 2009), our results show that small changes in molecular structure lead to extensive efficacy and potency differences, caused by yet unpredictable major changes in binding mode and receptor conformation. This could be elucidated by co-crystallization of hH₄R with structurally diverse inverse agonists. The recently published crystal structure of the β₂ adrenoceptor, co-crystallized with the inverse agonist carazolol (Cherezov et al., 2007; Rasmussen et al., 2007), shows that this approach is possible.

Acknowledgement

We would like to thank Mrs. Gertraud Wilberg and Mrs. Astrid Seefeld for their excellent technical assistance and the reviewers for their helpful critique.
References


Sander K, Kottke T, Tanrikulu Y, Proschak E, Weizel L, Schneider EH, Seifert R, Schneider G and Stark H (2009) 2,4-Diaminopyrimidines as histamine H₄ receptor ligands--


Footnotes:

This work was supported by the Research Training Program (Graduiertenkolleg) “Medicinal Chemistry – Ligand-Receptor Interactions” of the German Research Foundation (Deutsche Forschungsgemeinschaft, GRK 760). This work was supported by the European Cooperation in the Field of Scientific and Technical Research (COST) action #BM0806 (“Recent advances in histamine receptor H₄R research”), funded by the European Comission (7th European Framework Program).
Legends for Figures

Fig. 1. Comparison of S9 cell data with binding data from literature (Venable et al., 2005) and relationships between inverse agonist pKi, pEC50 and pKb values.

All data shown were determined in Sf9 cell membranes, co-expressing hH4R, Gαi2 and Gβ1γ2.

A, Correlation between the pKi values determined by radioligand binding in Sf9 cell membranes and the previously reported results (Venable et al., 2005). B, Correlation of pEC50 values (steady-state GTPase assays, inverse agonist mode) with pKi values (radioligand competition binding). C, Correlation of pKb values (steady-state GTPase assay, antagonist mode) with pKi values. D, Correlation of pEC50 values (steady-state GTPase assays, inverse agonist mode) with pKb values (steady-state GTPase assay, antagonist mode). The filled circles in B and D show the inverse agonists that have significantly higher pKi and pKb values, compared to the pEC50 value.

Fig. 2. Effects of selected inverse agonists in the steady-state GTPase assay.

Four compounds with efficacies from 0 to -0.62 were investigated in agonist mode (no pre-stimulation). The concentration-response curve of THIO is shown as reference. All experiments were performed with Sf9 insect cell membranes co-expressing hH4R, Gαi2 and Gβ1γ2. GTPase activity was determined as described in the Materials and Methods section.

Fig. 3. Binding mode at hH4R. A, binding mode of (2) at hH4R. There is an interaction between Asp3.32 and the hydrogen of the positively charged terminal amine moiety of the piperazine. A bivalent hydrogen bond interaction is found between the side chain of the non charged Glu5.46 and the carbonyl moiety and the NH of the indole moiety, respectively. The indole moieties of the ligand and of Trp6.48 establish a hydrophobic, aromatic interaction. B, View from the extracellular side. The additional Trp6.48 (green) and TMVII (green) represent the conformation without compound (2). In the ligand-receptor complex TMVII is slightly
shifted away from TMVI. C, compound (2) in the binding-pocket (grey). The dashed circles indicate the regions in the binding pocket with space left for substituents in position R5 and R7. D, compound (11) docked into the binding pocket of (2); Both chlorine fill the additional spaces optimum (indicated by black arrows). E, compound (20) docked in its first tautomeric form into the binding pocket of (2); the additional methyl group fits optimum into the additional space of the binding pocket (indicated by a black arrow). F, compound (20) docked in its second tautomeric form into the binding pocket of (2); the additional methyl group (indicated by a black arrow) is too large and thus does not fit optimally in the additional space of the binding pocket (indicated by a dashed circle); the ligand is shifted away from TMVI (brown arrow).

**Fig. 4. Dependency of the efficacy of compounds (2) – (9) from molar volume.**

The molar volume of the R5-substituted indol-derivatives 2 – 9 was correlated with inverse agonistic efficacy. Since all compounds differ only in the R5 substituent, the correlation shows that inverse agonistic efficacy becomes lower with increasing volume of the R5 substituent.
Table 1: Binding affinities ([3H]HA competition binding assays) and functional data (steady-state GTPase assays) for indole-derived H4R-ligands.

The highlighted pK_b- or pK_i-values are significantly (p<0.001) higher than the corresponding pEC_{50}-values.

<table>
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<th>R4</th>
<th>R5</th>
<th>R6</th>
<th>R7</th>
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<th>efficacy</th>
<th>pK_b</th>
<th>pK_i</th>
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<td>6.37 ± 0.03</td>
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<td>H</td>
<td>H</td>
<td>H</td>
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<td>6.58 ± 0.14</td>
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<td>H</td>
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<td>7.40 ± 0.13</td>
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</tr>
<tr>
<td>4</td>
<td>H</td>
<td>Cl</td>
<td>H</td>
<td>H</td>
<td>7.11 ± 0.16</td>
<td>-0.29 ± 0.02</td>
<td>7.65 ± 0.10</td>
<td>7.73 ± 0.04</td>
</tr>
<tr>
<td>5</td>
<td>H</td>
<td>Br</td>
<td>H</td>
<td>H</td>
<td>7.20 ± 0.28</td>
<td>-0.37 ± 0.04</td>
<td>7.31 ± 0.15</td>
<td>7.30 ± 0.05</td>
</tr>
<tr>
<td>6</td>
<td>H</td>
<td>CH_3</td>
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<td>H</td>
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<td>-0.32 ± 0.02</td>
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<td>6.96 ± 0.05</td>
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<tr>
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<td>H</td>
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<td>H</td>
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<td>H</td>
<td>H</td>
<td>6.48 ± 0.17</td>
<td>-0.42 ± 0.03</td>
<td>6.68 ± 0.10</td>
<td>6.91 ± 0.05</td>
</tr>
<tr>
<td>9</td>
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<td>CF_3</td>
<td>H</td>
<td>H</td>
<td>n.a.</td>
<td>n.a. (0.07 ± 0.05)</td>
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<td>5.63 ± 0.04</td>
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<td>6.97 ± 0.06</td>
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<td>Cl</td>
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<td>7.01 ± 0.06</td>
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<td>H</td>
<td>Br</td>
<td>H</td>
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<td>-0.42 ± 0.04</td>
<td>6.34 ± 0.12</td>
<td>6.35 ± 0.04</td>
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<td>14</td>
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<td>H</td>
<td>Br</td>
<td>n.a.</td>
<td>n.a. (0.03 ± 0.05)</td>
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<td>6.50 ± 0.04</td>
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<td>H</td>
<td>NH_2</td>
<td>6.95 ± 0.24</td>
<td>-0.37 ± 0.04</td>
<td>7.24 ± 0.16</td>
<td>7.28 ± 0.03</td>
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<tr>
<td>16</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>CH_3</td>
<td>7.46 ± 0.24</td>
<td>-0.29 ± 0.03</td>
<td>8.14 ± 0.22</td>
<td>7.36 ± 0.04</td>
</tr>
</tbody>
</table>

1 all compounds except for (1) are methylated at the piperazine moiety
2 steady-state GTPase assay, agonist mode
3 steady-state GTPase assay, antagonist mode (with 100 nM HA)
4 radioligand competition assay with 10 nM [3H]HA, n = 3, duplicates
5 n.a. = neutral antagonist
6 mean ± SEM of the effect of the highest concentration (100 µM)
Table 2: Binding affinities ([³H]HA competition binding assays) and functional data (steady-state GTPase assays) for benzimidazole- and thienopyrrol-derived H₄R-ligands. The highlighted pKᵦ-value is significantly (p<0.001) higher than the corresponding pEC₅₀-value.

**Benzimidazol-Derivatives**

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<th>cpd.#</th>
<th>R₄</th>
<th>R₅</th>
<th>R₆</th>
<th>R₇</th>
<th>pEC₅₀</th>
<th>(\text{efficacy}^{2})</th>
<th>(\text{pK}_b)</th>
<th>(\text{pK}_i)</th>
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<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>5.91</td>
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<td>6.71 ± 0.09</td>
<td>6.54 ± 0.04</td>
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<td>H</td>
<td>H</td>
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<td>-0.40 ± 0.03</td>
<td>6.80 ± 0.08</td>
<td>6.82 ± 0.05</td>
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<td>19</td>
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<td>CF₃</td>
<td>H</td>
<td>H</td>
<td>n.a.</td>
<td>n.a. (-0.05 ± 0.02)</td>
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<td>5.97 ± 0.05</td>
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<td>H</td>
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**Thienopyrrol-Derivatives**

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<th>pEC₅₀</th>
<th>(\text{efficacy}^{2})</th>
<th>(\text{pK}_b)</th>
<th>(\text{pK}_i)</th>
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<td>6.38 ± 0.03</td>
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<td>25</td>
<td>Cl</td>
<td>CH₃</td>
<td>7.37</td>
<td>-0.21 ± 0.02</td>
<td>7.11 ± 0.10</td>
<td>7.65 ± 0.05</td>
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</tbody>
</table>

¹ steady-state GTPase assay, agonist mode  
² steady-state GTPase assay, antagonist mode (with 100 nM HA)  
³ radioligand competition assay with 10 nM [³H]HA, n = 3, duplicates  
⁴ n.a. = neutral antagonist
Table 3: Comparison of EC$_{50}$ values from functional assays and K$_i$ values from radioligand binding assays of inverse agonists described in the literature.

<table>
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<tr>
<th>Ref</th>
<th>Receptor</th>
<th>Compound</th>
<th>Functional Parameter</th>
<th>EC$_{50}$ (inverse agonism)</th>
<th>inverse agonistic efficacy</th>
<th>reference, efficacy = -1</th>
<th>Radioligand used</th>
<th>K$_i$ (binding)</th>
<th>Ratio (EC$_{50}$/K$_i$)</th>
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<tbody>
<tr>
<td>(Smits et al., 2008)</td>
<td>Histamine h$_4$R</td>
<td>quinazoline 54</td>
<td>cAMP (CRE-galactosidase reporter gene)</td>
<td>145 nM$^1$</td>
<td>-1.49</td>
<td>thiopemamide</td>
<td>$[^{3}H]$HA</td>
<td>27 nM$^1$</td>
<td>5.4</td>
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<td></td>
<td>quinazoline 55</td>
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<td>69 nM$^1$</td>
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<td></td>
<td></td>
<td>7.6 nM$^1$</td>
<td>9.1</td>
</tr>
<tr>
<td>(Alewijnse et al., 1998)</td>
<td>Histamine h$_2$R</td>
<td>cimetidine</td>
<td>cAMP</td>
<td>400 ± 100 nM</td>
<td>-0.88 ± 0.04</td>
<td>ranitidine</td>
<td>$[^{125}I]$APT$^2$</td>
<td>660 ± 220 nM</td>
<td>0.6</td>
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<td>famotidine</td>
<td>cAMP</td>
<td>144 ± 64 nM</td>
<td>-0.88 ± 0.05</td>
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<td>16 ± 3 nM</td>
<td>9.0</td>
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<tr>
<td>(Chidiac et al., 1994)</td>
<td>β$_2$AR</td>
<td>alprenolol</td>
<td>cAMP</td>
<td>6.7 ± 1.1 nM</td>
<td>-0.68 ± 0.02</td>
<td>no reference compound$^3$</td>
<td>$[^{125}I]$CYP$^4$</td>
<td>0.32 ± 0.06 nM</td>
<td>21</td>
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<td>pindolol</td>
<td>cAMP</td>
<td>36 ± 8 nM</td>
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<td>0.71 ± 0.01 nM</td>
<td>51</td>
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<tr>
<td>(Ross et al., 1999)</td>
<td>Cannabinoid CB$_2$R</td>
<td>AM630 (6-iodopravadoline)</td>
<td>$[^{35}S]$GTP$\gamma$S binding</td>
<td>76.6 nM</td>
<td>47% inhib. of basal $[^{35}S]$GTP$\gamma$S binding</td>
<td>effect of AMD comparable to SR144528$^5$</td>
<td>$[^{3}H]$-CP55940$^6$</td>
<td>31.2 ± 12.4 nM</td>
<td>2.5</td>
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<tr>
<td>(Roberts et al., 2004)</td>
<td>Dopamine D$_2$R</td>
<td>Butoaclamol</td>
<td>$[^{35}S]$GTP$\gamma$S binding</td>
<td>0.5 nM$^1$</td>
<td>20% inhibition of basal $[^{35}S]$GTP$\gamma$S binding</td>
<td>no reference compound</td>
<td>$[^{3}H]$Spiperone$^7$</td>
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<td>$[^{3}H]$NPA$^8$</td>
<td>0.16 nM$^1$</td>
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</table>

1 calculated from logarithmic values
2 $[^{125}I]$APT = $[^{125}I]$Iodoaminopentidetide ($[^{125}I]$-N-[2-(4-amino-3-iodobenzoamido) ethyl]-N'cyano-N'-[3-(1-piperidinylmethyl) phenoxy]propylguanidin)
3 in this paper, inverse agonistic efficacy was defined as “fraction of spontaneous receptor activity that can be inhibited by a given agent”
4 $[^{125}I]$CYP = $[^{125}I]$Cyanopindolol ($[^{125}I]$-4-[2-hydroxy-3-(propan-2-ylamino)propoxy]-3-iodo-1H-indole-2-carbonitrile)
5 N-[(1S)-endo-1,3,3-trimethyl bicyclo [2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide
6 tritiated (1R,3R,4R)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol
7 neutral D$_2$R antagonist (tritiated 8-[4-(4-Fluorophenyl)-4-oxobutyl]-1-phenyl-1,3,8-triaza spiro[4,5]decan-4-one)
8 D$_2$R agonist (N-propylapomorphine)
Figure 1

A. $pK_i$ (J & J) vs. $pK_i$ (Sf9 cells), slope = 1.12 ± 0.09, $r^2 = 0.88$

B. $pK_i$ vs. $pEC_{50}$, slope = 0.69 ± 0.12, $r^2 = 0.62$

C. $pK_i$ vs. $pK_b$, slope = 0.80 ± 0.12, $r^2 = 0.66$

D. $pK_i$ vs. $pEC_{50}$, slope = 0.57 ± 0.17, $r^2 = 0.36$
Figure 2
Figure 4

Graph showing the relationship between molar volume (cm$^3$) and efficacy. The line has a slope of $0.013 \pm 0.003$ and an $r^2$ value of 0.78.