

***In vitro* pharmacology of clinically used CNS active
drugs as inverse H₁ receptor agonists**

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H₁R, histamine H₁ receptor; GPCR, G-protein coupled receptor; R-SAT, Repetitive Selection and Amplification Technology; CNS, central nervous system; BBB, blood brain barrier; FMH, S-(+)- α -fluoromethylhistidine; HTMT, 6-[2-(4-imidazolyl)ethylamine]-N-(4-trifluoromethylphenyl)-heptanecarboxamide dimaleate; PEA, 2-(2-aminoethyl)-pyridine; 5-HT, 5-hydroxytryptamine.

ABSTRACT

The human histamine H₁ receptor (H₁R) is a prototypical G-protein coupled receptor (GPCR) and an important, well-characterised target for the development of antagonists to treat allergic conditions. Many neuropsychiatric drugs are also known to potently antagonise this receptor, underlying aspects of their side effect profiles. We have used the cell-based R-SAT assay to further define the clinical pharmacology of the human H₁R by evaluating > 130 therapeutic and reference drugs for functional receptor activity. Based on this screen we have previously reported on the identification of 8*R*-lisuride as a potent stereospecific partial H₁R agonist (Bakker et al, 2004, Mol. Pharmacol. 65:538-549). In contrast, herein we report on a large number of varied clinical and chemical classes of drugs that are active in the central nervous system (CNS) that display potent H₁R inverse agonist activity. Absolute and rank order of functional potency of these clinically relevant brain-penetrating drugs may possibly be used to predict aspects of their clinical profiles, including propensity for sedation.

INTRODUCTION

Antagonists of the histamine H₁ receptor (H₁R) have proven effective in controlling many of the symptoms of the human allergic response. Classical H₁R antagonists, known as “first generation” anti-histamines may act as sedatives upon crossing the blood brain barrier (BBB) interacting with H₁R_s expressed in the central nervous system (CNS). In fact, sedation and performance impairment are undesirable and potentially dangerous side effects of first generation anti-histamines and are a major limitation of their use. Although many of the first generation anti-histamines exhibit additional anticholinergic properties that may contribute to their sedative properties, selective H₁R antagonists acting in the CNS might be exploited as sleeping aids. Subsequent development of anti-histamines focussed on H₁R antagonists that do not cross the BBB resulting in what are now termed “second generation” anti-histamines (Zhang et al., 1997). Another advantage of these second-generation anti-histamines is their increased selectivity for H₁R_s over other related monoaminergic receptor subtypes (Walsh et al., 2001). In contrast to second-generation anti-histamines, compounds developed to treat neuropsychiatric disease are specifically designed to enter the CNS and target various monoaminergic G-protein coupled receptors and small molecule re-uptake transporters. Radioligand binding studies have demonstrated that these compounds lack target specificity, and may act at multiple receptor and transporter sites simultaneously (Hill and Young, 1978; Richelson, 1978; Richelson and Nelson, 1984a; Cusack et al., 1994; Richelson and Souder, 2000). Notably, many of these compounds have been shown to possess high H₁R affinity (Tran et al., 1978; Richelson and Nelson, 1984a; Bymaster et al., 1996; Richelson and Souder, 2000). Examples of such molecules include antipsychotic drugs like clozapine and tricyclic anti-depressant drugs like amitriptyline. Since interactions with H₁R_s in brain can produce clinically significant adverse effects

including sedation (Sekine et al., 1999; Bakker et al., 2002; Simons, 2002), and possibly alterations in body weight (Kroeze et al., 2003; Roth and Kroeze, 2006), an improved understanding of the full extent of the H₁R mediated actions of neuropsychiatric drugs as a class may provide critical insights into their clinical profiles.

Drugs with anti-histaminergic activity have been traditionally classified as pharmacological antagonists of histamine at the H₁R, acting by competitively binding to the receptor, thus blocking H₁R mediated responses (Hill et al., 1997; Zhang et al., 1997). However, the techniques previously used to assess H₁R activity of commonly used therapeutics lack the ability to discriminate the functional nature of these interactions. More recent studies, utilising functional assays, have shown that some anti-histamines possess negative intrinsic activity at the H₁R, which has led to the reclassification of these agents as H₁R inverse agonists (Weiner et al., 1999; Bakker et al., 2000; Bakker et al., 2001). These observations raise important questions as to the critical physiological role of basal H₁R signalling and potential pharmacological importance of negative intrinsic *versus* neutral antagonistic activity of the multitude of clinically useful compounds that interact with H₁Rs.

We have utilised the cell based functional assay R-SAT (Receptor Selection and Amplification Technology), to further explore the clinical pharmacology of a variety of CNS drugs as inverse agonists at the human H₁R. We demonstrate a strong correlation between the affinity of known histaminergic drugs at the H₁R as determined by radioligand binding, to the inverse agonist potency determined by functional R-SAT and NF-κB assays (Bakker et al., 2001). Subsequently, extensive R-SAT based analysis of >

130 clinically relevant neuropsychiatric drugs revealed that many of these drugs are potent H₁R inverse agonists, while none were found to be true neutral antagonists.

METHODS

Materials

Cell culture media, penicillin, and streptomycin were obtained from Life Technologies (Merelbeke, Belgium). Calf serum (Life Technologies). Cyto-SF₃ (Kemp laboratories, Frederick, MD.) [³H]mepyramine (20 Ci/mmol) and *myo*-[2-³H]inositol (21 Ci/mmol) was purchased from NEN (Zaventem, Belgium). pNF-κB-Luc was obtained from Stratagene (La Jolla, USA), pSI was obtained Promega (Madison, WI), lipofectamine (Qiagen, Dusseldorf, Germany).

The sources of many of the drugs used in this study have been reported previously (Wellendorph et al., 2002; Bakker et al., 2004). The chemical names corresponding to the code names or non-standard abbreviations mentioned in Table 3 are: CPP ((±)-2-carboxypiperazine-4-yl)propyl-1-phosphonic acid), DOI ((±)-2,5-dimethoxy-4-iodoamphetamine hydrochloride), DS-121 (S-(-)-3-(3-cyanophenyl)-N-n-propyl piperidine), JL-18 (8-methyl-6-(4-methyl-1-piperazinyl)-11H-pyrido[2,3-b][1,4]benzodiazepine), LY 53,857 (6-methyl-1-(methylethyl)-ergoline-8β-carboxylic acid 2-hydroxy-1-methylpropyl ester maleate), mCPP (m-chlorophenylpiperazine), MDL 10097 ((±)2,3-dimethoxyphenyl-1-[2-(4-piperidine)-methanol]), MK 212 (6-chloro-2-(1-piperazinyl)pyrazine), SB 206553 (5-methyl-1-(3-pyridylcarbonyl)-1,2,3,5-tetrahydropyrrolo[2,3-f]indole), SCH 12679 (N-methyl-1-phenyl-7,8-dimethoxy-2,3,4,5-tetrahydro-3-benzazepine maleate), SCH 23390 (7-chloro-8-hydroxy-3-methyl-5-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine), SKF 38393 (6-phenyl-4-azabicyclo[5.4.0]undeca-7,9,11-triene-9,10-diol), SKF 83566 ((-)-7-bromo-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-3-benzazepine).

Gifts of acrivastine (The Wellcome Foundation Ltd, United Kingdom), astemizole (Janssen Pharmaceutica NV, Beerse, Belgium), cyproheptadine hydrochloride (MSD,

Haarlem, The Netherlands), d-chlorpheniramine maleate (A. Beld, Nijmegen, The Netherlands), diphenhydramine hydrochloride (Brocades, The Netherlands), levocabastine (Janssen Pharmaceutica, Beerse, Belgium), loratadine (Schering Plough, Bloomfield, USA), mainserin hydrochloride and mirtazepine (Org 3770, (\pm)-1,2,3,4,10,14b-hexahydro-2-methylpyrazino-[2,1-a]pyrido[2,3-c][2]benzazepine) (Organon NV, the Netherlands), pcDEF₃ (Dr. J. Langer), ranitidine dihydrochloride (Glaxo, UK), and of the cDNA encoding the human histamine H₁R (Dr. H. Fukui (Fukui et al., 1994)) are greatly acknowledged.

Molecular Cloning

The genes coding for the human H₁R and the G α q subunit were cloned as previously described (Burstein et al., 1995; Bakker et al., 2004). All receptor and G-protein constructs were fully sequence-verified by dideoxy chain termination methods. The sequence of the human H₁R used in this study corresponds to GenBank accession #D14436. All plasmid DNA used for transfections was prepared using resin based mega-prep purifications following the manufacture's protocols (Qiagen Inc. Dusseldorf, Germany).

Cell culture and transfection

COS-7 African green monkey kidney cells were maintained at 37°C in a humidified 5% CO₂/95% air atmosphere in Dulbecco's modified essential media (DMEM) containing 2mM L-glutamine, 50 IU/mL penicillin, 50 μ g/mL streptomycin and 5% (v/v) FCS. COS-7 cells were transiently transfected using the DEAE-dextran method as previously described (Bakker et al., 2001). The total amount of DNA transfected was maintained constant by addition of pcDEF₃. NIH-3T3 cells were cultured in DMEM supplemented

with 2mM L-glutamine, 1% penicillin and streptomycin and 10% bovine calf serum and maintained at 37°C in a humidified 5% CO₂/95% air atmosphere. NIH-3T3 cells were transiently transfected using the Superfect transfection reagent (Qiagen Inc, Dusseldorf, Germany) following the manufacture's protocols.

Receptor selection and amplification technology (R-SAT) assays

R-SAT assays were performed as described previously (Weiner et al., 2001; Bakker et al., 2004). On forming a monolayer, NIH-3T3 cells normally stop growing due to contact inhibition. In R-SAT assays the activation of pathways, ie through the activation of GPCRs, that promote cell growth result in NIH-3T3 cells being able to overcome their contact inhibition and proliferate. These stimulatory effects can be readily quantified using a marker gene, which allows graded responses to be measured, permitting precise determinations of ligand potency and efficacy. Briefly, on day one NIH-3T3 cells were plated into 96 well cell culture plates at a density of 7500 cells/well. On day two cells were transfected with 10-25 ng/well of H₁R DNA, with or without 5 ng/well of plasmid DNA encoding the various G α subunits, and 20 ng/well of plasmid DNA encoding β -galactosidase. On day three, the media was replaced with DMEM supplemented with 1% penicillin and streptomycin, 2% Cyto-SF₃ and varying drug concentrations. After five days of cell culture, media was removed and the cells were incubated in phosphate-buffered saline containing 3.5mM *O*-nitrophenyl- β -D-galactopyranoside, and 0.5% Nonidet P-40 detergent. The 96-well plates were incubated at room temperature for up to 8h, and the resulting colorimetric reaction was measured by spectrophotometric analysis at 420nm on an automated plate reader (Biotek Instruments Inc., Burlington, VT). Data were analysed by a non-linear, least squares curve-fitting procedure using Graphpad

Prism[®] (GraphPad Software, Inc., San Diego, CA). All data shown are expressed as mean \pm S.E.M..

Reporter-gene assay

COS-7 cells transiently co-transfected with pNF κ B-Luc (125 μ g/1 \cdot 10⁷ cells) and either pcDEF₃ or pcDEF₃hH₁ (25 μ g/1 \cdot 10⁷ cells) were seeded in 96 well blackplates (Costar) in serum free culture medium and incubated with drugs. After 48hrs, cells were assayed for luminescence by aspiration of the medium and the addition of 25 μ L/well luciferase assay reagent (0.83mM ATP, 0.83mM D-luciferin, 18.7mM MgCl₂, 0.78 μ M Na₂H₂P₂O₇, 38.9mM Tris (pH 7.8), 0.39% (v/v) glycerol, 0.03% (v/v) Triton X-100 and 2.6 μ M DTT). After 30min luminescence was measured for 3 sec/well in a Victor² multilabel counter (Perkin Elmer). All data shown are expressed as mean \pm S.E.M..

H₁R binding studies

Cells used for radioligand binding-studies were harvested 48h after transfection and homogenised in ice-cold H₁R-binding buffer (50mM Na₂/K-phosphate buffer (pH 7.4)). The cell homogenates were incubated for 30 min at 25°C in a total volume of 400 μ L H₁R-binding buffer with 1nM [³H]mepyramine. The non-specific binding was determined in the presence of 1 μ M mianserin. The incubations were stopped by rapid dilution with 3mL ice-cold H₁R-binding buffer. The bound radioactivity was separated by filtration through Whatman GF/C filters that had been treated with 0.3% polyethyleneimine. Filters were washed twice with 3mL buffer and radioactivity retained on the filters was measured by liquid scintillation counting. Binding data were evaluated by a non-linear, least squares curve-fitting procedure using Graphpad Prism[®] (GraphPad Software, Inc., San Diego, CA). Proteins concentrations were determined according to

Bradford (Bradford, 1976), using BSA as a standard. All data shown are expressed as mean \pm S.E.M..

RESULTS

Signalling characteristics of the human H₁R as determined by R-SAT

Plasmid DNA encoding the human H₁R was transiently transfected into NIH-3T3 cells as part of the R-SAT assay. Titration of the amount of H₁R DNA used for transfection revealed robust functional responses to histamine over a 100-fold dose range of receptor DNA, from 0.5 to 50 ng of DNA per well of a 96 well cell culture plate (Figure 1, Table 1). Histamine yielded an average biological response of 11.4±0.8 fold in H₁R expressing cells and was without effect in cells transfected with the marker gene alone. Transfection of the cells with increasing amounts of cDNA encoding the H₁R results in an increase in observed potencies for histamine, which reached a plateau pEC₅₀ of 7.3±0.2 at 10 ng DNA/per well. As depicted in Figure 1 and Table 1, mepyramine started to display negative intrinsic activity at the H₁R in cells transfected with 10 ng/well of cDNA encoding the H₁R. When expressed alone, a maximum of 6% of the total H₁R response corresponds to basal, agonist independent, signalling. As reported previously, constitutive receptor activity can be modulated by the expression of appropriate α subunits of guanine nucleotide binding proteins (G proteins) (Burstein et al., 1995; Burstein et al., 1997; Leurs et al., 2000; Bakker et al., 2001; Weiner et al., 2001), and this approach was therefore used in the present study to augment H₁R basal signalling properties. As depicted in Figure 1 and Table 1, co-transfection of a cDNA encoding G α_q (20 ng/well) enhanced the biological responses observed for the H₁R under all conditions studied, and yielded an average biological response of 9.6±0.9 fold for histamine. Agonist potencies were increased upon co-transfection of G α_q as compared to receptor alone, ranging from 11 to 26 fold more potent than that observed without G α_q co-expression. The observed potencies for histamine reached a plateau pEC₅₀ of 8.5±0.2 at 5 ng receptor DNA/per well in the G α_q co-expression experiments. Co-expression of

H₁Rs and G α_q resulted in an increased basal response and a concomitant reduction in the fold response upon histamine stimulation of the cells (Figure 1B). Mepyramine behaved as an inverse agonist under all conditions studied, but does not display a significant change in inverse agonist potency when increasing amounts of cDNA encoding the H₁R are utilised. Constitutive H₁R signalling was detectable in all co-expression experiments, ranging from 10% to 30% of the total biological response. Figure 1C depicts the relationship between the amount of transfected DNA and constitutive receptor signalling observed under these experimental conditions.

Constitutive H₁R activity is not due to endogenous histamine

We have previously reported the use of S-(+)- α -fluoromehtylhistidine (FMH), an irreversible inhibitor of histidine decarboxylase (Watanabe et al., 1990), together with serum-free assay conditions, to confirm that constitutive H₁R activity is not due to contamination with endogenous histamine (Bakker et al., 2000; Bakker et al., 2001). To avoid a similar confounding factor in the R-SAT assays, synthetic serum, devoid of trace monoamines, replaced calf serum during cell culture. Moreover, in agreement with our previous findings in COS-7 cells, the addition of as much as 100 μ M FMH to the cell culture media did not attenuate the basal H₁R mediated signalling or the observed negative intrinsic activity displayed by mepyramine observed in this assay (data not shown).

Evaluation of R-SAT for determining functional H₁R responses

Agonist Responses - Based on the potencies observed for histamine during the cDNA-titration studies, 10ng H₁R DNA per well was chosen as the most appropriate assay condition to evaluate potential agonist activity of ligands at the human H₁R. The

histamine induced R-SAT responses were competitively antagonised by the classical H₁R inverse agonist mepyramine. Schild-plot analysis of the competitive antagonism by mepyramine of the histamine-induced proliferation resulted in a pA₂ value for mepyramine of 8.3 (slope = 1.05±0.03, r² = 0.997). A series of known histaminergic agonists were tested for functional activity at the human H₁R, where the most potent agonist was histamine itself with an EC₅₀ of 35 nM. Histamine yielded the largest fold responses, consistent with its designation as a full agonist (intrinsic activity, α, of 1). N^α-methylhistamine and 2-(2-aminoethyl)-pyridine (PEA) behaved as full agonists, with EC₅₀ values of 120 nM and 1.32 μM, respectively, whereas HTMT (6-[2-(4-imidazolyl)ethylamine]-N-(4-trifluoromethylphenyl)-heptanecarboxamide dimaleate) displayed only weak partial agonist activity (pEC₅₀ = 6.2±0.2, α=0.27±0.04). In contrast, both enantiomers of the H₃R preferring agonist α-methylhistamine displayed only weak partial agonist activity with EC₅₀ values greater than 10 μM, while the H_{3/4}-receptor selective agonists imetit and imnepip, and the H₃R antagonist/H₄R agonist clobenpropit displayed no intrinsic activity at the H₁R (data not shown).

Inverse Agonist Responses - Based on the degree of basal signalling, and the potencies observed for mepyramine during the titration studies, 10 ng H₁R cDNA/well co-transfected with 20 ng Gα_q cDNA/well was chosen as the most appropriate assay condition to evaluate potential inverse agonist activity of ligands at the human H₁R. Mepyramine and astemizole consistently yielded the largest degree of inhibition of basal signalling, consistent with their designation as full inverse agonists (α = -1). As reported in Table 2, all 11 of the known H₁R antagonists that were tested in this manner behaved as inverse agonists. Ketotifen was the most potent, with an IC₅₀ of 0.21 nM. The rank order of potencies for these compounds was ketotifen > levocabastine > mepyramine >

astemizole > triprolidine > chlorpheniramine > tripeleminamine > acrivastine > diphenhydramine > loratidine. All of these compounds displayed high potency for the human H₁R, ranging from 0.21 to 126 nM, and all, with one notable exception, behaved as full inverse agonists. Interestingly, loratidine displayed partial efficacy ($\alpha = -0.77 \pm 0.03$; Table 2). The H₂R-selective inverse agonists cimetidine and ranitidine, as well as the H₃R inverse agonist clobenpropit, and H_{3/4} receptor preferring antagonists thioperamide and iodophenpropit, all lacked activity as inverse agonists at the H₁R (Table 2).

Correlation between assays - We have previously reported the potencies of a number of histaminergic drugs as H₁R inverse agonists as determined by the NF- κ B assay (Bakker et al., 2001). Table 2 reports the potencies of many of these histaminergic compounds as determined by this assay, as well as the affinities of many of these ligands for the H₁R as determined by radioligand binding experiments. Comparison of the functional potencies of these compounds between assays reveals a close correlation ($r^2 = 0.92$, slope = 0.72).

Evaluation of the functional H₁R activity of various therapeutics using R-SAT

Examination for H₁R agonist activity - We evaluated a library of > 130 clinically relevant therapeutic drugs for functional activity at the human H₁R using R-SAT (see Table 3 for a complete list of compounds tested). We controlled for both endogenous receptor and non-receptor mediated effects of the tested drugs on cellular growth by assaying all drugs against cells expressing the β -galactosidase marker gene alone, and cells expressing either related or unrelated receptors (e.g. 5-HT_{2A} or NK-1 receptors, data not shown). None of the compounds reported herein displayed non-specific potent

amplification or repression of cellular growth when tested in this manner (data not shown).

All compounds were initially screened for H₁R agonist activity. Only three compounds, lisuride, terguride, and methergine displayed reasonable potency as H₁R agonists. We have recently reported the detailed agonist pharmacology of these compounds (Bakker et al., 2004).

Examination for H₁R inverse agonist activity - After the evaluation of the various CNS drugs for H₁R agonist activity, all compounds were subsequently tested for H₁R inverse agonist activity. In contrast to the finding that only a few compounds display H₁R agonist activity, most of the tested compounds potently inhibited constitutive H₁R activity. Table 3 reports the H₁R inverse agonist potencies of all of these compounds as determined by the R-SAT assays and the inverse agonist behavior of several of the tested anti-psychotics, anti-depressants, and miscellaneous agents. Of this large dataset, only the H₁R inverse agonist potencies of the anti-psychotic agents that are listed in this large data set have been previously reported (Weiner et al., 2001). The majority of anti-psychotic agents tested possess potent H₁R inverse agonist properties. All behaved as full inverse agonists except for loxapine, risperidone, haloperidol, and the investigational agent MDL 10097. The dibenzodiazepine based agents (clozapine, loxapine, clothiapine, olanzapine, and perlapine) were amongst the most potent, the phenothiazine based agents (chlorpromazine, thioridazine, mesoridazine, etc.) displayed moderate potencies, whereas the butyrophenone based agents (haloperidol, trifluoperidol, fluspirilene, moperone, etc.) were amongst the least potent (Table 3). In addition to the anti-psychotics, many anti-depressant drugs also display this pharmacological activity. The tricyclic-based agents all display potent H₁R inverse agonism, with observed potencies

ranging from 0.25 nM for mirtazepine, to 200 nM for desipramine (Table 3). Lastly, of the various monoaminergic reference compounds tested, only a small number of serotonergic compounds displayed H₁R inverse agonist potencies, whereas the muscarinic, and dopaminergic receptor based compounds tested lack this activity (Table 3).

Examination for competitive H₁R antagonists - All compounds lacking intrinsic activity at the H₁R at concentrations up to 10 μ M were subsequently tested for their ability at concentrations up to 10 μ M to antagonise histamine induced R-SAT responses. Compounds were tested using agonist-biased assays with a 150 nM final concentration of histamine. We have described the identification of neutral H₁R antagonists in a separate study, and have shown that both inverse H₁R agonists and neutral H₁R antagonists are able to yield inhibitory actions using such an agonist-biased assay setup (Govoni et al., 2003). Hence both inverse H₁R agonists and neutral H₁R antagonists can be used as a positive control in these experiments, and herein we have chosen to use the readily available inverse H₁R agonist mepyramine for this purpose. Screening in this manner failed to identify any compounds that behaved as neutral antagonists of the human H₁R (data not shown). A list of all of the compounds tested in this manner can be found in Table 3.

DISCUSSION

That human H₁R antagonists have clinical utility in the treatment of allergic and inflammatory conditions has been appreciated for some time and antihistamines currently are among the most widely prescribed medications in the world (Woosley, 1996; Zhang et al., 1997; Handley et al., 1998). The development of such agents has been a major focus of drug discovery, and has yielded a number of widely used antihistamines. These compounds are thought to act primarily by competing with endogenous histamine, blocking histamine induced H₁R mediated activation of appropriate second messenger signalling-pathways (Zhang et al., 1997). Recent studies have demonstrated that many competitive antagonists, of a wide variety of different receptor types, are actually inverse agonists that possess the intrinsic ability to decrease agonist independent, constitutive receptor responses (Kenakin, 2001; Seifert and Wenzel-Seifert, 2002). Some classically defined H₁R antagonists have also recently been reclassified as inverse agonists based on the application of functional assays, that, unlike radioligand binding techniques, can differentiate competitive antagonists from inverse agonists (Weiner et al., 1999; Bakker et al., 2000; Bakker et al., 2001; Weiner et al., 2001; Wu et al., 2004; Sakhalkar et al., 2005). In the present study we set out to determine the functional activity of a large series of clinically useful agents at the human H₁R using the functional, cell based, R-SAT assay. R-SAT assays generate physiologically predictive responses that demonstrate strong correlations to the known *in vitro* pharmacology of multiple GPCRs, and is particularly suitable for screening large series of compounds due to the throughput necessary to perform such studies (Weiner et al., 2001; Croston, 2002; Wellendorph et al., 2002).

The development and application of radioligand binding methodologies allowed for the analysis of H₁R affinities of many clinically useful drugs and enabled the correlation between high H₁R affinity and the propensity for sedation for brain penetrating drugs (Sekine et al., 1999; Bakker et al., 2002; Simons, 2002). Validation of the R-SAT based H₁R pharmacology reported herein is demonstrated by the close correlation between the results obtained in this assay, and the previously reported H₁R pharmacology, including rank orders of affinity (Bakker et al., 2000; Bakker et al., 2001) and *in vitro* and *in vivo* potencies (Sekine et al., 1999) of many histaminergic compounds (see Table 2).

The broad functional screening reported herein has demonstrated that all the herein tested H₁R antagonists, despite their various molecular structures, possess negative intrinsic activity and are actually H₁R inverse agonists. This observation concurs with our previous observations on H₁R inverse agonism and suggests that perhaps negative intrinsic activity may be necessary for their therapeutic effectiveness.

We also demonstrate a strong correlation between antagonist affinities, and potencies of these agents as H₁R inverse agonists in these two assays. Thus, absolute and relative H₁R inverse agonist potencies can be used to predict the propensity of a compound to produce sedation (Sekine et al., 1999; Bakker et al., 2002; Simons, 2002) as well as other H₁R-mediated effects such as weight gain (Kroeze et al., 2003; Roth and Kroeze, 2006) if it is known that these properties are primarily related to the H₁R effects of the compound and that the drug will enter the CNS. For instance, the potent H₁R inverse agonist activity of the antipsychotic perlapine is consistent with its robust sedative effects clinically (Allen and Oswald, 1973; Stille et al., 1973), as are the potent inverse agonist activity of clozapine (The Parkinson Study Group, 1999). Similarly, the high potency H₁R inverse agonist activity of tricyclic antidepressants is consistent with prior

binding affinity data (Richelson, 1978; Richelson and Nelson, 1984a; Richelson and Nelson, 1984b; Cusack et al., 1994; Bymaster et al., 1996; Richelson, 2001). We have, in contrast to prior studies, tested a larger set of clinically useful compounds, and have found that many serotonergic compounds possess inverse agonist activity at human H₁Rs (Table 4 and discussion below).

Constitutive, basal, or spontaneous activity of the receptor, in the context of receptor pharmacology, is receptor-mediated signalling in the absence of agonist. It is most commonly seen in systems with high levels of receptor expression where inverse agonists inhibit both basal and agonist-induced receptor signalling. While the detection of constitutive GPCR activity is therefore system dependent, ie dependent on for instance receptor and G-protein expression levels. For the H₁R we have been able to readily detect constitutive activity, as well as the inverse agonistic characteristics of a variety of ligands previously known as H₁R antagonists, when measuring either the accumulation of inositolphosphates (Bakker et al., 2000), the activation of the transcription factor nuclear factor kappa-B in a reporter-gene assay (Bakker et al., 2001), as well as in R-SAT assays (Weiner et al., 1999; Bakker et al., 2004; and herein), when using heterologous expression systems. As inverse agonists are able to induce a response they potentially also display physiological activity in the absence of elevated levels of (endogenous) extracellular agonist. Since neutral antagonists and inverse agonists may have physiologically distinct actions *in vivo*, an H₁R neutral antagonist may differ from existing agents with respect to efficacy, tolerance, and perhaps propensity to induce clinically relevant side effects (Govoni et al., 2003). Constitutive GPCR activity is typically more readily observed in receptor over-expression systems compared to native systems. In line with these observations, to date, there have been no reports directly

showing *in vivo* constitutive activity of the H₁R. The development of high affinity H₁R ligands that lack intrinsic activity, and the subsequent utilisation of these compounds in *in vivo* studies will be necessary to fully assess these hypotheses.

We have previously reported on the identification of the neutral H₁R antagonists histabudifen and histapendifen (Govoni et al., 2003). These findings resulted from the screening of a large variety of structurally diverse ligands for their activity at the human H₁R and while many antagonists were found to possess negative intrinsic activity, only very few ligands failed to display any intrinsic activity at the H₁R. Unfortunately, the affinity of the currently known neutral H₁R antagonists is too poor for the evaluation of their therapeutic efficacy and potential side effects such as potential induction of weight gain due to antagonizing the action of histamine at the H₁R.

In conclusion, we have screened a large number of CNS drugs for their intrinsic activity at the human H₁R and found the vast majority of these drugs to display pronounced H₁R inverse agonistic properties. Exceptions are the drugs 8*R*-lisuride and 8*R*-terguride we identified to possess H₁R agonistic properties (Bakker et al., 2004), and the drugs that were found not to interact with the H₁R as assessed by their ability to modulate the H₁R-mediated effects of histamine and mepyramine in functional competition experiments. These data may help to understand the propensity of the identified H₁R inverse agonists to induce side effects, including weight gain and sedation, and prompt for the development of high affinity neutral H₁R antagonist to evaluate their clinical effectiveness and side effects.

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FOOTNOTES

- a) Unnumbered footnotes:

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LEGENDS FOR FIGURES

Figure 1. Signalling properties of human H₁Rs. Functional responses for histamine (■), and mepyramine (□) observed in R-SAT assays in 3T3 cells transfected with H₁R cDNA amounts of 0.5 ng/well (A), and 10 ng/well (B). The effects of co-transfection of 20ng/well of Gα_q are indicated for histamine (●) and mepyramine (○) for both receptor cDNA amounts (dashed lines). No drug values for each experimental condition are denoted at the left hand side of the figure. Data are reported as a percentage of the total response determined by Histamine Response / Mepyramine Response. C, Graphic depiction of the relationship between constitutive activity of the human H₁R and concentration of receptor DNA used for transfections as part of the R-SAT assay. Percentage constitutive activity is calculated as: (Basal, No Drug, Response – Mepyramine Response) / (Histamine Response – Mepyramine Response). Solid squares represent receptor expressed alone (■), while open squares depict co-expression with Gα_q at 20 ng per well (□). The values are expressed as means ± SEM of 3 to 8 separate experiments from representative nine-point concentration response curves each performed in duplicate.

Table 1. Constitutive activity of the human H₁R as determined by R-SAT. The amount of receptor DNA utilized in the R-SAT assays, the pEC₅₀ and pIC₅₀ values of histamine and mepyramine, respectively, and the percentage constitutive activity for the human H₁R is reported. The effects of co-expression of the murine Gα_q subunit are also reported. The percentage constitutive activity is calculated as (Basal, No Drug, Response – Mepyramine Response) / (Histamine Response – Mepyramine Response). The values are expressed as means ± SEM of 3 to 8 separate experiments, each performed in duplicate.

DNA (ng/well)	Histamine		Mepyramine	Constitutive activity (%)
	Fold	pEC ₅₀	pIC ₅₀	
0.5 H ₁	10±2	6.7±0.2	- ^a	- ^a
1.0 H ₁	12±3	7.1±0.1	- ^a	- ^a
5.0 H ₁	13±2	7.1±0.1	- ^a	- ^a
10 H ₁	14±2	7.3±0.2	- ^a	- ^a
25 H ₁	11±1	7.2±0.1	8.3±0.2	4±1
50 H ₁	9±1	7.3±0.2	8.7±0.4	6±2
0.5 H ₁ + 20 Gα _q	10±2	8.0±0.1	8.2±0.2	10±3
1.0 H ₁ + 20 Gα _q	12±2	8.2±0.2	8.5±0.2	16±4
5.0 H ₁ + 20 Gα _q	11±2	8.5±0.2	8.7±0.1	29±4
10 H ₁ + 20 Gα _q	11±1	8.5±0.2	8.6±0.1	31±5
25 H ₁ + 20 Gα _q	9±1	8.5±0.2	8.6±0.1	29±3
50 H ₁ + 20 Gα _q	5±1	8.4±0.1	8.6±0.1	29±6
^a Could not be determined.				

Table 2. H₁R Inverse agonist pharmacology of known histaminergic ligands as determined by radioligand binding, as well as NF-κB and R-SAT functional assays. The pK_i and pIC₅₀ at the human H₁R and their intrinsic activities (α) are reported. The values are expressed as means ± SEM of separate experiments, each performed in triplicate.

Compound	[³ H]mepyramine ^a	R-SAT assay ^b		NF-κB assay ^c	
	pK _i	pIC ₅₀	α	pIC ₅₀	α
<i>Clinically relevant H₁R ligands</i>					
Acrivastine	7.5±0.1	7.7±0.2	-0.96±0.02	7.4±0.1	-0.65±0.06
Astemizole	8.7±0.1	8.4±0.1	-1.00 ^d	8.1±0.2	-1.00 ^d
D-chlorpheniramine	8.2±0.1	8.1±0.2	-0.96±0.01	7.8±0.1	-0.92±0.06
Cyproheptadine	9.3±0.1	9.4±0.1	-0.81±0.08	8.4±0.1	-0.97±0.02
Diphenhydramine	7.9±0.1	7.4±0.1	-0.93±0.03	7.2±0.2	-0.71±0.10
Doxepine	9.9±0.1	9.1±0.1	-0.96±0.03	9.4±0.3	-0.82±0.05
Ketotifen	10.4±0.1	9.7±0.2	-0.99±0.05	9.3±0.1	-0.93±0.05
Levocabastine	7.9±0.1	9.0±0.1	-0.95±0.02	8.0±0.1	-0.97±0.02
Loratadine	6.8±0.1	6.9±0.1	-0.77±0.03	6.4±0.2 ^e	-0.97±0.06 ^e
Mepyramine	8.7±0.1	8.6±0.1	-1.00±0.01	7.9±0.1	-0.89±0.01
Mianserine	9.1±0.1	9.0±0.1	-0.99±0.01	8.7±0.1	-0.96±0.05
Tripelennamine	8.0±0.1	7.8±0.1	-1.01±0.04	7.4±0.1	-0.75±0.02
Tripolidine	8.5±0.1	8.4±0.1	-0.99±0.03	8.4±0.1	-0.63±0.06
<i>Other histaminergic ligands</i>					
Cimetidine	<4	<4		n.d.	
Ranitidine	<4	<4		<4	
Clobenpropit	5.2±0.1	<4		<4	
Thioperamide	<4	<4		<4	

^a Determined by displacement of [³H]mepyramine binding.

^b Experiments performed on NIH-3T3 cells transiently expressing the human histamine H₁ receptor as well as Gα_q (see text).

^c Experiments performed on COS-7 cells transiently transfected with cDNA encoding the human histamine H₁ receptor.

^d The intrinsic activity (α) of astemizole was set to -1.00.

^e Loratadine was found to inhibit luciferase activity in the reporter-gene assay in mock transfected COS-7 cells at concentrations higher than 1μM, therefore the pIC₅₀ value and the intrinsic activity could not be determined reliably in this assay (Bakker et al., 2001).

n.e. No effect.

n.d. Not determined.

Table 3. H₁R inverse agonist activity of clinically relevant drugs. The pIC₅₀ and intrinsic activity (α) values of identified histamine H₁R inverse agonists are reported, and the values are expressed as means ± SEM of 3-8 separate experiments, each performed in triplicate. N.D. denotes not determined.

	pIC ₅₀	α		pIC ₅₀	α
<i>ANTIPSYCHOTICS</i>			<i>ANTIDEPRESSANTS</i>		
Perlapine	9.7 ± 0.1	-1.01 ± 0.05	Mirtazepine	9.6 ± 0.1	-0.90 ± 0.04
Clozapine	9.4 ± 0.1	-1.03 ± 0.03	Doxepin	9.2 ± 0.1	-0.96 ± 0.03
Octoclothebin	8.6 ± 0.1	-1.01 ± 0.02	Amitypriline	8.9 ± 0.2	-0.95 ± 0.05
Clothiapine	8.5 ± 0.1	-0.95 ± 0.02	Trimipramine	8.7 ± 0.1	-0.93 ± 0.01
Loxapine	8.3 ± 0.1	-0.79 ± 0.06	Nortryptiline	7.9 ± 0.2	-1.05 ± 0.04
Quetiapine	8.3 ± 0.1	-0.99 ± 0.01	Imitramine	7.5 ± 0.1	-0.90 ± 0.05
JL-18	8.3 ± 0.1	-0.97 ± 0.04	Protrypriline	7.3 ± 0.1	-1.04 ± 0.05
Cis-flupentixol	8.1 ± 0.1	-0.92 ± 0.05	Clomipramine	6.9 ± 0.2	-0.96 ± 0.02
Promazine	7.9 ± 0.1	-0.97 ± 0.02	Desipramine	6.7 ± 0.1	-0.92 ± 0.05
Thiothixene	7.9 ± 0.2	-1.01 ± 0.04	Trazodone	6.3 ± 0.1	-0.82 ± 0.05
Telfudazine	7.8 ± 0.2	-1.03 ± 0.01	Buspirone	6.0 ± 0.2	-0.80 ± 0.05
Olanzapine	7.8 ± 0.1	-0.99 ± 0.02	Flouxetine	5.9 ± 0.2	-0.62 ± 0.03
Pimozide	7.8 ± 0.2	-1.03 ± 0.03	Zimelidine	5.3 ± 0.1	N.D.
Mesoridazine	7.7 ± 0.1	-0.92 ± 0.07	<i>SEROTONERGICS</i>		
Trans-flupentixol	7.6 ± 0.1	-0.96 ± 0.03	Cyproheptadine	9.4 ± 0.1	-0.81 ± 0.08
Prothypendyl	7.6 ± 0.1	-0.97 ± 0.04	Mianserin	9.0 ± 0.1	-0.99 ± 0.01
Perazine	7.5 ± 0.1	-0.97 ± 0.02	Methiothepin	8.8 ± 0.2	-0.96 ± 0.03
Fluphenazine	7.4 ± 0.1	-0.97 ± 0.02	Ritanserin	7.0 ± 0.1	-0.93 ± 0.02
Amoxapine	7.4 ± 0.1	-0.94 ± 0.04	Ketanserin	6.6 ± 0.1	-0.90 ± 0.01
Chlorpromazine	7.4 ± 0.1	-0.94 ± 0.05	Pirenpirone	6.4 ± 0.1	-0.74 ± 0.09
Thioridazine	7.3 ± 0.1	-0.91 ± 0.04	Cinanserin	6.1 ± 0.4	-0.98 ± 0.04
Thiospirone	7.1 ± 0.1	-0.98 ± 0.02	LY 53,857	5.9 ± 0.2	-0.65 ± 0.04
Chlorprothizene	7.1 ± 0.2	-0.91 ± 0.04	Metergoline	5.8 ± 0.2	-0.79 ± 0.09
Fluspiriline	6.8 ± 0.3	-0.88 ± 0.09	mCPP	5.7 ± 0.2	-0.74 ± 0.05
Trifluoperazine	6.5 ± 0.2	-0.94 ± 0.06	<i>DOPAMINERGICS</i>		
MDL 10097	6.5 ± 0.1	-0.74 ± 0.07	Prochlorperazine	6.7 ± 0.2	-0.94 ± 0.05
Amperozide	6.4 ± 0.1	-0.88 ± 0.03	<i>MUSCARINICS</i>		
Sertindole	6.2 ± 0.3	-0.93 ± 0.05	Benztropine	7.7 ± 0.2	-0.91 ± 0.02
Moperone	6.1 ± 0.2	-0.83 ± 0.08			
Spiperone	5.9 ± 0.2	-0.98 ± 0.08			
Trifluoperidol	5.9 ± 0.2	-0.85 ± 0.09			
Haloperidol	5.6 ± 0.1	-0.55 ± 0.06			
Butaclamol	5.6 ± 0.3	-0.89 ± 0.03			
Risperidone	5.5 ± 0.1	-0.73 ± 0.06			

Compounds that failed to show intrinsic activity and competitive antagonism at the human H₁ receptor at concentrations up to 10μM: 5-Hydroxymethylenedioxyamphetamine, 5-methoxytryptophan, 5-Hydroxyl-tryptophan, Alaprocate, Alpha methyl 5-HT, Apomorphine, Atropine, Bromocriptine, Bromperidol, Bupropion, Carbamazepine, Chlorzoxazone, CPP, Dihydroergochristine, DOI, Dopamine, Domperidone, DS-121, Ebalzotran, Eltoprazine, Ethinimate, Eticlopride, Fluvoxamine, Himbacine, Hupericin, Imidazole, Imidazole ether, Indatraline, Isomaltane, L-tryptophan, Mazindol, Melperone, Memantine, Mescaline, Mesulergine, Methoxamine, Metoclopramide, Minaprine, MK 212, Molindone, Naloxone, Nomifensine, Oxymetazoline, PCP, Pergolide, Phenelzine, Physostigmine, Pindolol, Pirenzepine, Pitrazepine, Phenylephrine, Prazosin, Quipazine, Raclopride, Ranitidine, Rauwolscine, Remoxipride, Rolipram, SB 206553, SCH 12679, SCH 23390, Serotonin, SKF 38393, SKF 83566, Spiroxatrine, Sulpiride, Sultopride, Thioperamide, Tiapride, Timolol, Tranuylcypromide, Verapamil, Viloxazine, Zimelidine, Zolpidem, Zopiclone.

Figure 1

