Two Novel and Selective Non-Imidazole Histamine H3 Receptor Antagonists

Timothy A. Esbenshade, Kathleen M. Krueger, Thomas R. Miller, Chae Hee Kang,
Lynne I. Denny\textsuperscript{a}, David G. Witte, Betty B. Yao, Gerard B. Fox, Ramin Faghih,
Youssef L. Bennani\textsuperscript{b}, Michael Williams\textsuperscript{c}, and Arthur A. Hancock.

Neuroscience Research
Global Pharmaceutical Research Division
Abbott Laboratories
Running Title:
In vitro pharmacological properties of A-304121 and A-317920

Corresponding Author: Timothy A. Esbenshade, Ph.D.
Neuroscience Research
Abbott Laboratories
R4MN, AP9A
100 Abbott Park Road
Abbott Park, IL 60064

Phone: (847)-935-4727
FAX: (847)-937-9195
e-mail: Tim.Esbenshade@Abbott.com

Text Page Count: 40
Table Count: 4
Figure Count: 6
Reference Count: 40
Abstract Word Count: 250
Introduction Word Count: 741
Discussion Word Count: 1487

Abbreviations: H3R, H3 receptor; H3LR, full length H3 receptor; (R)-α−MeHA, (R)-α-methylhistamine; GPCR, G-protein coupled receptor; cAMP, cyclic 3',5'-adenosine monophosphate, FLIPR, Fluorometric Imaging Plate Reader; [3H]-NAMH, [3H]-N-α-methylhistamine; HEK, human embryonic kidney; EFS, electric field stimulation; TE, 50 mM Tris/5 mM EDTA; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethene sulfonic acid; DPBS, Dulbecco’s phosphate buffered saline; IBMX, 3-isobutyl-1-methylxanthine; PEI, polyethylenimine

Section: Neuropharmacology
Abstract

Histamine H₃ receptor (H₃R) antagonists enhance neurotransmitter release and are being developed for the treatment of a variety of neurological and cognitive disorders. Many potent histamine H₃R antagonists contain an imidazole moiety that limits receptor selectivity and the tolerability of this class of compounds. Here we present the in vitro pharmacological data for two novel piperazine amide ligands, A-304121 (4-(3-((2R)-2-aminopropanoyl-1-piperazinyl)propoxy)phenyl)(cyclopropyl)methanone) and A-317920 (N-((1R)-2-(4-(3-(4-(cyclopropylcarbonyl)phenoxy)propyl)-1-piperazinyl)-1-methyl-2-oxoethyl)-2-furamide), and compare them to the imidazole H₃R antagonists ciproxifan, clobenpropit, and thioperamide. Both A-304121 and A-317920 bind potently to recombinant full-length rat H₃R (pKi values = 8.6 and 9.2, respectively) but have lower potencies for binding the full-length human H₃R (pKi values = 6.1 and 7.0, respectively). A-304121 and A-317920 are potent antagonists at rat H₃R in reversing R-α-Methylhistamine [(R)-α−MeHA] inhibition of forskolin-stimulated cAMP formation (pKb values = 8.0 and 9.1) but weak antagonists at human H₃Rs in cyclase (pKb values = 5.2 and 6.5) and calcium mobilization (pKb values = 5.8 and 7.2) assays in cells co-expressing Gαq/15-protein. Both compounds potently antagonize native H₃Rs by blocking histamine inhibition of potassium-evoked [³H]-histamine release from rat brain cortical synaptosomes (pKb values = 8.9 and 9.2) and (R)-α−MeHA reversal of electric field stimulated guinea pig ileum contractions (pA₂ values = 7.0 and 9.7). A-304121 and A-317920 are also more efficacious inverse agonists in reversing basal [³⁵S]-GTPγS binding at the human H₃R (pEC₅₀
values = 5.7 and 7.0) than are the imidazole antagonists. These novel and selective piperazine amides represent useful leads for the development of H₃R antagonist therapeutic agents.
The histamine H₃R is one of four histamine receptor subtypes (H₁-H₄), all members of the larger G-protein coupled receptor (GPCR) superfamily of receptors. This receptor was originally described as a central autoreceptor that modulates the release of histamine (Arrang et al., 1983). Subsequent work has shown that the H₃R is also a central heteroreceptor that can modulate the release of a wide variety of neurotransmitters including acetylcholine, dopamine, serotonin, and norepinephrine, among others (Blandina et al., 1998). Since these neurotransmitters are involved in vigilance, attention, and cognition enhancement, it has been postulated that the H₃R may be a potential therapeutic target for disorders associated with deficits in these central effects, such as attention deficit/hyperactivity disorder, Alzheimer’s Disease, mild cognitive impairment, and schizophrenia (Leurs et al., 1998).

The recent cloning of rat and human histamine H₃Rs (Lovenberg et al., 1999; Lovenberg et al., 2000) has significantly impacted the search for potential therapeutic H₃R antagonists to treat CNS disorders. Additionally, the cloning of the H₃R has revealed a number of unique properties associated with this receptor. Multiple splice isoforms for human and rat H₃Rs have been identified that appear to be differentially expressed in the brain (Coge et al., 2001; Drutel et al., 2001). Although no major pharmacological differences have yet been noted for these isoforms using antagonists, agonists do show increased potencies for the short isoform (Wieland et al., 2001). Activation of recombinant H₃Rs has been shown to
inhibit adenylate cyclase activity presumably mediated through a G\(\alpha_i/o\)-protein pathway (Lovenberg et al., 1999; Drutel et al., 2001). The rat H\(_3\)R has also been shown to activate MAPK and arachidonic acid release in an isoform selective manner (Drutel et al., 2001). Profound species differences in the antagonist pharmacology of the rat and human H\(_3\)Rs have been observed (Ligneau et al., 2000; Lovenberg et al., 2000; Yao et al., 2001) due to variations at amino acids 119 and 122 within the third transmembrane domain of the receptor. Both native and heterologously expressed recombinant H\(_3\)Rs are constitutively active [Rouleau et al., 2002; Wieland et al., 2001; Morisset et al., 2000] and several previously characterized H\(_3\)R antagonists have subsequently been shown to be inverse agonists, perhaps a desirable quality for therapeutic agents.

A large number of H\(_3\)R antagonists have been synthesized since the original discovery of this receptor but none are yet approved for clinical use. Until very recently, these compounds were primarily imidazole derivatives represented by agents such as thioperamide (Arrang et al., 1987), ciproxifan (Ligneau et al., 1998), clobenpropit (Barnes et al., 1993), and GT-2331 (Tedford et al., 1998). Many of these compounds were originally defined with high affinity for the rat H\(_3\)R but were later found to have lower affinity for the human H\(_3\)R including thioperamide, ciproxifan, and GT-2331 (Ligneau, et al., 2000; Lovenberg et al., 2000; Esbenshade et al., 2001; Ireland-Denny et al., 2001; Yao et al., 2001). Additionally, subsequent studies have shown that as a class, the imidazole H\(_3\)R antagonists are not as selective for the human H\(_3\)R as originally believed,
demonstrating appreciable binding affinities for serotonin 5HT3 (Leurs et al., 1995), \( \alpha_2 \)-adrenergic, and histamine H\(_4\)R (Esbenshade et al., 2001; Liu et al., 2001). Not only has clobenpropit been shown to have relatively high binding affinity for the histamine H\(_4\)R, but it is also a partial agonist at this receptor (Liu et al., 2001). Potential interaction of imidazole H\(_3\)R antagonists with cytochrome P\(_{450}\) enzymes is also of note since metyrapone, a cytochrome P\(_{450}\) inhibitor, markedly improves the specific H\(_3\)R binding of radiolabeled thioperamide and clobenpropit (Alves-Rodrigues et al., 1996; Harper et al., 1999). In addition, thioperamide has been shown to bind cytochrome P\(_{450}\) enzymes and inhibit adrenal steroidogenesis (Labella et al., 1992). Interestingly, the imidazole moiety is found in other drug molecules that have been shown to inhibit this important metabolic pathway (Halpert et al., 1994). Thus, it is desirable to synthesize potent and selective non-imidazole H\(_3\)R antagonists as potential therapeutic agents in man. Recent reports from our laboratory (Faghih et al., 2002a; Faghih et al., 2002b) and from other groups (Ganellin et al., 1998; Walczynski et al., 1999a; Walczynski et al., 1999b; Linney et al., 2000; Lazewska et al., 2001; Meier et al., 2001) have described the properties of novel non-imidazole H\(_3\)R antagonists. Herein, we describe the in vitro pharmacological profile of two non-imidazole, aryloxyalkyl piperazine-based H\(_3\)R antagonists, A-304121 and A-317920 (Fig. 1). An accompanying report (Fox et al., 2003) presents the in vivo behavioral data of these two novel compounds.
METHODS

Chemicals. A-304121 (4-(3-(2R)-2-aminopropanoyl-1-piperazinyl)propoxy)phenyl)(cyclopropyl)methanone), A-317920 (N-((1R)-2-(4-(4-(cyclopropylcarbonyl)phenoxy)propyl)-1-piperazinyl)-1-methyl-2-oxoethyl-)-2-furamide), (Figure 1), and ciproxifan were synthesized at Abbott Laboratories. 

[^3]H-N-α-methylhistamine, 45-90 Ci/m mole,[^3]H-pyrilamine, 20-30 Ci/m mole,[^3]H-tiotidine, 70-90 Ci/m mole,[^3]H-prazosin, 75-80 Ci/m mole,[^3]H-rauwolscine, 75 Ci/m mole,[^3]H-histidine, 40-60 Ci/m mole, and[^35]S-GTPγS, 1,250 Ci/m mole were obtained from Perkin Elmer Life Science Products and[^3]H-histamine, 30-60 Ci/m mole, and[^3]H-LY-278584 (1-methyl-N-(8-methyl-8-azabicyclo[3.2.1]-oct-3-yl)-1H-indazole-3-carboxamide), 60-85 Ci/m mole, were from Amersham Biosciences. Phentolamine was purchased from Novartis Pharmaceutical Corp., (R)-α-methylhistamine and clobenpropit were purchased from Tocris (UK), and serotonin and thioperamide were purchased from Sigma (USA).

Animals: Animals for experiments conducted in house were housed in AAALAC approved facilities at Abbott Laboratories in a temperature-regulated environment with lights on between 6:00 and 18:00 hours. Male, Sprague-Dawley rats (weighing 200-250 g on arrival) and male, Hartley guinea pigs (weighing from 150-200 g on arrival), were supplied by Charles River (Portage, MI). Male, beagle dogs were obtained from Marshall Farms (North Rose, NY). The animals were acclimated to laboratory conditions for at least one week before testing. All in-
house testing was conducted according to protocols approved by Abbott’s Institutional Animal Care and Use Committee.

**H₃R cloning and cell membrane preparation.** The human histamine H₃R gene was cloned using human thalamus poly-A RNA (Clontech, Palo Alto, CA) with RT-PCR methods using primers designed according to the published human H₃R gene sequences (Lovenberg *et al.*, 1999); GenBank accession number AF140538). The full-length (H₃L) human histamine H₃R cDNA encoding 445 amino acids was cloned into the pCIneo expression vector. A partial rat histamine H₃R gene was identified by homology searching using the InCyte Pharmaceutical (Palo Alto, CA) database. This unique clone shared significant homology to the published human histamine H₃LR sequence. RACE (rapid amplification of cDNA ends) was performed with thalamus RNA from Long Evans rat (Charles River Laboratories, Wilmington, MA) using primers designed according to the InCyte clone and the PCR product was cloned into the pCIneo expression vector.

HEK and C6 cells were grown in DMEM media containing high glucose that was supplemented with 10% fetal bovine serum and 20 mM L-glutamine. Transfection of HEK and C6 cells was performed with Lipofectamine according to the protocol provided by the vendor (Invitrogen, Carlsbad, CA) and cell lines were selected using Geneticin. Cells from stable clonal lines were harvested and homogenized in TE buffer (50 mM Tris-HCl, 5 mM EDTA, pH7.4) using a polytron.
at 20,000 rpm for 2 x 10 sec bursts in the presence of protease inhibitors (1 mM benzamidine, 2 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin, Sigma, St. Louis, MO), followed by centrifugation at 40,000 x g. The membrane pellets were further purified by repeating the homogenization and centrifugation steps as described above. Final membrane preparations were obtained by re-homogenizing the pellets in 6.25 volumes (weight/volume) of TE buffer and were frozen at −70 °C until used.

Human (Analytical Biological Services, Wilmington, DE), rat (Pelfreez, Rogers, AR), dog, or guinea pig brain cerebral cortices expressing H₃R were homogenized in cold TE buffer containing protease inhibitors. The homogenate was centrifuged at 40,000 x g for 20 min at 4 °C. This step was repeated and the resulting pellet was resuspended in TE buffer in a final volume of three times the wet weight of the tissue. Aliquots were frozen at −70 °C until needed.

Cells from stable clonal lines expressing the human histamine H₁R (Fukui H et al., 1994) or H₂R (Gantz et al., 1991) were harvested and homogenized in TE buffer as described above and final membrane preparations were obtained by re-homogenizing the pellets in 6.25 volumes of TE buffer and frozen at −70 °C until used. Membranes were prepared from HEK cells transiently transfected with the pClNeo expression vector harboring the human histamine H₄R (Liu et al., 2001) as described above for the H₃R.
Radioligand Binding Assays. For H3R binding, membrane preparations were incubated with \([^3]H\)-N-\(\alpha\)-methylhistamine (\([^3]H\)-NAMH) (0.5 – 1.0 nM) in the presence or absence of increasing concentrations (from 5 to 11 concentrations over a five log unit range) of ligands for competition binding. The binding reactions were carried out for 30 min at 25 °C in a final volume of 0.5 ml TE buffer. Nonspecific binding was defined with 30 \(\mu\)M thioperamide. Radioligand binding assays for cloned human histamine H1R and H2R were performed as described (Esbenshade and Hancock, 2000) using \([^3]H\)-mepyramine and \([^3]H\)-tiotidine, respectively. In brief, H1R membranes were incubated with increasing concentrations of test compound in the presence of 0.7 nM \([^3]H\)-mepyramine at 25 °C for 30 min in a total volume of 0.5 ml 50 mM Na/K PO\(_4\) buffer, pH=7.4. Nonspecific binding was defined with 2.0 \(\mu\)M promethazine. H2R membranes were incubated with increasing concentrations of test compound in the presence of 0.6 nM \([^3]H\)-tiotidine at 25 °C for 45 min in a total volume of 0.5 ml 50 mM Na/K PO\(_4\) buffer, pH=7.4. Nonspecific binding was defined with 100 \(\mu\)M cimetidine. Binding to human histamine H4R (Liu et al., 2001) transiently expressed in HEK cells was performed essentially as described. Competition binding assays were performed with increasing concentrations of test compound in the presence of 20 nM \([^3]H\)-histamine incubated at 25 °C for 1 hr in a total volume of 0.5 ml 50 mM Tris, 5 mM EDTA, pH=7.4. Nonspecific binding was defined with 0.5 \(\mu\)M clobenpropit. All binding reactions were terminated by filtration under vacuum onto polyethylenimine (0.3%) pre-soaked Unifilters (Perkin Elmer Life Sciences, Boston, MA) or Whatman GF/B filters (for human
cortex H₃R and human H₄R) followed by 3 brief washes with 4 ml of ice-cold TE buffer. Bound radiolabel was determined by liquid scintillation counting.

For the binding to α₂-adrenergic receptors, assays were performed using [³H]-rauwolscine binding to cloned human α₂a and α₂c-receptors expressed in mouse fibroblast cells (LTK⁻) membranes. Competition binding assays were performed with increasing concentrations of test compound in the presence of 200 pM [³H]-rauwolscine in 25 mM glycyl-glycine (pH = 7.4) and samples were incubated 120 minutes at 0 °C. All assays were terminated by filtration under vacuum through Unifilter plates. Membranes for 5HT₃-serotonin binding assays were prepared from rat frontal cortex and 5HT₃-receptor competition binding assays were performed with increasing concentrations of test compound in the presence of 500 pM [³H]-LY278584 in 10 mM HEPES buffer (pH = 7.5 at 37 °C) and samples were incubated 2 hr at 0 °C. Nonspecific binding was defined with 10 µM quipazine. Binding was terminated by filtration under vacuum through Whatman GF/B filters.

For all of the radioligand competition binding assays, IC₅₀ values and Hill slopes were determined by Hill transformation of the data as previously described (Esbenshade and Hancock, 2000) and pKi values were determined by the generalized Cheng-Prusoff equation (Cheng and Prusoff, 1973). Data are presented as the mean pKi ± standard error of the mean. For compounds where the Hill slope was less than 0.8, the data were reanalyzed using GraphPad Prism (San Diego, CA) and the best fit to a one- or two-site binding curve determined.
Adenylate Cyclase Assay. C6 cells or HEK cells stably expressing the full-length human or rat H₃LR were plated the day before the assay at 75,000-100,000 cells per well in a 96-well plate coated with either Collagen IV or polyethylenimine (PEI). Medium was removed and cells were incubated with Dulbecco’s phosphate buffered saline with calcium (DPBS, Invitrogen, Carlsbad, CA) for 10 min followed by DPBS containing 1 mM 3-isobutyl-1-methylxanthine (IBMX) for 20 min at 37 °C with 5% CO₂. Upon removal of buffer, cells were incubated with H₃R antagonists for 2 min prior to the addition of 30 nM (R)-α−MeHA. After an additional 5 min incubation, 10 µM forskolin was added to provide a final volume of 150 µl. Cells were hydrolyzed after 10 min by the addition of 20 µl of 1 N HCl and subsequent shaking for 10 min. After the addition of 20 µl of 1 N NaOH, the level of cAMP was determined by scintillation proximity assay (Amersham Biosciences, Piscataway, NJ). Data were normalized to the amount of cAMP produced in control wells and are expressed as the % forskolin-stimulated cAMP response. Experiments were run in triplicate and data were analyzed using GraphPad Prism (San Diego, CA) to obtain IC₅₀ values and Hill slopes. pKᵦ values were determined by the generalized Cheng-Prusoff equation (Cheng and Prusoff, 1973) and are presented as the mean ± S.E.M.

Measurement of Intracellular Calcium Levels. Functional activity of human H₃LR was determined in a stable HEK-293 cell line co-expressing the receptor and Gαᵦ₃/₅ by measuring agonist-evoked increases in intracellular calcium (Coward et al., 1999). Fluo-4, a calcium-sensitive fluorescent dye, was used as an indicator of
intracellular calcium levels. Relative fluorescence was measured in a 96-well format by the Fluorometric Imaging Plate Reader (FLIPR, Molecular Devices, Sunnyvale, CA). Confluent cells grown in 96-well black-walled PEI-treated tissue culture plates were loaded with 8 µM Fluo-4-AM (acetoxymethylester) in DPBS at room temperature for 1-2 hr. Before measuring fluorescence, each plate was washed 3 times. Increasing concentrations of H₃R antagonists were added at 10 sec intervals followed by addition of 30 nM (R)-α−MeHA 5 min later. Raw fluorescence data were corrected by subtracting fluorescence values just prior to addition of test compounds from fluorescence values at all time points. Corrected responses were then measured by selecting peak fluorescence values within the period of drug exposure. Peak response values were then expressed as a percentage of the reference peak response for 30 nM (R)-α−MeHA in the absence of H₃R antagonists. Experiments were performed in duplicate and data were analyzed using GraphPad Prism (San Diego, CA) to obtain IC₅₀ values and Hill slopes. pKᵣ values were determined by the generalized Cheng-Prusoff equation (Cheng and Prusoff, 1973) and are presented as the mean ± S.E.M.

Electric field stimulated (EFS) guinea-pig ileum. The modulation of EFS guinea pig ileum by H₃R antagonists was determined as previously described (Ireland-Denny et al., 2001). A 20 cm section of ileum, obtained approximately 10 cm proximal to the ileocecal junction, was removed from male guinea-pigs and sectioned into 2 cm segments, cleaned and placed in warm (37°C) Krebs-Henseleit bicarbonate buffer (g/L; MgSO₄, 0.141, KCl, 0.35, KH₂PO₄, 0.16, NaCl, 6.9, D-glucose, 2.0,
NaHCO$_3$, 2.1, Sigma, St. Louis, MO) containing 2.6 mM CaCl$_2$, 1 µM mepyramine, and 10 µM ranitidine. One end of the segment was then mounted onto a stationary rod containing parallel platinum electrodes aligned on each side of the tissue, and the other end connected to a Grass FT03 transducer at a basal preload tension of 1g. After a 10 min equilibrium period in heated 10 ml tissue baths, the tissues were electrically stimulated (supramaximal voltage $\sim$15V, 0.1hz frequency, 0.5 msec duration) and rinsed every 10 min for 1 hour. The intensity of the stimulus was then decreased every 5 min by 2 V until the threshold voltage for EFS contraction could be established. The experiment was then performed at a test voltage of 1.5 times the observed threshold voltage. The tissues were stimulated for an additional hour at the test voltage (7-8V) before the control agonist (R)-$\alpha$–MeHA response curve was determined by cumulatively adding logarithmically increasing doses to the baths. The concentration of the (R)-$\alpha$–MeHA necessary to cause a 50% inhibition in the EFS contraction (EC$_{50}$) was calculated using an Excel-based program, AGANTG (Zielinski and Buckner, 1998), and expressed as the negative logarithm (pD$_2$). H$_3$R antagonists were tested by adding various concentrations to the tissue baths 30 min prior to the generation of (R)-$\alpha$–MeHA concentration response curves. The potency of the antagonists (pA$_2$) to inhibit the (R)-$\alpha$–MeHA response was calculated according to the method of Schild (Schild, 1947) using AGANTG.

**Rat Cerebral Cortical Histamine Release Assay.**
Functional modulation of histamine release from rat cerebral cortical synaptosomes by histamine H₃R ligands was determined essentially as previously described (Arrang et al., 1985). Freshly dissected rat cerebral cortices were homogenized in cold 4 mM HEPES containing 0.32 M sucrose (pH = 7.3) using a Potter-Elvehjem homogenizer with a teflon pestle and centrifuged at 800 x g for 10 min to remove debris. Synaptosomes were isolated by centrifugation at 10,000 x g for 20 min, washed in a modified Krebs-Ringer’s Bicarbonate buffer (g/L; MgCl₂-6H₂O, 0.1, KCl, 0.34, Na₂HPO₄, 0.1, NaH₂PO₄, 0.18, NaCl, 7.0, D-glucose, 1.8, NaHCO₃, 1.26, Sigma, St. Louis, MO), and incubated with 1.2 µM [³H]-histidine for 30 min at 37 °C under a constant stream of 95% O₂/5% CO₂. Synaptosomes were washed extensively by repeated centrifugation to remove unincorporated radioactivity and were subsequently resuspended in the Krebs-Ringer’s Bicarbonate buffer. Aliquots (containing approximately 2 mg protein) were added to microcentrifuge tubes containing the agonist histamine (1 µM) either alone or together with H₃R antagonists and the mixture was incubated for 2 min at 37 °C in the presence of 5% CO₂ to allow the ligand to bind. Potassium (15 mM final concentration as KCl) was subsequently added and the samples were incubated for an additional 2 min. Samples were placed on ice and immediately centrifuged (4 °C) at 20,000 x g for 20 min. The supernatant was removed and chromatographic separation of [³H]-histidine and [³H]-histamine was performed utilizing Amberlite CG-50. Basal release values (obtained in buffer without additional potassium) were subtracted from each sample and the data expressed as a percentage of the maximum potassium-stimulated release for each assay.
Data were analyzed from duplicate experiments using GraphPad Prism (San Diego, CA) to obtain IC$_{50}$ values and Hill slopes. pK$_b$ values were determined by the generalized Cheng-Prusoff equation (Cheng and Prusoff, 1973) and are presented as the mean ± S.E.M.

**Inverse Agonism: [35S]-GTP$\gamma$S Binding Assay.** Membranes from HEK cells expressing the human H$_3$LR were prepared by homogenization in cold buffer containing 50 mM Tris-HCl (pH 7.4), 5mM EDTA, 10 mM MgCl$_2$, 1 mM benzamidine, 2 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 µg/ml pepstatin. The homogenate was centrifuged at 40,000 x g for 20 min at 4 °C and the resulting pellet was resuspended in 50mM Tris HCl (pH 7.4), 5mM EDTA, 10 mM MgCl$_2$, and homogenized. Glycerol and BSA where added to a final concentration of 10% glycerol and 1% BSA. Membranes were diluted in GTP$\gamma$S assay buffer (25 mM HEPES, 2.5 mM MgCl$_2$, and 75 mM NaCl, pH 7.4) and 10 µg of membrane protein was incubated in a 96-well deep-well block in the presence of 5.0 µM unlabeled GDP, approximately 0.5 nM of [35S]-GTP$\gamma$S, and increasing concentrations of test compounds. Samples were incubated at 37 °C for 20 minutes and the assays were terminated by the addition of cold buffer (50 mM Tris-HCl, 75 mM NaCl, and 2.5 mM MgCl$_2$, pH 7.6) and subsequent harvesting onto a Packard Unifilter 96-well GF/B plate followed by extensive washing. Microscint 20 (Perkin Elmer Life Sciences, Boston, MA) was added to the samples and bound [35S]-GTP$\gamma$S was determined utilizing the Topcount (Perkin Elmer Life Sciences, Boston, MA). The percentage of [35S]-GTP$\gamma$S bound in each sample was calculated as a percentage.
of that bound to control samples incubated in the absence of the H₃R antagonists (basal). Data were analyzed from experiments performed in triplicate using GraphPad Prism (San Diego, CA) to obtain pEC₅₀ values and Hill slopes.
RESULTS.

**Histamine H₃R Binding.**

H₃R binding affinities were determined for A-304121, A-317920, thioperamide, ciproxifan, and clobenpropit by determining displacement of specific [³H]-NAMH binding from H₃R binding sites in membranes prepared from C6 cells expressing the full length recombinant rat and human H₃LR as well as brain cortical membranes prepared from rat, human, dog, and guinea pig (Table 1). All five compounds potently bound with pKᵢ values greater than 8.0 to both the recombinant rat H₃LR as well as rat brain cortical membrane H₃Rs with a fairly similar rank order of potency at the recombinant rat H₃LR (clobenpropit > A-317920 = ciproxifan > A-304121 = thioperamide) and native rat H₃R (clobenpropit > A-317920 = ciproxifan > A-304121 > thioperamide). In contrast, all compounds except clobenpropit (pKᵢ = 9.4) were markedly less potent at both the recombinant human full length H₃LR and human brain cortical H₃Rs with pKᵢ values ranging from 6.1 to 7.2. Again, the compounds demonstrated a similar rank order of potency (clobenpropit > A-317920 = ciproxifan = thioperamide > A-304121) for the recombinant human H₃LR and native H₃R in human brain. The compounds were overall more potent for H₃Rs in dog and guinea pig brain membranes in comparison to human H₃Rs with pKi values > 7 for all compounds. Rank order of potencies for this set of five compounds for the guinea pig brain cortex was clobenpropit > A-317920 = ciproxifan > thioperamide > A-304121 and for the dog
brain cortex was clobenpropit > A-317920 = ciproxifan = thioperamide > A-304121. Hill slopes for all of the antagonist displacement curves approached unity, indicating the compounds recognized a single H₃R binding site in each membrane preparation. For those membrane preparations where the Hill slopes for 304121 (guinea pig and dog cortex) and 317920 (dog cortex) were < 0.8, comparison of the nonlinear regression curve fits to one- and two-binding site models revealed that the data were best fit by a one-binding site model.

**H₃R Binding Selectivity Profile.**

The binding affinities of A-304121, A-317920, ciproxifan, thioperamide, and clobenpropit at the three other histamine receptor subtypes (H₁, H₂, H₄) were determined as were binding affinities at other biogenic amine receptors including the rat serotonin 5HT₃ and human α₂a and α₂c-adrenergic receptors (Table 2). A-304121 exhibited no binding affinity at concentrations up to 10 µM for any of the other three histamine receptor subtypes (pKᵢ < 5) and A-317920 likewise had no affinity for either the histamine H₂R or H₄R (pKᵢ < 5) and low affinity for the histamine H₁R (pKᵢ = 5.4). Ciproxifan and thioperamide demonstrated no affinity for either the histamine H₁R or H₂R (pKᵢ values < 5) and clobenpropit exhibited low affinity (pKᵢ values = 5.2 and 5.6, respectively) for these receptors. However, thioperamide and clobenpropit demonstrated appreciable histamine H₄R binding affinity (pKᵢ values ~ 7.3) whereas the binding affinity of ciproxifan was lower (pKᵢ value = 5.7). Both A-304121 and A-317920 had no affinity (pKᵢ values < 5) for the α₂A-adrenergic and 5HT₃ receptor binding sites and low affinity (pKᵢ values = 5.5
and 5.6, respectively) for the α2C-adrenergic receptor. In contrast, clobenpropit, ciproxifan, and thioperamide exhibited varying degrees of higher binding affinities for the serotonin 5HT3- (pKi values = 8.1, 6.5, and 5.6, respectively) and the α2A- (pKi values = 7.8, 7.4, 6.9, respectively), and α2C-adrenergic (pKi values = 7.8, 7.2, 6.5, respectively) receptor binding sites with a similar rank order of potency for these receptors of clobenpropit > ciproxifan > thioperamide in parallel with their rat histamine H3R potencies.

**Functional Antagonism at Recombinant H3Rs.**

A-304121 and A-317920 inhibited the (R)-α-MeHA-mediated reversal of forskolin-stimulated cAMP accumulation in both C6 cells expressing the full length human (Figure 2, top panel) and rat (Figure 2, bottom panel) H3LRs in a concentration-dependent manner. Similar to the results observed in the binding assays, both A-304121 and A-317920 were more potent at the rat H3L (pKb values = 8.0 and 9.1, respectively) than the human H3L (pKb values = 5.3 and 6.5, respectively; Table 3). Clobenpropit and ciproxifan were equipotent as antagonists at the rat H3L in the adenylate cyclase assay (Figure 2, bottom panel) with pKb values of 9.0 and 9.2, respectively (Table 3), whereas thioperamide was less potent (pKb = 7.6). In comparison to the rat H3L potencies, all three imidazole H3R antagonists were less potent in antagonizing the cAMP response by the human H3L (Figure 2, top panel) with clobenpropit demonstrating the greatest potency (pKb = 8.2) whereas the potencies of ciproxifan and thioperamide were considerably lower (pKb values = 6.6 and 6.1, respectively; Table 3). Like those results seen in the adenylate...
cyclase experiments, A-304121 and A-317920 also inhibited (R)-α−MeHA-stimulated increases in intracellular calcium in a concentration dependent manner in HEK cells co-expressing the human histamine H\textsubscript{3}LR with the chimeric G\alpha\textsubscript{qi5}−protein (Figure 3) with respective pK\textsubscript{b} values of 6.0 and 7.3 (Table 3). Clobenpropit more potently antagonized the effects of (R)-α−MeHA (pK\textsubscript{b} value = 8.9) in this assay (Figure 3) than any of the other H\textsubscript{3}R antagonists including ciproxifan and thioperamide (pK\textsubscript{b} values = 6.8 for both; Table 3), consistent with its enhanced binding affinity for the human H\textsubscript{3}R.

Effects of H\textsubscript{3}R antagonists in Models of Neurotransmitter Release.

In the electric field stimulated guinea pig ileum model, activation of H\textsubscript{3}Rs by (R)-α−MeHA inhibits the electrically evoked release primarily of acetylcholine from nerve terminals that causes contraction of the tissue. Increasing concentrations of A-304121 caused dextral shifts of the concentration response curves for (R)-α−MeHA-mediated reversal of electric field stimulated contractions of guinea pig ileum (Figure 4, top left panel). Although (R)-α−MeHA was not able to fully overcome the antagonism of the highest concentration tested of A-304121 (3,000 nM), Schild analysis of the data (Figure 4, top right panel) revealed a pA\textsubscript{2} value of 7.1 (Table 3) with a slope of −1.08, consistent with competitive antagonist activity. A-317920 also behaved as a competitive antagonist but was more potent than A-304121 in this model, producing rightward shifts of the (R)-α−MeHA concentration response curves (Figure 4, bottom left panel) and generating a pA\textsubscript{2} value of 8.25
(Figure 4, bottom right panel; Table 3) and a slope of −1.0. The rank order of potency for all of the H₃R antagonists tested in this model was clobenpropit > thioperamide = A-317920 = ciproxifan > A-304121.

In the rat brain cortical synaptosome model of neurotransmitter release, activation of H₃Rs inhibits the release of [³H]-histamine caused by potassium-stimulated depolarization. Both A-304121 and A-317920 potently antagonized the histamine-mediated reversal of [³H]-histamine release from rat synaptosomes (Figure 5) in a concentration dependent manner with respective pKᵦ values of 8.6 and 9.3. All of the H₃R antagonists tested were potent in this model with a rank order of potency of A-317920 > ciproxifan = clobenpropit > A-304121 = thioperamide.

**Inverse Agonism: [³⁵S]-GTPγS Binding.**

A-304121 reduced basal [³⁵S]-GTPγS binding in membranes from HEK cells expressing the human H₃LR in a concentration dependent manner (Figure 6) with a pEC₅₀ value of 5.7 and a maximal inhibition of 16% from basal (Table 4). A-317920 more potently inhibited basal [³⁵S]-GTPγS binding than did A-304121 with a pEC₅₀ value of 7 and a maximal inhibition of 21% from basal (Table 4). Although clobenpropit, ciproxifan, and thioperamide were equally or more potent than A-304121 and A-317920 (rank order of potency of clobenpropit > thioperamide > ciproxifan = A-317920 > A-304121), these compounds were of lower inverse
Discussion.

Our *in vitro* and *in vivo* studies (see accompanying article by Fox, *et al.*, 2003) demonstrate that the non-imidazole compounds A-304121 and A-317920 are novel, competitive H₃R antagonists. These compounds potently and selectively bind to the rat H₃R with affinities comparable to those of the imidazole H₃R antagonists thioperamide and ciproxifan. Additionally, A-304121 and A-317920 have equal or greater rat or guinea pig H₃R affinities than several other non-imidazole series (Ganellin *et al.*, 1998; Walczynski *et al.*, 1999a; Walczynski *et al.*, 1999b; Linney *et al.*, 2000; Lazewska *et al.*, 2001; Meier *et al.*, 2001).

Like many of the imidazole H₃R antagonists, A-304121 and A-317920 are potent at rat H₃R, but are considerably less potent at human H₃R. It remains to be seen if this is also true of other non-imidazole H₃R antagonists described previously. The relatively low H₃R affinity of A-304121 is improved by the addition of the furanoyl moiety, creating A-317920 and increasing the potency at the rat H₃LₐR by 3-fold and at the human H₃LₐR by 8-fold with a pKᵢ value of 7.0, similar to that for ciproxifan and thioperamide. It has been shown that amino acids 119 and 122 critically determine the potency of imidazole H₃R antagonists at the human and rat receptors (Ligneau *et al.*, 2000) and are very important in determining the binding potencies of A-304121 and A-317920 (Yao *et al.*, 2001). Mutating the corresponding amino acids in the human H₃R to those amino acids in the rat (T119A and A122V) allows the resulting double mutant human H₃LₐR to bind to A-
304121 and A-317920 with equal affinity as that seen in the wild type rat H₃R (Yao et al., 2001). Indeed, A-304121 is thus far in our hands the H₃ antagonist with the greatest potency difference between the rat and human H₃R. Both A-304121 and A-317920 display similar binding affinities for the guinea pig and dog brain H₃Rs that are intermediate between those of rat and human. Interestingly, these two species share the same amino acid 119 (threonine) as human and the same amino acid 122 (valine) as rat, thus perhaps accounting for the pharmacological profile seen in dog and guinea pig. Combining the knowledge about the molecular properties of the H₃R along with continued insight into the chemical properties of H₃R antagonists that contribute to their distinctive binding potencies across species will allow for the optimization of compounds such as A-304121 and A-317920 with greater human H₃R potency.

Despite the relatively lower affinity of A-304121 and A-317920 for the human H₃R, these non-imidazole H₃R antagonists offer increased H₃R selectivity in comparison to the more conventional imidazole H₃R antagonists. Both compounds are more selective for the human H₃R versus other biogenic amine receptors than are clobenpropit, ciproxifan, and thioperamide. Indeed, A-304121 and A-317920 have little or no affinity for any of the three other human histamine receptor (H₁, H₂, and H₄) subtypes whereas all three of the imidazole H₃R antagonists tested have considerable binding potencies at the human H₄R. Both clobenpropit and thioperamide possess mid-nanomolar affinity for this receptor and clobenpropit demonstrated partial agonist activity (Liu et al., 2001). In addition to their
demonstrated low affinity for other histamine receptors, A-304121 and A-317920 have little or no affinity for over 80 rodent and human GPCRs and ligand activated ion channels (data not shown) including those for the biogenic amine serotonin 5HT3 and \( \alpha_{2A} \)-adrenergic receptors. The one receptor for which either compound exhibited significant binding affinity was the \( \alpha_{2C} \)-adrenergic receptor where A-304121 and A-317920 were approximately 4- and 25-fold selective for the human H3LR, respectively. In contrast, the imidazole H3R antagonists exhibit little or no selectivity against these receptors. Ciproxifan has equivalent binding affinities for the human histamine H3LR and human \( \alpha_{2A} \)-and \( \alpha_{2C} \)-adrenergic receptors and only 5-fold lower affinity for the rat 5HT3 receptor. Similarly, thioperamide was only 2-5-fold selective for the human H3LR versus the human \( \alpha_{2A} \)-and \( \alpha_{2C} \)-adrenergic receptors and 30-fold selective against the rat 5HT3 receptor. Because of its higher potency at the human histamine H3LR, clobenpropit is the most selective of the imidazole H3R antagonists, approximately 40-fold more potent at the human histamine H3LR than at the human \( \alpha_{2A} \)-and \( \alpha_{2C} \)-adrenergic receptors and only 20-fold more potent than at the rat 5HT3 receptor. Other imidazole H3R antagonists such as GT-2331, GT-2016, and iodophenpropit also exhibit this same lack of selectivity for these receptors (Esbenshade et al., 2001). Radiolabeled imidazole H3R antagonists have also been shown to interact with cytochrome P450 proteins (Alves-Rodrigues et al., 1996; Harper et al., 1999), an effect that can be minimized with the addition of metyrapone, a cytochrome P450 inhibitor. We have synthesized \[^{3}H\]-A-317920, an H3R radiolabeled antagonist that exhibits specific binding that is completely displaceable by H3R agonists and antagonists. In addition, it exhibits
low non-specific binding that is not altered by the addition of metyrapone (data not shown), unlike radiolabeled thioperamide and clobenpropit (Alves-Rodrigues et al., 1996; Harper et al., 1999), suggesting that this series of non-imidazole H₃R antagonists does not interact with cytochrome P₄₅₀ proteins to an appreciable extent.

A-304121 and A-317920 display well-behaved competitive antagonist properties in a variety of tissue and cell-based functional assays. These two novel compounds displayed all of the attributes associated with H₃R antagonists including the blockade of recombinant rat and human H₃Rs mediated signaling pathways as well as antagonism of H₃R mediated neurotransmitter release in two different classical H₃R assay paradigms, H₃R agonist-mediated inhibition of EFS guinea pig ileum contraction and release of [³H]-histamine from rat brain synaptosomes. In assays comparing the effect of H₃R antagonists to competitively inhibit (R)-α−MeHA-induced reversal of forskolin-stimulated cAMP accumulation in C6 cells expressing the rat H₃L R, the compounds exhibited a fairly similar rank order of potency as seen with their binding affinities with A-317920, clobenpropit, and ciproxifan possessing equivalent subnanomolar potencies that were about an order of magnitude greater than those for A-304121 and thioperamide. A similar pharmacological profile for these compounds was seen in the rat brain synaptosome model for the H₃R modulation of [³H]-histamine release. In the EFS guinea pig ileum model, A-304121 and A-317920 displayed antagonist potencies intermediate between those in the rat H₃R and human H₃R models, much like that
seen in the binding assays. Interestingly, the high degree of selectivity of A-304121 and A-317920 for the H3R in comparison to the H4R would suggest that indeed the EFS guinea pig ileum model is a very appropriate model for determining H3R antagonist potencies in contrast to suggestions that this tissue may be mediating H4R effects as well (Leurs et al., 2001). As predicted from their binding affinities, A-304121 and A-317920 are not as potent H3R antagonists at the human H3LR in both the adenylate cyclase and FLIPR assays. Again, clobenpropit is the most potent H3R antagonist in these assay systems with A-317920, ciproxifan, and thioperamide exhibiting comparable affinities and A-304121 showing the lowest affinity. For all of these functional assays, neither A-304121 nor A-317920 displayed any partial or full agonist activity when used alone, unlike results obtained with some imidazole H3R antagonists such as GT-2331 or proxyfan which exhibit various degrees of agonism dependent upon the assay system (Esbenshade et al., 2001).

With the cloning of the H3R and the discovery of the high degree of constitutive activity of the H3R in both recombinant and native systems, many H3R antagonists have been subsequently reclassified as inverse agonists because of their ability to reverse basal H3R activity. As has been previously shown, ciproxifan, thioperamide, and clobenpropit are inverse agonists at the human H3LR in reducing basal [35S]-GTPγS binding activity and/or enhancing cAMP formation (Morisset et al., 2000; Wieland et al., 2001). Likewise, A-304121 and A-317920 are also inverse agonists at the human H3LR and all the compounds tested display
comparable potencies as in radioligand binding assays. Surprisingly, both A-304121 and A-317920 appear to be more efficacious as human H3LR inverse agonists than the three imidazole H3R antagonists tested with A-317920 reversing the basal level of [35S]-GTPγS binding to a level over 2-fold greater level than that for the imidazole H3R antagonists. This suggests that the aryloxyalkyl piperazine pharmacophore may confer a different conformational change on the human H3LR that decreases the constitutive activity state of this receptor to a lower level than that achieved with the imidazole H3R antagonists. Since H3Rs may inhibit neurotransmitter release in the absence of endogenous histamine because of their inherent constitutive activity (Morisset et al., 2000), compounds that demonstrate greater inverse agonist efficacy (negative intrinsic activity) may in turn cause greater enhancement of neurotransmitter release and a potentially greater therapeutic effect. Thus, it is necessary to not only develop compounds that are highly potent and selective for the H3R, it is also important to understand the structural properties of such compounds that contribute to their efficacy as inverse agonists in order to develop potent and efficacious H3R inverse agonists as therapeutic agents.

H3R antagonists have been proposed as potential therapeutic agents for a variety of central nervous system disorders including attention deficit/hyperactivity disorder, Alzheimer’s disease, and schizophrenia because of the regulatory role this receptor has been shown to play in controlling the release of neurotransmitters important in vigilance, attention, and learning in various animal models. However,
the full potential of H₃R antagonists has not yet been realized since no potent, selective, and safe H₃R antagonist has been approved for treatment of such disorders even though many H₃R antagonists have been developed since the initial discovery of the H₃R in 1983 (Arrang et al., 1983). We believe that the optimization of non-imidazole H₃R selective antagonists such as A-304121 and A-317920 with good inverse agonist efficacy will lead to the development of potent, selective and efficacious human H₃R antagonists that will be important therapeutic agents in the treatment of a variety of neuropsychiatric disorders, and behavioral evidence supporting such a role is provided in the accompanying paper [Fox et al., 2003].
Acknowledgements

The authors thank Huaqing Liu for the chemical synthesis of A-304121 and A-317920.
References


(hexahydro-1H-1,4-diazepin-1-yl)benzothiazole derivatives as H₃-antagonists with H₁ blocking activities. *Farmaco* **54**(10): 684-94.


Footnotes:

a) Unnumbered footnotes:

Funded by Abbott Laboratories, Inc.

Portions of this work were previously presented at the 31st Annual European Histamine Research Society Meeting, May 22-26, 2002 and at the 32nd Annual Society for Neuroscience Meeting, November 2-7, 2002.

b) Reprint requests should be sent to:

Timothy A. Esbenshade, Ph.D.
Neuroscience Research
Abbott Laboratories
R4MN, AP9A
100 Abbott Park Road
Abbott Park, IL 60064

c) Numbered footnotes:

aPharmacia Corp., St. Louis, MO 63198

bAthersys, 3201 Carnegie Ave., Cleveland, OH 44115-2634

cNorthwestern University Medical School, 1130 Walden Lane, Lake Forest, IL 60045
FIGURE LEGENDS.

Figure 1. Chemical structures of A-304121 (4-(3-((2R)-2-aminopropanoyl-1-piperazinyl)propoxy)phenyl)(cyclopropyl)methanone) and A-317920 (N-((1R)-2-(4-(3-(4-(cyclopropylcarbonyl)phenoxy)propyl)-1-piperazinyl)-1-methyl-2-oxoethyl)-2-furamide).

Figure 2. A-304121 and A-317920 block (R)-α-MeHA reversal of forskolin-stimulated cAMP formation in a concentration-dependent manner in C6 cells stably expressing the full length human (top panel) and rat (bottom panel) histamine H₃Rs. Cells were incubated with increasing concentrations of the H₃R antagonists prior to the addition of 30 nM (R)-α-MeHA and 10 µM forskolin and cAMP formation determined as described in Methods. Data are expressed as a percentage of the maximal forskolin-stimulated cAMP response in the absence of H₃R antagonist and (R)-α-MeHA. Results represent the mean ± S.E.M. of 6-12 concentration response curves performed in triplicate.

Figure 3. A-304121 and A-317920 block (R)-α-MeHA-stimulated increases in intracellular calcium in a concentration-dependent manner in HEK cells stably co-expressing the full length human histamine H₃R and Gαq15-protein. Cells were incubated with increasing concentrations of the H₃R antagonists prior to the addition of 30 nM (R)-α-MeHA and fluorescence measured as described in Methods. Data are expressed as a percentage of the maximal (R)-α-MeHA-
stimulated response in the absence of $H_3$R antagonist. Results represent the mean $\pm$ S.E.M. of 5-37 concentration response curves performed in triplicate.

Figure 4. Schild analysis of A-304121 (top panels) and A-317920 (bottom panels) competitive antagonism of (R)-$\alpha$−MeHA-mediated reversal of electric field stimulated contracted guinea pig ileum. Tissues were incubated with varying concentrations of A-304121 and A-317920 for 30 min prior to generating the (R)-$\alpha$−MeHA concentration response curves as described in the Methods. The right panels show the Schild transformations of the shifts in (R)-$\alpha$−MeHA concentration response curves (left panels) for A-304121 and A-317920. Data for the (R)-$\alpha$−MeHA concentration response curves are expressed as a percentage of the maximal electrical field stimulated contractile response in the absence of (R)-$\alpha$−MeHA and $H_3$R antagonist. Results represent the mean $\pm$ S.E.M. of 15-17 (R)-$\alpha$−MeHA concentration response curves for each antagonist.

Figure 5. A-304121 and A-317920 block histamine reversal of potassium-stimulated $[^3H]$-histamine release in a concentration-dependent manner in rat brain cortical synaptosomes. Synaptosomes were incubated with increasing concentrations of the $H_3$R antagonists prior to the addition of 1 µM histamine and 15 mM potassium and $[^3H]$-histamine release was determined as described in Methods. Data are expressed as a percentage of the maximal potassium-stimulated $[^3H]$-histamine release in the absence of $H_3$R antagonist and histamine.
Results represent the mean ± S.E.M. of 2-6 concentration response curves performed in triplicate.

Figure 6. A-304121 and A-317920 decrease basal [35S]-GTPγS binding in a concentration-dependent manner in membranes from HEK cells stably expressing the full-length human histamine H₃R. Membranes were incubated with increasing concentrations of the H₃R antagonists and [35S]-GTPγS binding determined as described in Methods. Data are expressed as a percentage of the basal [35S]-GTPγS binding in the absence of H₃R antagonist. Results represent the mean ± S.E.M. of 2-4 concentration response curves performed in triplicate.
Table 1. Comparison of binding affinities of A-304121, A-317920, clobenpropit, ciproxifan, and thioperamide at various H₃Rs.

<table>
<thead>
<tr>
<th>H₃ Receptor</th>
<th>A-304121</th>
<th>A-317920</th>
<th>Clobenpropit</th>
<th>Thioperamide</th>
<th>Ciproxifan</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human H₃LR</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean pKᵢ + S.E.M.</td>
<td>6.12 ± 0.08</td>
<td>7.03 ± 0.04</td>
<td>9.44 ± 0.04</td>
<td>7.14 ± 0.06</td>
<td>7.20 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>(0.9 ± 0.06)</td>
<td>(0.89 ± 0.03)</td>
<td>(1.02 ± 0.03)</td>
<td>(0.93 ± 0.04)</td>
<td>(0.84 ± 0.04)</td>
</tr>
<tr>
<td><strong>Rat H₃LR</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean pKᵢ + S.E.M.</td>
<td>8.6 ± 0.1</td>
<td>9.15 ± 0.08</td>
<td>9.75 ± 0.01</td>
<td>8.44 ± 0.07</td>
<td>9.29 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>(0.88 ± 0.1)</td>
<td>(0.86 ± 0.02)</td>
<td>(1.09 ± 0.03)</td>
<td>(0.84 ± 0.06)</td>
<td>(0.88 ± 0.02)</td>
</tr>
<tr>
<td><strong>Human Brain Cortex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean pKᵢ + S.E.M.</td>
<td>6.09 ± 0.12</td>
<td>6.93 ± 0.08</td>
<td>9.11 ± 0.12</td>
<td>7.18 ± 0.008</td>
<td>7.05 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>(0.94 ± 0.08)</td>
<td>(0.92 ± 0.07)</td>
<td>(0.89 ± 0.06)</td>
<td>(1.01 ± 0.09)</td>
<td>(1.06 ± 0.07)</td>
</tr>
<tr>
<td><strong>Rat Brain Cortex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean pKᵢ + S.E.M.</td>
<td>8.90 ± 0.05</td>
<td>9.14 ± 0.04</td>
<td>9.45 ± 0.08</td>
<td>8.15 ± 0.06</td>
<td>9.20 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>(0.82 ± 0.03)</td>
<td>(0.92 ± 0.03)</td>
<td>(0.98 ± 0.03)</td>
<td>(0.90 ± 0.04)</td>
<td>(0.90 ± 0.03)</td>
</tr>
<tr>
<td><strong>G.P. Brain Cortex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean pKᵢ + S.E.M.</td>
<td>7.76 ± 0.08</td>
<td>8.62 ± 0.11</td>
<td>9.65 ± 0.2</td>
<td>8.34 ± 0.11</td>
<td>8.76 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>(0.75 ± 0.04)</td>
<td>(0.93 ± 0.09)</td>
<td>(1.14 ± 0.08)</td>
<td>(1.10 ± 0.08)</td>
<td>(0.89 ± 0.05)</td>
</tr>
<tr>
<td><strong>Dog Brain Cortex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean pKᵢ + S.E.M.</td>
<td>7.33 ± 0.09</td>
<td>8.37 ± 0.15</td>
<td>10.09 ± 0.11</td>
<td>8.17 ± 0.12</td>
<td>8.24 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>(0.76 ± 0.06)</td>
<td>(0.77 ± 0.04)</td>
<td>(1.11 ± 0.07)</td>
<td>(0.83 ± 0.05)</td>
<td>(0.88 ± 0.02)</td>
</tr>
</tbody>
</table>

aₙᵢ is Hill slope, n = 3-31 independent experiments performed in duplicate.

bInhibition curve best fit to one-site model when compared to two-site model (Graphpad Prism, San Diego, CA)
Table 2. Comparison of binding affinities of A-304121, A-317920, clobenpropit, ciproxifan, and thioperamide at additional HR subtypes and other biogenic amine receptors.

<table>
<thead>
<tr>
<th>Histamine Receptor</th>
<th>A-304121</th>
<th>A-317920</th>
<th>Clobenpropit</th>
<th>Thioperamide</th>
<th>Ciproxifan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human H&lt;sub&gt;1&lt;/sub&gt;R</td>
<td>&lt; 5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.40 ± 0.15 (0.99 ± 0.14)</td>
<td>5.56 ± 0.03 (1.18 ± 0.08)</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>Human H&lt;sub&gt;2&lt;/sub&gt;R</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
<td>5.24 ± 0.05 (1.32 ± 0.11)</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>Human H&lt;sub&gt;4&lt;/sub&gt;R</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
<td>7.38 ± 0.15 (0.77 ± 0.08)</td>
<td>7.32 ± 0.25 (0.62 ± 0.01)</td>
<td>5.73 ± 0.09 (0.81 ± 0.19)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Biogenic Amine Receptor</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Human α&lt;sub&gt;2A&lt;/sub&gt;-adrenergic</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
<td>7.76 ± 0.16 (1.24 ± 0.18)</td>
<td>6.90 ± 0.08 (0.99 ± 0.06)</td>
</tr>
<tr>
<td>Human α&lt;sub&gt;2C&lt;/sub&gt;-adrenergic</td>
<td>5.54 ± 0.16 (0.97 ± 0.06)</td>
<td>5.62 ± 0.27 (1.02 ± 0.16)</td>
<td>7.80 ± 0.08 (1.20 ± 0.11)</td>
<td>6.46 ± 0.11 (0.84 ± 0.13)</td>
</tr>
<tr>
<td>Rat 5HT&lt;sub&gt;3&lt;/sub&gt;-serotonergic</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
<td>8.13 ± 0.25 (0.64 ± 0.04)</td>
<td>5.64 ± 0.13 (0.84 ± 0.16)</td>
</tr>
</tbody>
</table>

<sup>a</sup>n<sub>H</sub> is Hill slope, n = 3-9 independent experiments performed in duplicate.

<sup>b</sup>Less than 30% inhibition of binding was detected at the maximal test concentration of 10 µM.
Table 3. Comparison of antagonist potencies of A-304121, A-317920, clobenpropit, ciproxifan, and thioperamide in histamine H₃R functional assays.

<table>
<thead>
<tr>
<th>Functional Assay²</th>
<th>A-304121</th>
<th>A-317920</th>
<th>Clobenpropit</th>
<th>Thioperamide</th>
<th>Ciproxifan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human H₃R FLIPR</td>
<td>5.95 ± 0.10 [5]</td>
<td>7.26 ± 0.14 [5]</td>
<td>8.91 ± 0.06 [37]</td>
<td>6.82 ± 0.06 [11]</td>
<td>6.84 ± 0.08 [12]</td>
</tr>
<tr>
<td>EFS Guinea Pig ileum (pA₂)</td>
<td>7.11 ± 0.29 [17]</td>
<td>8.25 ± 0.05 [15]</td>
<td>9.66 ± 0.49 [17]</td>
<td>8.44 ± 0.49 [20]</td>
<td>8.12 ± 0.56 [21]</td>
</tr>
</tbody>
</table>

All antagonist potencies shown represent the mean (± S.E.M.) of pKᵦ determinations for each of the assays except for the EFS guinea pig ileum model where the values represent the mean (± S.E.M.) of pA₂ determinations.
Table 4. Inverse agonist properties of A-304121, A-317920, clobenpropit, ciproxifan, and thioperamide at the histamine H₃LR as determined by reversing basal [³⁵S]-GTPγS binding.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Potency pEC₅₀ (Mean ± S.E.M.)</th>
<th>Efficacy Max. Inhibition (Mean ± S.E.M.)</th>
<th>Intrinsic Activityᵃ</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-304121</td>
<td>5.72 ± 0.07</td>
<td>15.7 ± 3.1</td>
<td>-0.75</td>
<td>3</td>
</tr>
<tr>
<td>A-317920</td>
<td>6.98 ± 0.09</td>
<td>20.8 ± 5.0</td>
<td>-1.0</td>
<td>3</td>
</tr>
<tr>
<td>Clobenpropit</td>
<td>8.07 ± 0.66</td>
<td>8.4 ± 1.8</td>
<td>-0.4</td>
<td>4</td>
</tr>
<tr>
<td>Ciproxifan</td>
<td>7.16 ± 0.19</td>
<td>6.5 ± 1.2</td>
<td>-0.3</td>
<td>3</td>
</tr>
<tr>
<td>Thioperamide</td>
<td>7.49 ± 0.34</td>
<td>9.9 ± 0.9</td>
<td>-0.5</td>
<td>3</td>
</tr>
</tbody>
</table>

ᵃIntrinsic activity is defined as the ratio of the efficacy of each compound relative to A-317920.
Figure 1, Esbenshade et al., JPET/2002/047183

![Chemical structures of A-304121 and A-317920](image-url)
Figure 2, Esbenshade et al., JPET/2002/047183

![Graph](image-url)

- **Antagonist Concentration [log M]**
- **% Forskolin Response**

- A-304121
- A-317920
- Thioperamide
- Ciproxifan
- Clobenpropit

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 4, Esbenshade et al., JPET/2002/047183
Figure 5, Esbenshade et al., JPET/2002/047183

![Graph showing the concentration-response relationship of various antagonists to histamine release.](image-url)
Figure 6, Esbenshade et al., JPET/2002/047183

Human $H_3LR$

% Basal GTP$_\gamma$S Binding

-11 -10 -9 -8 -7 -6 -5 -4

Antagonist Concentration (log M)

A-304121
A-317920
Thioperamide
Ciproxifan
Clobenpropit