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, 2000) has significantly impacted the search for potential therapeutic H3R antagonists to treat central nervous system disorders. Additionally, the cloning of the H3R has revealed a number of unique properties 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).

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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 cyclopromylmethanone] and A-317920 [N-((1R)-2-(4-(3-(4-(cyclopropylcarbonyl)phenoxo)propoxy)propoxy)propoxy)phenyl cyclopromylmethanone] and compare them with the imidazole H3R antagonists ciproxifan, clobenpropit, and thioperamide. Both A-304121 and A-317920 bind potently to recombinant full-length rat H3R (pK_i values = 8.6 and 9.2, respectively) but have lower potencies for binding the full-length human H3R (pK_i values = 6.1 and 7.0, respectively). A-304121 and A-317920 are potent antagonists at rat H3R in reversing R-alpha-methylhistamine ([R]-alpha-MeHA) inhibition of forskolin-stimulated cAMP formation (pK_i values = 8.0 and 9.1) but weak antagonists at human H3Rs in cyclase (pK_i values = 6.0 and 6.3) and calcium mobilization (pK_i values = 6.0 and 7.3) assays in cells co-expressing Ga(120) protein. Both compounds potently antagonize native H3Rs by blocking histamine release from rat brain cortical synaptosomes (pA2 values = 8.6 and 9.3) and (R)-alpha-MeHA reversal of electric field-stimulated guinea pig ileum contractions (pA2 values = 7.1 and 8.3). A-304121 and A-317920 are also more efficacious inverse agonists in reversing basal guanosine 5’-O-(3-thiotriphosphate) binding to 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.
ated 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 Go(protein pathway (Lovenberg et al., 1999; Drutel et al., 2001). The rat H3R has also been shown to activate mitogen-activated protein kinase 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 H3Rs have been observed (Ligneau et al., 2000; Lovenberg et al., 2001; Yao et al., 2003) due to variations at amino acids 119 and 122 within the third transmembrane domain of the receptor. Both native and heterologously expressed recombinant H3Rs are constitutively active (Morisset et al., 2000; Wieland et al., 2001; Rouleau et al., 2002), and several previously characterized H3R antagonists have subsequently been shown to be inverse agonists, perhaps a desirable quality for therapeutic agents.

A large number of H3R 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 H3R but were later found to have lower affinity for the human H3R 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., 2003). Additionally, subsequent studies have shown that as a class, the imidazole H3R antagonists are not as selective for the human H3R as originally believed, demonstrating appreciable binding affinities for serotonin 5-HT3 (Leurs et al., 1995), originally believed, demonstrating appreciable binding affinities for serotonin 5-HT3 (Leurs et al., 1995), and histamine H4R (Esbenshade et al., 2001; Liu et al., 2001). Not only has clobenpropit been shown to have relatively high binding affinity for the histamine H3R, but it is also a partial agonist at this receptor (Liu et al., 2001). Potential interaction of imidazole H3R antagonists with cytochrome P450 enzymes is also of note since metyrapone, a cytochrome P450 inhibitor, markedly improves the specific H3R binding of radiolabeled thioperamide and clobenpropit (Alves-Rodrigues et al., 1996; Harper et al., 1999). In addition, thioperamide has been shown to bind cytochrome P450 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 H3R antagonists as potential therapeutic agents in man. Recent reports from our laboratory (Faghih et al., 2002a,b) and from other groups (Ganellin et al., 1998; Walczynski et al., 1999a,b; Linney et al., 2000; Lazewska et al., 2001; Meier et al., 2001) have described the properties of novel non-imidazole H3R antagonists. Herein, we describe the in vitro pharmacological profile of two non-imidazole, aryloxyalkyl piperazine-based H3R antagonists, A-304121 [4-(3-((2R)-2-aminoaminoxyalkyl)piperazinyl)propoxy]phenyl)cyclopropylmethylene] and A-317920 [N-((1R)-2-(4-(3-(4-(cyclopropylcarbonyl)phenoxo)propyl)-1-piperazinyl)-1-methyl-2-oxo-ethyl)-2-furamide] (Fig. 1). An accompanying report (Fox et al., 2003) presents the in vivo behavioral data of these two novel compounds.

**Materials and Methods**

**Chemicals.** A-304121, A-317920 (Fig. 1), and ciproxifan were synthesized at Abbott Laboratories. [3H]N-α-methylhistamine, 45 to 90 Ci/mmol, [3H]pyrilamine, 20 to 30 Ci/mmol, [3H]tiotidine, 70 to 90 Ci/mmol, [3H]prazosin, 75 to 80 Ci/mmol, [3H]rauwolscine, 75 Ci/mmol, [3H]histidine, 40 to 60 Ci/mmol, and [35S]GTPγS, 1250 Ci/mm were obtained from PerkinElmer Life Sciences (Boston, MA) and [3H]histamine, 30 to 60 Ci/mmol, and [3H]LY-278584, 60 to 85 Ci/mm were purchased from Amersham Biosciences Inc. (Piscataway, NJ). Phentolamine was purchased from Novartis Pharmaceutical Corp. (Basel, Switzerland), (R)-α-methylhistamine and clobenpropit were purchased from Torcic Cookson Inc. (Bristol, UK), and serotonin and thioperamide were purchased from Sigma-Aldrich (St. Louis, MO).

**Animals.** Animals for experiments conducted in house were housed in Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) approved facilities at Abbott Laboratories in a temperature-regulated environment with lights on between 6:00 AM and 6:00 PM. Male Sprague-Dawley rats (weighing 200–250 g on arrival) and male Hartley guinea pigs (weighing 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 1 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 (BD Biosciences Clontech, Palo Alto, CA) with reverse transcription-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 H₃R 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 Dulbecco’s modified Eagle’s medium 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 genetin. Cells from stable clonal lines were harvested and homogenized in TE buffer (50 mM Tris-HCl, 5 mM EDTA, pH 7.4) using a polytron at 20,000 rpm for 2 × 10 s bursts in the presence of protease inhibitors (1 mM benzamidine, 2 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin; Sigma-Aldrich), followed by centrifugation at 40,000 × g for 40 min. 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 vol (w/v) 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 40,000g for 40 min at 4°C. This step was repeated, and the resulting pellet was resuspended in TE buffer in a final volume of 3 times the wet weight of the tissue. Aliquots were frozen at −70°C until needed.

Cells from stable clonal lines expressing the human histamine H₃R (Fukui et al., 1994a) or H₃R (Gantz et al., 1991) were harvested and homogenized in TE buffer as described above. Final membrane preparations were obtained by re-homogenizing the pellets in 6.25 vol (w/v) of TE buffer and were frozen at −70°C until used. Membranes were prepared from HEK cells transiently transfected with the pCIneo expression vector harboring the human histamine H₃R (Liu et al., 2001), as described above for the H₃R.

**Radioligand Binding Assays.** For H₃R binding, membrane preparations were incubated with [³H]-labeled N-α-methylhistamine ([³H]NMH) (0.5–1.0 nM) in the presence or absence of increasing concentrations (from 5 to 11 concentrations over a 5 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 of TE buffer. Nonspecific binding was defined with 30 μM thioperamide. Radioligand binding assays for cloned human histamine H₃R and H₂R were performed as described (Esbenshade and Hancock, 2000) using [³H]mepyramine and [³H]tiotidine, respectively. In brief, H₃R membranes were incubated with increasing concentrations of test compound in the presence of 0.7 nM [³H]mepyramine at 25°C for 30 min in a total volume of 0.5 ml of 50 mM sodium/potassium PO₄ buffer, pH 7.4. Nonspecific binding was defined as 20 μM promethazine. H₃R membranes were incubated with increasing concentrations of test compound in the presence of 0.6 nM [³H]tiotidine at 25°C for 45 min in a total volume of 0.5 ml of 50 mM sodium/potassium PO₄ buffer, pH 7.4. Nonspecific binding was defined with 100 μM cimetidine. Binding to human histamine H₃R (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 [³H]histamine incubated at 25°C for 1 h in a total volume of 0.5 ml of 50 mM Tris, 5 mM EDTA, pH 7.4. Nonspecific binding was defined with 0.5 μM clobenpropit. All binding reactions were terminated by filtration under vacuum onto polyethyleneimine (0.3%) presoaked Unifilters (PerkinElmer Life Sciences) or Whatman GF/B filters (Whatman, Clifton, NJ) for human cortex H₃R and human H₂R followed by three brief washes with 4 ml of ice-cold TE buffer. Bound radiolabel was determined by liquid scintillation counting.

For the binding to α₁-adrenergic receptors, assays were performed using [³H]rauwolscine binding to cloned human α₁A and α₁B receptors expressed in mouse fibroblast cells (LTK⁻) membranes. Competition binding assays were performed with increasing concentrations of test compound in the presence of 200 nM [³H]rauwolscine in 25 mM glycylglycine (pH 7.4), and samples were incubated 120 min at 0°C. All assays were terminated by filtration under vacuum through Unifilter plates. Membranes for 5-HT₂ serotonin binding assays were prepared from rat frontal cortex and 5-HT₃ 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 for 2 h 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 ± S.E.M. For compounds where the Hill slope was less than 0.8, the data were reanalyzed using GraphPad Prism (GraphPad Software, Inc., San Diego, CA), and the best fit to a one- or two-site binding curve was determined.

**Adenylate Cyclase Assay.** C6 cells or HEK cells stably expressing the full-length human or rat H₃R were plated the day before the assay at 75,000 to 100,000 cells per well in a 96-well plate coated with either collagen IV or polyethyleneimine. Medium was removed, and cells were incubated with Dulbecco’s phosphate-buffered saline with calcium (DPBS; Invitrogen) for 10 min followed by DPBS containing 1 mM 3-isobutyl-1-methylxanthine for 20 min at 37°C with 5% CO₂. Upon removal of buffer, cells were washed with DPBS and 2 μM forskolin for 2 min. A final addition of 30 nM (R)-o-MeHA elicited 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 Inc.). Data were normalized to the amount of cAMP produced in control wells and are expressed as the percentage of forskolin-stimulated cAMP response. Experiments were run in triplicate and data were analyzed using GraphPad Prism to obtain IC₅₀ values and Hill slopes. pKᵢ 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 H₃R was determined in a stable HEK-293 cell line coexpressing the receptor and Go₅₆₃₋₅₆ 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 Corp., Sunnyvale, CA). Confluent cells grown in 96-well black-walled polyethyleneimine-treated tissue culture plates were loaded with 8 μM Fluo-4/acetoxymethylene in DPBS at room temperature for 1 to 2 h. Before measuring fluorescence, each plate
was washed three times. Increasing concentrations of H₂R antagonists were added at 10-s intervals followed by addition of 30 nM (R)-α-MeHA 5 min later. Raw fluorescence data were corrected by subtracting fluorescence values just before the 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 H₂R antagonists. Experiments were performed in duplicate, and data were analyzed using GraphPad Prism to obtain IC₅₀ values and Hill slopes. pKᵢ 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 H₂R 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 (0.141 g/l MgSO₄, 0.35 g/l KCl, 0.16 g/l KH₂PO₄, 6.9 g/l NaCl, 2.0 g/l D-glucose, 2.1 g/l NaHCO₃ Sigma-Aldrich) containing 2.6 mM CaCl₂, 1 μM mepyramine, and 10 μM marnitide. 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 was connected to a Grass FT03 transducer at a basal preload tension of 1 g. After a 10-min equilibrium period in heated 10-ml tissue baths, the tissues were electrically stimulated (supramaximal voltage −15 V, 0.1 Hz frequency, 0.5 ms duration) and rinsed every 10 min for 1 h. 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× the observed threshold voltage. The tissues were stimulated for an additional hour at the test voltage (7 to 8 V) before the control agonist (R)-α-MeHA response curve was determined by cumulatively adding logarithmically increasing doses to the baths. The concentration of the (R)-α-MeHA necessary to cause a 50% inhibition in the EFS contraction (EC₅₀) was calculated using an Excel-based program, AGANTG (Zielinski and Buckner, 1998), and expressed as the negative logarithm (pD₂). H₂R antagonists were tested by adding various concentrations to the tissue baths 30 min before the generation of (R)-α-MeHA concentration-response curves. The potency of the antagonists (pD₂) to inhibit the (R)-α-MeHA response was calculated according to the method of Schindler and AGANTG.

**Rat Cerebral Cortical Histamine Release Assay.** Functional modulation of histamine release from rat cerebral cortical synaptosomes by histamine H₂R ligands was determined essentially as previously described (Arrang et al., 1985). Freshly dissected rat cerebral cortical 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 g for 10 min to remove debris. Synaptosomes were isolated by centrifugation at 10,000 g for 20 min, washed in a modified Krebs-Ringer bicarbonate buffer (0.1 g/l MgCl₂, 6H₂O, 0.34 g/l KCl, 0.1 g/l NaH₂PO₄, 0.18 0.34 g/l NaHPO₄, 7.9 g/l NaCl, 1.5 g/l D-glucose, 1.26 g/l NaHCO₃ Sigma-Aldrich), and incubated with 1.2 μM [³H]histidine for 30 min at 37°C under a constant stream of 5% O₂/5% CO₂. Synaptosomes were washed extensively by repeated centrifugation to remove unincorporated radioactive precursors and were subsequently resuspended in the Krebs-Ringer bicarbonate buffer. Aliquots (containing approximately 2 mg of protein) were added to microcentrifuge tubes containing the agonist histamine (1 μM) either alone or together with H₂R 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 20,000g for 20 min. The supernatant was removed and chromatographic separation of [³H]histidine and [³H]histamine was performed using Amberlite CG-50. Basal release values (obtained in buffer without additional potassium) were subtracted from each sample, and the data were expressed as a percentage of the total [³H]histamine present. Samples were analyzed from duplicate experiments using GraphPad Prism to obtain IC₅₀ values and Hill slopes. pKᵢ 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·S Binding Assay.** Membranes from HEK cells expressing the human H₃R were prepared by homogenization in cold buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 10 mM MgCl₂, 1 mM benzamidine, 2 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin. The homogenate was centrifuged at 40,000 rpm for 20 min at 4°C, and the resulting pellet was resuspended in 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 10 mM MgCl₂ and homogenized. Glycerol and bovine serum albumin were added to a final concentration of 10% glycerol and 1% bovine serum albumin. Membranes were diluted in TGP·S assay buffer (25 mM HEPES, 2.5 mM MgCl₂, 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·S, and increasing concentrations of test compounds. Samples were incubated at 37°C for 20 min and the assays were terminated by the addition of cold buffer (50 mM Tris-HCl, 75 mM NaCl, and 2.5 mM MgCl₂, pH 7.6) and subsequent harvesting onto a Packard Unifilter 96-well GF/B plate followed by extensive washing. Microscint 20 (PerkinElmer Life Sciences) was added to the samples, and bound [³⁵S]GTP·S was determined using the Topcount (PerkinElmer Life Sciences). The percentage of [³⁵S]GTP·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 to obtain pEC₅₀ values and Hill slopes.

**Results**

**Histamine H₃R Binding.** H₃R binding affinities were determined for A-304121, A-317920, thiopramide, 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 H₃R 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 H₃R as well as rat brain cortical membrane H₃Rs with a fairly similar rank order of potency at the recombinant rat H₃R (clobenpropit, > A-317920 = ciproxifan > A-304121 = thiopramide) and native rat H₃R (clobenpropit > A-317920 = ciproxifan > A-304121 > thiopramide). In contrast, all compounds except clobenpropit (pKᵢ = 9.4) were markedly less potent at both the recombinant human full-length H₃R 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 = thiopramide > A-304121) for the recombinant human H₃R 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 ranging from 6.1 to 7.2. Rank order of potencies for this set of five compounds for the guinea pig brain cortex was clobenpropit > A-317920 = ciproxifan > thiopramide > A-304121 and clobenpropit > A-317920 = ciproxifan = thiopramide > A-304121 for the dog brain cortex. Hill slopes for all 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 A-304121 (guinea pig and dog cortex) and A-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, H3) were determined as were binding affinities at other biogenic amine receptors including the rat serotonin 5-HT3 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 (pK<sub>i</sub> < 5) and A-317920 likewise had no affinity for either the histamine H3R or H2R (pK<sub>i</sub> < 5) and low affinity for the histamine H1R (pK<sub>i</sub> = 5.4). Ciproxifan and thioperamide demonstrated no affinity for either the histamine H1R or H3R (pK<sub>i</sub> values < 5) and clobenpropit exhibited low affinity (pK<sub>i</sub> values = 5.2 and 5.6, respectively) for these receptors. However, thioperamide and clobenpropit demonstrated appreciable histamine H3R binding affinity (pK<sub>i</sub> values ~7.3), whereas the binding affinity of ciproxifan was lower (pK<sub>i</sub> value = 5.7). Both A-304121 and A-317920 had no affinity (pK<sub>i</sub> values <5) for the α2a-adrenergic and 5-HT<sub>3</sub> receptor binding sites and low affinity (pK<sub>i</sub> 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 5-HT<sub>3</sub>- (pK<sub>i</sub> values = 8.1, 6.5, and 5.6, respectively) and the α2c- (pK<sub>i</sub> values = 7.8, 7.4, 6.9, respectively), and α2c-adrenergic (pK<sub>i</sub> 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 (Fig. 2, top panel) and rat (Fig. 2, bottom panel) H3RRs 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 H3R (pK<sub>i</sub> values = 8.0 and 9.1, respectively) than the human H3R (pK<sub>i</sub> values = 5.3 and 6.5, respectively; Table 3). Clobenpropit and ciproxifan were equipotent as antagonists at the rat H3R in the adenylate cyclase assay (Fig. 2, bottom panel) with pK<sub>i</sub> values of 9.0 and 9.2, respectively (Table 3), whereas thioperamide was less potent (pK<sub>i</sub> = 7.6). Compared with the rat H3R potencies, all three imidazole H3R antagonists were less potent in antagonizing the cAMP response by the human H3R (Fig. 2, top panel) with clobenpropit demonstrating the greatest potency (pK<sub>i</sub> =

### 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>Receptor Type</th>
<th>A-304121</th>
<th>A-317920</th>
<th>Clobenpropit</th>
<th>Thiorperamide</th>
<th>Ciproxifan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histamine Receptor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human H3R</td>
<td>&lt;5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.40 ± 0.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.56 ± 0.03</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Human H2R</td>
<td>&lt;5</td>
<td>5.24 ± 0.05</td>
<td>5.32 ± 0.08</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Human H1R</td>
<td>&lt;5</td>
<td>7.38 ± 0.15</td>
<td>7.32 ± 0.25</td>
<td>5.73 ± 0.09</td>
<td>0.81 ± 0.19</td>
</tr>
<tr>
<td>Biogenic Amine Receptor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human α&lt;sub&gt;2&lt;/sub&gt;a-adrenergic</td>
<td>&lt;5</td>
<td>7.76 ± 0.16</td>
<td>6.90 ± 0.08</td>
<td>7.37 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>Human α&lt;sub&gt;2&lt;/sub&gt;c-adrenergic</td>
<td>5.54 ± 0.16</td>
<td>5.62 ± 0.27</td>
<td>7.80 ± 0.08</td>
<td>6.46 ± 0.11</td>
<td>7.20 ± 0.13</td>
</tr>
<tr>
<td>Rat 5-HT&lt;sub&gt;3&lt;/sub&gt;-serotonergic</td>
<td>&lt;5</td>
<td>8.13 ± 0.25</td>
<td>5.64 ± 0.13</td>
<td>6.52 ± 0.19</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Hill slope; n = 3 to 9 independent experiments performed in duplicate.

<sup>b</sup> Mean pK<sub>i</sub> ± S.E.M.

<sup>c</sup> Mean pK<sub>i</sub> ± S.D.

<sup>d</sup> Mean pK<sub>i</sub> ± S.E.M. is shown in parentheses.
8.2), whereas the potencies of ciproxifan and thioperamide were considerably lower (pK₈ 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 coexpressing the human histamine H₃R with the chimeric Goₛ₉₆⁺₆ protein (Fig. 3) with respective pK₈ values of 6.0 and 7.3 (Table 3). Clobenpropit more potently antagonized the effects of (R)-α-MeHA (pK₈ value = 8.9) in this assay (Fig. 3) than any of the other H₃R antagonists including ciproxifan and thioperamide (pK₈ values = 6.8 for both; Table 3), consistent with its enhanced binding affinity for the human H₃R.

Effects of H₃R Antagonists in Models of Neurotransmitter Release. In the electric field-stimulated guinea pig ileum model, activation of H₃Rs 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 (Fig. 4, top left panel). Although (R)-α-MeHA was not able to fully overcome the antagonism of the highest concentration tested of A-304121 (3000 nM), Schild analysis of the data (Fig. 4, top right panel) revealed a pA₂ 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 (Fig. 4, bottom left panel) and generating a pA₂ value of 8.25 (Fig. 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.

### 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 Assay</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>
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<tr>
<td>EFS guinea pig ileum (pA₂)</td>
<td>7.11 ± 0.28 [17]</td>
<td>8.25 ± 0.05 [16]</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 pK₈ 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.*
In the rat brain cortical synaptosome model of neurotransmitter release, activation of H$_3$Rs inhibits the release of $[^3]$H$[$histamine caused by potassium-stimulated depolarization. Both A-304121 and A-317920 potently antagonized the histamine-mediated reversal of $[^3]$H$[$histamine release from rat synaptosomes (Fig. 5) in a concentration-dependent manner with respective $pK_a$ values of 8.6 and 9.3. All of the H$_3$R antagonists tested were potent in this model with a rank order of potency of A-317920 > ciproxifan = clobenpropit > A-304121 = thioperamide.

**Inverse Agonism: $[^35]$S$[$GTP$\gamma$S Binding.** A-304121 reduced basal $[^35]$S$[$GTP$\gamma$S binding in membranes from HEK cells expressing the human H$_3$R in a concentration-dependent manner (Fig. 6) with a $pEC_{50}$ value of 5.7 and a maximal inhibition of 16% from basal (Table 4). A-317920 more potently inhibited basal $[^35]$S$[$GTP$\gamma$S binding than did A-304121 with a $pEC_{50}$ 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 agonist intrinsic activity than A-304121 and A-317920 with rank order of intrinsic activity of A-317920 > A-304121 > thioperamide = clobenpropit = ciproxifan.

**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 H$_3$R antagonists. These compounds potently and selectively bind to the rat H$_3$R with affinities comparable to those of the imidazole H$_3$R antagonists thioperamide and ciproxifan. Additionally, A-304121 and A-317920 have equal or greater rat or guinea pig H$_3$R affinities than several other non-imidazole series (Ganellin et al., 1998; Walczynski et al., 1999a,b; Linney et al., 2000; Lazewska et al., 2001; Meier et al., 2001). Like many of the imidazole H$_3$R antagonists, A-304121 and A-317920 are potent at rat H$_3$R, but are considerably less potent at human H$_3$R. It remains to be seen if this is also true of other non-imidazole H$_3$R antagonists described previously. The relatively low H$_3$R affinity of A-304121 is improved by the addition of the furanyol moiety, creating A-317920 and increasing the potency at the rat H$_3$R by 3-fold and at the human H$_3$R by 8-fold with a $pK_i$ 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 H$_3$R 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., 2003). Mutating the corresponding amino acids in the 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 with continued insight into the chemical properties of H3R antagonists, which 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 compared with 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 other three human histamine receptor subtypes (H1, H2, and H4) whereas all three of the imidazole H3R antagonists tested have considerable binding potencies at the human H3R. 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 humanGPCRs and ligand-activated ion channels (data not shown) including those for the biogenic amine serotonin 5-HT3 and α2-adrenergic receptors. The one receptor for which either compound exhibited significant binding affinity was the α2-adrenergic receptor where A-304121 and A-317920 were approximately 4- and 25-fold selective for the human H3R, respectively. In contrast, the imidazole H3R antagonists exhibit little or no selectivity against these receptors. Ciproxifan has equivalent binding affinities for the human histamine H3R and human α2- and α2-adrenergic receptors and only 5-fold lower affinity for the rat 5-HT3 receptor. Similarly, thioperamide was only 2- to 5-fold selective for the human H3R versus the human α2- and α2-adrenergic receptors and 30-fold selective against the rat 5-HT3 receptor. Because of its higher potency at the human histamine H3R, clobenpropit is the most selective of the imidazole H3R antagonists, approximately 40-fold more potent at the human histamine H3R than at the human α2- and α2-adrenergic receptors and only 20-fold more potent than at the rat 5-HT3 receptor. Other imidazole H3R antagonists such as GT-2331, GT-2016, and iodopenpropit 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 [3H]A-317920, an H3R-radiolabeled antagonist that exhibits specific binding that is completely displaceable by H3R agonists and antagonists. In addition, it exhibits low nonspecific 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 H3R-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 H3R, the compounds exhibited a fairly similar rank order of potency as seen with their binding affinities with A-317920, clobenprogip, 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 compared with the H2R 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 H3R effects as well (Leurs et al., 2001). As predicted from their binding affinities, A-304121 and A-317920 are not as potent H2R antagonists at the human H3R in both the adenylate cyclase and FLIPR assays. Again, clobenpropit is the most potent H2R 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 H3R 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 H3R 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 H3R 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 H3R that decreases the constitutive activity state of this receptor to a lower level than that achieved with the imidazole H3R antagonists. Since H2Rs 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, but 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. Behavioral evidence supporting such a role is provided in the accompanying paper (Fox et al., 2003).

Acknowledgments

We thank Huaqing Liu for the chemical synthesis of A-304121 and A-317920.

References


### Table 4

<table>
<thead>
<tr>
<th>Compound</th>
<th>Potency pEC50 (Mean ± S.E.M.)</th>
<th>Efficacy Maximal Inhibition (Mean ± S.E.M.)</th>
<th>Intrinsic Activity</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>
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<tr>
<td>Clobenpropit</td>
<td>8.07 ± 0.66</td>
<td>8.4 ± 1.8</td>
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<tr>
<td>Ciproxifan</td>
<td>7.16 ± 0.19</td>
<td>6.5 ± 1.2</td>
<td>-0.3</td>
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<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>

* Intrinsic activity is defined as the ratio of the efficacy of each compound relative to A-317920.
release mediated by a novel class (H₃) of histamine receptor. *Nature (Lond)* **302**:832–837.


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