Title: Pharmacological properties and pro-cognitive effects of ABT-288, a potent and selective histamine H₃ receptor antagonist


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Running Title:
Pharmacological properties of ABT-288

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Abbreviations: H3R, H3 receptor; (R)-α−MeHA, (R)-α-methylhistamine; GPCR, G-protein coupled receptor; [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; PEI, polyethylenimine; PFC, prefrontal cortex

Section: Neuropharmacology
Abstract

Blockade of the histamine H₃ receptor (H₃R) enhances central neurotransmitter release, making this an attractive target for the treatment of cognitive disorders. Here we present the \textit{in vitro} and \textit{in vivo} pharmacological profiles for the H₃R antagonist ABT-288 (2-[4'-(3aR,6aR)-5-Methyl-hexahydro-pyrrolo[3,4-b]pyrrol-1-yl)-biphenyl-4-yl]-2H-pyridazin-3-one). ABT-288 is a competitive antagonist with high affinity and selectivity for human and rat H₃Rs (Kᵢₛ = 1.9 and 8.2 nM, respectively) that enhances the release of acetylcholine and dopamine in rat prefrontal cortex. In rat behavioral tests, ABT-288 improved acquisition of a five-trial inhibitory avoidance test in rat pups (0.001–0.03 mg/kg), social recognition memory in adult rats (0.03–0.1 mg/kg), and spatial learning and reference memory in a rat water maze test (0.1-1.0 mg/kg). ABT-288 attenuated methamphetamine-induced hyperactivity in mice. \textit{In vivo} rat brain H₃R receptor occupancy of ABT-288 was assessed in relation to rodent doses and exposure levels in behavioral tests. ABT-288 demonstrated a number of other favorable attributes, including good pharmacokinetics and oral bioavailability of 37-66%, with a wide CNS and cardiovascular safety margin. Thus, ABT-288 is a selective H₃R antagonist with broad pro-cognitive efficacy in rodents and excellent drug-like properties to support its advancement to the clinical area.
INTRODUCTION

Histamine is a biogenic amine that is recognized as an important neurotransmitter involved in a number of physiological responses in humans. The variety of effects mediated by histamine is the result of the activation of H₁, H₂, H₃, and H₄ receptor subtypes. The specificity of histamine responses are dependent upon the differential pharmacology, molecular biology, signal transduction characteristics, and localization exhibited by these four receptors (Hough, 2001; Bakker et al., 2002; Haas et al., 2008). H₁ and H₂ receptor antagonists have been very successful therapeutic agents for the treatment of allergic reactions and gastric ulcers, respectively.

Histamine is an important neurotransmitter in the CNS, regulating a variety of functions including attention, cognition, sleep, and weight homeostasis (Passani et al., 2004; Esbenshade et al., 2008; Brioni et al., 2011). Histamine is released from histaminergic neurons that originate in the hypothalamic tuberomammillary nucleus and project throughout most of the brain including cortex, striatum, thalamus, hippocampus, locus coeruleus and spinal cord. The H₃R was originally described as a presynaptic autoreceptor that when stimulated, inhibits the release of histamine in the brain (Arrang et al., 1983). It also functions as a heteroreceptor that can inhibit the release of other neurotransmitters (Schlicker et al., 1988; Schlicker et al., 1989; Clapham and Kilpatrick, 1992; Schlicker et al., 1993; Blandina et al., 1996; Baldi et al., 2005). The H₃R receptor is localized in rodent and human brain regions, with high expression in both species in the prefrontal cortex, hippocampus, and hypothalamus, regions associated with
histaminergic innervation and responsible for learning, memory and sleep (Martinez-Mir et al., 1990; Lovenberg et al., 1999; Drutel et al., 2001).

It is well established that histamine H₃R antagonists can enhance the release of multiple neurotransmitters involved in vigilance, alertness, and cognition enhancement including histamine by blockade of H₃ autoreceptors (Arrang et al., 1983) as well as the release of acetylcholine, norepinephrine, dopamine, and serotonin via blockade of H₃ heteroreceptors (Arrang et al., 1983; Blandina et al., 1998). These findings, in addition to the wide CNS projection of histaminergic neurons, localized expression of H₃Rs in cortical and limbic system areas, has made this receptor an attractive drug target to treat cognitive dysfunctions such as Alzheimer’s disease (AD), attention deficit hyperactivity disorder (ADHD), and cognitive deficits of schizophrenia (CDS) (Esbenshade et al., 2006; Esbenshade et al., 2008; Browman et al., 2009; Celanire et al., 2009; Passani et al., 2009; Brioni et al. 2011).

Considerable efforts have been expended to develop H₃R antagonists. First, imidazole-based H₃R antagonists such as thioperamide (Arrang et al., 1987), ciproxifan (Ligneau et al., 1998), clobenpropit (Barnes et al., 1993), and cipralisant (Tedford et al., 1998) were made and while useful pharmacological tools, they suffered pharmacokinetic and metabolic issues that halted their development as human therapeutics (Esbenshade et al., 2008). More recently, novel selective non-imidazole H₃R antagonists (Esbenshade et al., 2008; Celanire et al., 2009, Leurs et al., 2011), including pitolisant (BF2.649), GSK189254, GSK207040, MK-0249, JNJ-17216498, and irdabisant (CEP-26401) have
been advanced to early human trials for a variety of indications. Although preclinical data for some of these compounds are limited, pitolisant has been reported to increase acetylcholine and dopamine release in the rat cortex, to reverse the cognitive deficits induced by scopolamine in the object recognition model, and to exhibit antipsychotic potential in animal models (Ligneau et al., 2007a; Ligneau et al., 2007b, Schwartz, 2011). Both GSK189254 and GSK207040 have been shown to enhance neurotransmitter release in rat cortex (Medhurst et al., 2007; Southam et al., 2009). GSK189254 reversed scopolamine-induced amnesia in an inhibitory avoidance assay and has shown efficacy in water maze and object recognition tests (Medhurst et al., 2007) while irdabiasant improved performance in the rat social recognition model of short-term memory (Raddatz et al., 2012). We previously reported that ABT-239 (Esbenshade et al., 2005; Fox et al., 2005) is a potent and selective H₃R antagonist, but cardiovascular safety issues precluded advancement to the clinic.

In this report, we show the in vitro pharmacological profile and broad preclinical efficacy of the H₃R antagonist ABT-288 (Fig. 1), a compound with high affinity and selectivity for human histamine H₃Rs.
METHODS

Chemicals.  ABT-288 (2-[4’-((3aR,6aR)-5-Methyl-hexahydro-pyrrolo[3,4-b]pyrrol-1-yl)-biphenyl-4-yl]-2H-pyridazin-3-one) (Cowart, et al., WO2007/100990A2, Fig. 1), ABT-239 (4-(2-[(2R)-2-methylpyrrolidinyl]ethyl]-benzofuran-5-yl)benzonitrile), [3H]A-349821 (24-36 Ci/mmole) and ciproxifan were synthesized at Abbott Laboratories. [3H]-N-α-methylhistamine, 45-90 Ci/mmole, [3H]-pyrilamine, 20-30 Ci/mmole, [3H]-tiotidine, 70-90 Ci/mmole, [3H]-histidine, 40-60 Ci/mmole, and [35S]-GTPγS, 1,250 Ci/mmole were obtained from Perkin Elmer Life Science Products (Boston, MA) and [3H]-histamine, 30-60 Ci/mmole, 60-85 Ci/mmol, was from Amersham Biosciences (Piscataway, NJ). (R)-α-methylhistamine ((R)-α−MeHA) was purchased from Tocris (UK), and thioperamide was purchased from Sigma (USA).

Animals: Male spontaneously hypertensive rat (SHR) pups for repeated acquisition avoidance studies were obtained from Harlan at postnatal day 7 and housed in Abbott Laboratories facilities until use on postnatal days 20 to 24 (body weights ranged from 35–50 g). Pups were housed up to 12 per cage (average of two litters) and fostered with Long-Evans lactating females (two per cage), largely to avoid the poor maternal care of SHR females and possible associated effects on brain and cognitive development (Fox et al., 2002a). Adult (350–450 g) and juvenile (75–100 g) male Sprague-Dawley rats for social recognition studies, adult (250–350 g) male Sprague-Dawley rats for in vitro tissue assays and general observation studies, and adult Long-Evans rats for water maze studies (300–400g) were obtained from Charles River Breeding Laboratories, whereas Sprague-Dawley rats for microdialysis studies (350–380 g) were purchased
from Elevage Janvier (LeGenest Saint Isle, France). Male CD-1 mice were obtained from Charles River Breeding Laboratories (Portage, MI) at approximate 20 to 25 g body weight for methamphetamine hyperactivity studies. All rodents were housed in a quiet room under conditions of 12 h lights on/12 h lights off (on at 6:00 AM), with food and water available ad libitum. Rats for EEG studies were housed singly. Male, Hartley guinea pigs (weighing from 150-200 g on arrival), were supplied by Charles River (Portage, MI). All testing occurred during the light phase, and all experiments were conducted in accordance with Abbott Animal Care and Use Committee and National Institutes of Health Guide for Care and Use of Laboratory Animals guidelines in facilities accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. The animals were acclimated to laboratory conditions for at least one week before testing in a temperature-regulated environment with lights on between 6:00 and 18:00 hours.

**Radioligand Binding Assays.** For H₃R competition binding, membrane preparations were made from HEK cells expressing the full-length (445 amino acids) human histamine H₃R (Lovenberg, et al., 1999), the full-length rat histamine H₃R and brain cerebral cortices from human, (Analytical Biological Services, Wilmington, DE), rat and mouse (Pelfreez, Rogers, AR) as previously described (Esbenshade et al., 2005). Membranes were incubated with [³H]-N-α-methylhistamine ([³H]-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 competing ligands for 30 min at 25 °C in a final volume of 0.5 ml 50 mM Tris/5 mM EDTA, pH 7.4 buffer as previously described.
(Esbenshade et al., 2005; Strakhova and Esbenshade, 2006). Nonspecific binding was defined with 10 μM thioperamide. Cloned human histamine H₁, H₂, and H₄ receptor radioligand binding assays were performed as described (Esbenshade et al., 2005; Strakhova and Esbenshade, 2006) using [³H]-mepyramine, [³H]-tiotidine, and [³H]-histamine, respectively. All binding reactions were terminated by vacuum filtration onto polyethylenimine (0.3%) pre-soaked Unifilter plates (Perkin Elmer Life Sciences, Boston, MA) or Whatman GF/B filters (for human cortex H₃R, and human H₄ receptor) followed by 3 brief washes with 2 ml of ice-cold TE buffer. Liquid scintillation counting was used to determine bound ligand.

IC₅₀ values and Hill slopes were determined by Hill transformation for all of the radioligand competition binding data as previously described (Strakhova and Esbenshade, 2006). Kᵢ values were determined by the Cheng-Prusoff equation (Cheng and Prusoff, 1973) and the data are presented as the mean Kᵢ with 95% confidence intervals. The data were reanalyzed using GraphPad Prism (San Diego, CA) and best fit to a one-site binding curve.

Receptor Occupancy Studies Receptor occupancy by ABT-288 was assessed by an established in vivo method employing the H3 antagonist ligand [³H]A-349821 as radiotracer (Miller et al, 2009). Various doses of unlabeled ABT-288 were administered i.p. in a volume of 1 ml/kg, and [³H]A-349821 (1.5 μg/kg, i.v.) was subsequently administered 30 min later. After an additional 30 min post-dosing, the rats were anesthetized with CO₂ and decapitated and blood and brain tissues were
collected for quantification of unlabeled ABT-288 and [³H]A-349821 levels, respectively. The brain tissues were solubilized with Solvable™ (PerkinElmer) for liquid scintillation counting to determine [³H]A-349821 levels in cortex and cerebellum tissue on a per-mg tissue basis. H₃ receptor occupancy by unlabeled antagonists was reflected as a decrease in the [³H]A-349821 cortex: cerebellum distribution ratio, relative to control; the % H₃ receptor occupancy was calculated from the [³H]A-349821 distribution ratio of each individual rat using the following formula, where cortex and cerebellum are abbreviated ctx and cb, respectively.

\[
\% \text{ H₃ receptor occupancy} = \\
100 - \left(\frac{\text{ctx}: \text{cb ratio} - 1}{\text{average control ctx}: \text{cb ratio} - 1}\right) \times 100
\]

The average % receptor occupancy values (± s.e. mean) were plotted versus the antagonist dose, and an ED₅₀ for receptor occupancy was determined by nonlinear regression using the equation for sigmoidal dose-response. Similarly, the % receptor occupancy values from individual rats were plotted versus ABT-288 levels, and an ED₅₀ was determined and reported as ng/ml plasma. In all cases, GraphPad Prism software was used for nonlinear regression analysis.

**Electric Field Stimulated (EFS) guinea-pig ileal segments.** The reversal of (R)-α-MeHA-mediated inhibition of EFS (test voltage ∼7-8V, 0.1Hz frequency, 0.5 msec duration) elicited twitches of guinea pig ileal segments by H₃R antagonists was determined as previously described (Ireland-Denny et al., 2001; Esbenshade et al., 2005). Various concentrations of H₃R antagonists were added to the tissue baths 30 min prior to the
generation of \((R)-\alpha\)-MeHA cumulative concentration response curves and the potency (pA₂) of the antagonists to inhibit the \((R)-\alpha\)-MeHA response was calculated according to the method of Schild (Schild, 1947) as described previously (Ireland-Denny et al., 2001; Esbenshade et al., 2005).

**Rat Cerebral Cortical Histamine Release Assay.** The ability of H₃R antagonists to reverse histamine-mediated inhibition of \[^{3}H\]-histamine release from rat cerebral cortical synaptosomes evoked by potassium (15 mM) was determined as previously described (Arrang et al., 1985; Esbenshade et al., 2005). Synaptosomes (approximately 2 mg protein) were added to microcentrifuge tubes containing 1 \(\mu\)M of the agonist histamine (EC₅₀ = 34 nM) either alone or together with ABT-288 and the mixture was incubated for two minutes at 37°C. Potassium (15 mM final concentration) was subsequently added and the samples were incubated for an additional two minutes before the incubation was terminated. Values for the basal release of \[^{3}H\]-histamine (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. Experiments were performed in triplicate and data were analyzed using GraphPad Prism to obtain IC₅₀ values and Hill slopes. pKᵦ values were determined by the generalized Cheng-Prusoff equation (Cheng and Prusoff, 1973; Leff and Dougall, 1993) which are presented as the mean ± S.E.M.

**[^{35}S]-GTPγS Binding Assay.** HEK cell membranes expressing the human H₃R or C6 cell membranes expressing the rat H₃R were prepared by homogenization in cold buffer
containing 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 10 mM MgCl₂, and protease inhibitors. The homogenate was centrifuged two times at 40,000xg for 20 min at 4°C and the resulting pellet was resuspended in buffer containing 50 mM Tris HCl (pH 7.4), 5 mM EDTA, and 10 mM MgCl₂. Glycerol and bovine serum albumin (BSA) were added to a final concentration of 10% glycerol and 1% BSA prior to freezing the membranes. The inverse agonist activity of H₃R antagonists was determined as described previously (Esbenshade et al., 2005). In brief, membranes were diluted in GTPγS 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]-GTPγS, and various concentrations of H₃R antagonists. Samples were subsequently incubated at 37 °C for 20 min. For assays to determine antagonist activity, (R)-α-MeHA (30 nM for human and 300 nM for rat H₃R), was added in addition to the assay components described above and the samples were incubated at 37°C for 5 min. 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 by vacuum filtration onto a Packard Unifilter 96-well GF/B plate (Perkin Elmer Life Sciences). After extensive washing, the plates were dried, Microscint 20 was added to the samples, and the amount of bound [³⁵S]-GTPγS was determined utilizing the Topcount (Perkin Elmer Life Sciences, Boston, MA). The Bound [³⁵S]-GTPγS in each sample was expressed as a percentage of that bound to control samples incubated in the absence of histamine H₃R ligands. Triplicate determinations were obtained at each concentration and the data were analyzed using GraphPad Prism (San Diego, CA) to obtain EC₅₀ values for inverse agonism. The potency (pA₂) of antagonists to inhibit the
(R)-α-MeHA response was calculated according to the method of Schild (Schild, 1947). The mean ± S.E.M. of at least three independent experiments is reported.

Microdialysate Levels of Acetylcholine, Dopamine, and Histamine. For pain prophylaxis, animals were dosed pre-operatively with rimadyl (5 mg/kg, i.p.). Individual male Sprague-Dawley rats (290–320 g body weight) anesthetized with pentobarbital (60 mg/kg i.p.; Narcoren®, Rhone-Merieux, Lyon, France) were mounted in a stereotaxic frame (David Kopf Instruments, Tujunga, CA), and microdialysis guide cannulas (CMA/12; Axel Semrau GmbH, Germany) were implanted into the medial prefrontal cortex (anterior-posterior: 2.5; medial-lateral: 0.6; tip of the 3-mm long active membrane = 3.2 mm below the brain surface, according to Paxinos and Watson, 1998). The guide cannulas were secured with dental cement (Technovit powder, Technovit polymerization starter fluid; Kulzer GmbH, Dormagen, Germany) and four anchor screws into the skull. The rats were allowed to recover from surgery for 5 to 7 days.

The day before the experiment, each animal was transferred into a system allowing for free movement (CMA/120 Axel Semrau GmbH, Germany, consisting of a plastic bowl, wire attachment, counter balance arm, swivel assembly connecting in-/outlet of the probe with the perfusion pump). Next, a CMA/12 microdialysis probe (3 mm membrane length) was slowly lowered into the final position. The probe was perfused with Ringer solution (147 mM NaCl, 4.0 mM KCl and 2.4 mM CaCl₂, containing 0.1 μM neostigmine for the acetylcholine microdialysis study), for about one hour (CMA/102 microdialysis pump, Axel Semrau GmbH, Germany; 1.5 μL/min). The probe was perfused again 24 hours later for at least one hour before microdialysate fractions were collected every 20 minutes. On the day of the experiment, ABT-288 or its vehicle (2 mL/kg) was
administered intraperitoneally. Six fractions before and six fractions after the intraperitoneal administration of the test compound or vehicle were analyzed for microdialysate levels of dopamine or acetylcholine by HPLC with electrochemical detection.

**Assay of Microdialysate Acetylcholine Levels.** Ten μL of each microdialysate fraction was injected onto a reversed phase column (MF 8908 Acetylcholine SepStik Kit; microbore column, particle size 10 mm, 530 x 1.0 μm coupled to an immobilized enzyme reactor 50 x 1.0 mm, particle size 10 μm, containing acetylcholinesterase and choline oxidase; BAS, Lafayette, IN) using a refrigerated autosampler (HTC PAL twin injector autosampler system, Axel Semrau, Germany). The mobile phase consisted of 50 mmol/L Na₂HPO₄ (pH 8.5) and 5 mL/L Kathon. Flow rate was 0.14 mL/min (Rheos Flux pump, Axel Semrau GmbH, Germany), and the sample run time was less than 15 minutes. Acetylcholine and choline were measured via an electrochemical detector (LC-4C, BAS, Lafayette, IN) with a platinum working electrode set at +500 mV versus an Ag/AgCl reference electrode. The system was calibrated by standard solutions (acetylcholine, choline) containing 1 pmol/10 μL injection. Acetylcholine was identified by its retention time and peak height with an external standard method using chromatography software (Chrom Perfect®; Justice Laboratory Software, Denville, NJ). Microdialysis (area under curve for time 0-120 min was calculated by integration of the area by the trapezoidal rule) data were evaluated for significance using one way analysis of variance (ANOVA) followed by Dunnett’s pair wise comparison post hoc test.
using GraphPad Prism version 4.0 software (Graph-Pad Software, Inc., San Diego, CA.).

**Assay of Microdialysate Dopamine Levels.** Ten μL of each microdialysate fraction was injected onto a reversed phase column (Nucleosil C18, Macherey & Nagel, Germany 125 x 4.0 mm, 3 μm particle size) using a refrigerated autosampler (HTC PAL twin injector autosampler system, Axel Semrau GmbH, Germany). The mobile phase consisted of 0.15 mol/L NaH$_2$PO$_4$, 1 mmol/L Na$_2$ EDTA, 0.23 mmol/L sodium octylsulfate, 5 mM NaCl, and 4% isopropanol, pH 3.7. Flow rate was set to 0.75 mL/minute (Rheos flux pump, Axel Semrau GmbH, Germany), and the sample run time was less than 15 minutes. Dopamine was measured via an electrochemical detector (Decade II, Axel Semrau GmbH, Germany) with a carbon glass working electrode (VT-03, Antec Netherlands) and an ISAAC reference electrode. Oxidation currents were measured at a working potential of + 600 mV. The system was calibrated by standard solutions (dopamine, DOPAC, HVA) containing 1 pmol/10 μL injection. Dopamine was identified by its retention time and peak height with an external standard method using chromatography software. The chromatographic software and microdialysis statistics used were the same as above.

**Assay of Microdialysate Histamine Levels.** Animals were dosed pre-operatively with banamine (2.5 mg/kg, s.c.), anesthetized with isoflurane, and stereotaxically implanted with stainless steel microdialysis guide cannulae (CMA/12) above the medial PFC with the following coordinates (Paxinos and Watson, 1998): anterior-posterior: +2.8, medial-
lateral: +0.9, dorsal-ventral: -0.6. The cannulae were fixed in place using skull screws and dental cement, the wound closed with 4-0 non-absorbable suture, and the animals allowed to recover for 3 to 7 days. The afternoon before the experiment, the animals were placed into individual cages acclimated to handling. Microdialysis probes (CMA/12 Elite; 3-mm exposed probe) were inserted into the guide cannula, rats were tethered to the liquid swivel lines, and perfused overnight with artificial CSF at a flow rate of 1.5 µl/min. The following morning, 3 hours of 20-minute fractions, 1.5 µL/min, were collected, of which the last 4 were averaged and used for the baseline level of histamine. At Time 0, animals were dosed with vehicle or ABT-288 (0.3 and 1 mg/kg, i.p). Eleven sequential 20-minute fractions, 1.5 µL/min, were collected and kept at 4°C. Animals were sacrificed and the brains examined histologically for verification of appropriate probe placement. Only animals with the desired probe placement were used for analysis. Samples were analyzed for histamine by LC/MS using an Applied Biosciences Sciex 5000 mass spectrometer with a SIELC Primesep 200 multidimensional column. No probe recovery adjustment was made. The microdialysis data calculations and statistics used were the same as above.

**Five-Trial Inhibitory Avoidance in SHR Pups Studies:** In this behavioral paradigm, hypothesized to test aspects of both attention and impulsivity, vehicle treated rat pups showed an inability to withhold a natural response to transfer from the light into a preferred dark chamber. In the dose response study, SHR rat pups were treated s.c. (5.0 ml/kg with ABT-288 or vehicle) 30 minutes prior to being placed into the brightly illuminated compartment of a two compartment chamber (Hamilton Kinder, San Diego),
with a retractable door between the light and dark compartments opened. After animals transferred through the open door to the dark compartment, the door closed automatically and a 0.1 mA scrambled current was applied to a 31 bar stainless steel grid floor for one second. At this point the pup was removed and returned to its home cage and littermates for one minute inter-trial interval, and the transfer latency was noted. The same pup was returned to the bright compartment, and the process repeated for five trials. Plasma samples were collected from a separate set of pups dosed with ABT-288 with samples collected 40 to 45 minutes post injection.

To assess the effect of drug on shock response thresholds, a separate experiment was conducted in which pups were treated with ABT-288 30 minutes prior to test. Pups were placed in the light compartment with the door between the two compartments closed, and the current was slowly ramped up until the pup produced sustained vocalizations, after which the current was immediately ramped down until vocalizations ceased. These time points are referred to as iMAX and iMIN, respectively. To assess efficacy with repeated dosing, SHR pups were dosed for five days with an efficacious dose of ABT-288 (0.01 mg/kg, s.c.). To further explore any effects of repeated dosing on efficacy, a second repeated dosing study was conducted in which the maximally effective dose of ABT-288 (0.03 mg/kg, s.c.) was administered for four days, and on Day 5 the pups were dosed with 0.01 mg/kg s.c. of ABT-288 and tested in the task 30 minutes later as described above in the acute assays. Data were analyzed by summing the responses from learning trials number two to five. Statistical significance was determined using two tailed non-parametric Mann-Whitney U tests to compare drug
Adult Social Memory. Adult rats were trained in a social recognition memory test to recall prior exposure to a conspecific juvenile as previously described (Fox et al., 2005). Briefly, adult rats (350–450 g) were separated into fresh test cages and allowed to habituate for 30 min. An unfamiliar juvenile was introduced, and overall investigation duration (grooming, sniffing, and close following) was recorded for a 5-min period. Both rats were then removed to their respective holding cages. After 90 min, the adult was replaced into the test cage, the same juvenile reintroduced 30 min later, and overall investigation duration was again recorded during a second 5-min period. ABT-288 or saline vehicle was administered i.p. to the adult rat immediately after the first exposure period. To control for nonspecific effects, immediately after the second investigation period, a new, unfamiliar juvenile was introduced to the same adult rat for a third 5-min period, and overall investigation duration was recorded once again. Social memory was quantified by determining, for each adult rat, the ratio of investigation duration (RID) of the second to the first investigation periods. Nonspecific effects were assessed by determining the RID (third to the first investigation periods) for the unfamiliar juvenile. To assess efficacy with repeated dosing, rats were dosed for five days with ABT-288 (0.03 mg/kg, i.p.) and tested according to the protocol described above for this task. Trial 2 testing took place two hours after the last injection, which was immediately after Trial 1 as described above. Group sizes were n = 15 to 16. Data were analyzed using a one-way ANOVA with Dunnett’s post hoc analyses, comparing the response of drug-treated
groups to the response of vehicle controls (Graph Pad Prism 4.0, GraphPad Software, Inc. San Diego, CA).

**Two-Choice Discrimination Water Maze.** Adult male Long-Evans rats (Charles River Breeding Laboratories) were trained in a two-choice, visual discrimination water maze test of spatial learning and reference memory as previously described (Komater et al., 2005). This version of the water maze is known to be sensitive to age, septal lesions, and scopolamine-induced impairments and is proposed as a model that may be pertinent to cognitive deficits associated with Alzheimer’s disease. Two daily habituation sessions were conducted in which each rat was trained to find a visible escape platform in a pool (180 cm diameter and 60 cm high) filled to a depth of 37 cm with water made opaque with powdered milk. Water temperature was maintained at 26°C. On the second day of habituation training, a latency to escape measure was obtained in order to assure that animals are assigned to groups without swim speed bias. After a two-day rest period, spatial discrimination testing was conducted using two visually similar platforms, each covered in aluminum foil. The platforms remained in the same position through five days of training. However, only one of the platforms provided escape; the other (incorrect), made of Styrofoam, floated and did not support the weight of the animal. Each rat received six trials/day from one of the two starting positions that were alternated from trial to trial, and the number of contacts with the incorrect platform (errors) was recorded for each trial and averaged per day. Drugs or vehicle control were administered i.p. each day prior to training in a volume of 1.0 ml/kg. ABT-288 (0, 0.01, 0.1 or 1 mg/kg) or ABT-239 (1 mg/kg) was administered 15 minutes prior to
scopolamine (0.3 mg/kg) and the animals tested 15 minutes later. Data were compared over days using a two way repeated measures analysis of variance (ANOVA). Subsequent one-way ANOVAs were used to evaluate errors for each of the five training days, with Dunnett’s post hoc analyses (Graph Pad Prism 4.0, GraphPad Software, Inc. San Diego, CA).

**Methamphetamine – Induced Hyperactivity.** To evaluate the efficacy of ABT-288 in reversing methamphetamine induced hyperactivity, mice were injected with ABT-288 (0.1, 0.3 or 1 mg/kg, i.p.) or vehicle and placed separately into one of 16 acrylic open field environments (42 (length) x 42 (base) x 40 cm (height); Piper Plastics, Libertyville, IL) situated inside Versamax/Digiscan monitors, each equipped with 32 horizontal and 16 vertical infrared sensors (AccuScan Instruments, Inc., Columbus, OH) in a dimly illuminated test room. The mice were allowed to habituate while baseline activity data were recorded for a period of 60 minutes at which point they were injected with methamphetamine (1.0 mg/kg i.p.) and monitored for a further 90 minutes. Data were analyzed using a one way analysis of variance (ANOVA) and Dunnett’s post hoc test (Graphpad Prism Version 4.0, GraphPad Software, Inc., San Diego, CA).

**Electroencephalogram Slow-Wave Activity.** EEG recording electrodes were bilaterally implanted under pentobarbital anesthesia (50 mg/kg i.p.; Abbott Laboratories) over the parietal cortex (-2.0 mm AP, 4.0 mm L) in 400 gm male rats (Charles River Laboratories) maintained 12-hour light:12-hour dark schedule. A reference electrode was placed 11 mm posterior to bregma, and a miniature connector was affixed to the
skull. Implanted rats were allowed 2 weeks of recovery from the surgery before use. EEG (sampling rate 200 Hz) was recorded from previously habituated rats inside sound-attenuating chambers. Before experiments began, a flexible cable was attached to the implanted miniature connector that allowed the rats unrestricted movement during the recording sessions. Standard EEG amplifiers (Grass Instrument Division, Astro-Med) and a computer-based system (Stellate Systems, Montreal, Canada) were used to acquire and analyze data. The average EEG amplitude in microvolts was determined using fast Fourier transform analysis and was broken down into an analysis of the 1- to 4-Hz slow-wave band activity. Dose response effects on EEG were determined for i.p. administration of ABT-288 and compared with saline vehicle. The treatments were administered in a random order on different days with one treatment per day and 3 days between each treatment. On one of these treatment days, the rat would receive a vehicle control treatment. This within-subjects design allowed each rat to serve as its own control. EEG recordings were conducted during the light cycle and begun within 5 min after injection, and recording sessions lasted for 120 min. A total of eight rats were used in these studies.

Cardiovascular Parameters

**HERG Binding and Function** Human embryonic kidney (HEK-293) cells, stably transfected with the hERG channel, were obtained from Dr. C. W. January, Cardiology Division, University of Wisconsin. For \[^3\text{H}\]-dofetilide competition binding to hERG channels, membrane preparations were incubated with \[^3\text{H}\]-dofetilide in the presence or
absence of increasing concentrations of ABT-288 for 45 min at 37 °C in a final volume of 0.2 ml Tris buffer as previously described (Diaz et al., 2004). Isolated whole-cell patch clamp electrophysiology experiments to determine IC$_{50}$ values for functional inhibition of hERG channel activity was determined as previously described (Diaz et al., 2004).

*Anesthetized Dog Model* Male beagle dogs, weighing 9.4-11.5 kg, were anesthetized with pentobarbital (35.0 mg/kg, intravenously) and immediately placed on a constant intravenous infusion of pentobarbital (6.0 mg/kg/hour). Once anesthetized, the dogs were intubated with a cuffed endotracheal tube and ventilated with room air by means of a mechanical respiration pump (Harvard Apparatus, Model 613). Expiratory CO$_2$ was monitored with an end-tidal CO$_2$ monitor (Criticare Systems; Model POET TE) and maintained at 4-5% CO$_2$. Electrocardiogram limb leads were attached to the animals, and a lead II ECG was recorded. A Swan-Ganz catheter (5.5 F) was advanced into the pulmonary artery via the right jugular vein for measurement of cardiac output utilizing a cardiac output computer (Abbott Laboratories, Oximetric 3). Central venous and pulmonary artery pressures were measured through the proximal and distal ports of the catheter, respectively. A dual tip micromanometer catheter (Millar, Model SPC-770, 7F) was advanced into the left ventricle of the heart via the right carotid artery for measurement of left ventricular and aortic blood pressure. Polyethylene catheters were inserted into the right femoral vein and artery for infusion of test agents and collection of blood samples, respectively. Systemic vascular resistance was calculated as [(mean arterial pressure-mean central venous pressure)/cardiac output]. Pulmonary vascular
resistance was calculated as \[ \frac{\text{pulmonary arterial pressure} - \text{central venous pressure}}{\text{cardiac output}} \]. Body temperature was monitored throughout the experiment.

The primary hemodynamic variables were computed using commercial software and a signal processing workstation (Ponemah, Gould Instrument Systems, Inc. The electronic Lead II ECG record was assessed for changes in QT-interval (QTc, corrected for heart rate using Fridericia’s and Van de Water’s formulae) and the PR-interval via the Ponemah system with manual over-reads conducted at 15-minute intervals (Polakowski et al, 2009). ABT-288 was dissolved in vehicle (PEG 200, High-dose series or PEG 400, Low Dose Series, Sigma) and administered by intravenous infusion (0.02 mL/kg/min) in two sets of experiments at doses of 0.035, 0.106, and 0.352 mg/kg/min (1.06, 3.17, and 10.56 mg/kg/30 minutes; High Dose Series) and 0.004, 0.012 and 0.04 mg/kg/min (0.12, 0.36 and 1.20 mg/kg/30 minutes; Low Dose Series). Animals were monitored for one hour following administration of the third dose. Blood samples were collected into standard blood tubes containing heparin, beginning at time 0 and at 15-minute intervals throughout the experimental protocol and stored on ice. Hematocrit was determined at 30-minute intervals using microcapillary tubes. Note that whole blood samples were used in the high dose series for drug concentration measurements, while plasma was used for the low dose series.

Data are expressed as the group mean ± SEM. Statistical analysis was performed by comparison of values from ABT-288 and vehicle-treated dogs at each time point for each variable, using two-sided t-tests. In addition, for each parameter at each treatment
and post-treatment time point, two-sample, two-sided t-tests were used to compare changes from baseline for drug-treated vs. vehicle-treated dogs.

A statistical analysis was performed for each parameter at 15-minute intervals coinciding with the 10 time points whereby plasma concentrations of ABT-288 were determined. The effect of antagonists on each parameter was analyzed based on the change from baseline within each group during antagonist infusion and during the post-treatment period. Specifically, a repeated measure analysis of variance model was used to analyze the data. The model factors include treatment group, time point, and treatment group by time point interaction. Since the readings at the 10 time points (repeated measures) for each animal were correlated, a heterogeneous autoregression covariance matrix was used to account for the correlation of repeated measurements. The change in each hemodynamic parameter in antagonist treated animals vs. vehicle was also evaluated at each time point when the ANOVA model indicated a significant treatment group by time point interaction (p<0.05). All statistical tests and comparisons were assessed at a 0.05 level of significance.

**Pharmacokinetic Analysis.** ABT-288 was selectively removed from the plasma or brain homogenate utilizing protein precipitation with acetonitrile at neutral pH. The samples were vortexed vigorously followed by centrifugation. The supernatant was transferred and evaporated to dryness with a gentle stream of nitrogen over low heat (~35°C). The samples were reconstituted by vortexing with mobile phase. ABT-288 and internal standard were separated from c-extracted contaminants on a 5 cm x 3 mm 3 μ Luna CN
column with an acetonitrile:0.1% trifluoroacetic acid (30:70, by volume) mobile phase at a flow rate of 0.4 ml/min with a 25 μl injection. ABT-288 was quantified using MRM detection, m/z 373.3->343.2 using a turbo ionspray source on a PE Sciex mass spectrometer. The limits for quantification were approximately 1 ng/ml for plasma and 10 ng/g for brain samples. For more sensitive analysis of plasma and brain samples obtained from the H₃ receptor occupancy and behavioral studies, a 5 cm x 3 mm 5 μ Aquasil C18 column was employed with a 10 μl injection volume. All other parameters were the same as previously described. The limits for quantification were approximately 0.08 ng/ml for plasma and 0.25 ng/g for brain samples under these conditions.
RESULTS.

Histamine Receptor Binding.

The H₃R binding affinities for ABT-288, ABT-239 and ciproxifan were determined for the full length recombinant rat or human H₃Rs as well as for H₃Rs present in rat, mouse, and human brain cortical membranes (Table 1). All three compounds exhibited high affinity for the recombinant rat H₃R and rodent brain cortical membrane H₃Rs with Kᵢ values less than 10 nM. ABT-288 was 4-8-fold more potent at human recombinant (Kᵢ = 1.9 nM) and brain cortical (0.64 nM) H₃Rs than at the corresponding rat receptors in contrast to the more rat selective H₃R antagonist ciproxifan (Table 1). ABT-288 recognized a single high affinity H₃R binding site bound by the agonist radioligand [³H]-NAMH as demonstrated by displacement curve Hill slopes that approached unity in each of the membrane preparations tested. ABT-288 exhibited no binding at concentrations up to 10 μM to human H₁-, H₂-, and H₄ receptors providing a greater than 5,000-fold H₃R selectivity versus other histaminergic receptors whereas the imidazole ciproxifan was 20-fold selective for the human H₃R compared to the human H₄R (Table 1). Additionally, a Cerep binding screen of 85 other GPCR and ion channel targets revealed good selectivity for ABT-288 with greater than 50% displacement of specific binding seen only at the 5-HT₁B (52%) and sigma (85%, Kᵢ = 760 nM) receptors, Na+-channel (51%), and 5-HT transporter (58%).

Functional Antagonism and Inverse Agonism at Recombinant H₃Rs.
ABT-288 demonstrated competitive H₃R antagonism in Schild experiments, shifting the concentration response curves for \((R)\)-α-MeHA-mediated stimulation of \([^{35}\text{S}]\text{GTPγS}\) binding by human (Fig. 2, top panel) and rat H₃Rs in a parallel, dextral manner with Schild slopes approaching unity and respective pA₂ values of 8.7 and 8.2 (Table 2). In these studies, while the basal \([^{35}\text{S}]\text{GTPγS}\) binding was decreased up to 50% in a concentration dependent manner by the inverse agonist activity of ABT-288 described below, the maximal agonist response to \((R)\)-α-MeHA in the presence of ABT-288 was still achieved, indicating the competitive antagonism properties of this compound. The pA₂ values compared favorably to those for ABT-239 at both receptors as was seen in the binding assays whereas ciproxifan exhibited lower antagonist potency at human H₃Rs. ABT-288 is also an inverse agonist, reversing basal \([^{35}\text{S}]\text{GTPγS}\) binding mediated by human and rat H₃Rs in a concentration dependent manner with respective EC₅₀ values of 3.78 and 7.2 nM, equipotent with ABT-239 at both receptors and more potent than ciproxifan at the rat H₃R (Table 2).

**Effects of ABT-288 in In Vitro Models of Neurotransmitter Release.**

ABT-288 exhibited competitive antagonism by shifting the concentration response curves for \((R)\)-α-MeHA-mediated reversal of EFS contractions of guinea pig ileal segments with a pA₂ value of 9.1, comparable to the potencies exhibited by both ABT-239 and ciproxifan (Table 2). In another model of neurotransmitter release, the activation of presynaptic H₃Rs inhibits the release of \(^{3}\text{H}\)-histamine from rat brain synaptosomes caused by potassium-stimulated depolarization. This histamine-
mediated reversal of [³H]-histamine release from rat synaptosomes was inhibited by ABT-288 in a concentration dependent manner with a $K_b$ value of 3.2 nM (Fig. 2, bottom panel), a somewhat greater antagonist potency in this model than that shown by ABT-239 (Table 2).

**Effects of ABT-288 on In Vivo Neurotransmitter Release.** ABT-288 dose dependently increased microdialysate acetylcholine (Fig. 3, top panel) and dopamine (Fig. 3, middle panel) levels in the medial prefrontal cortex (mPFC). Acetylcholine levels peaked at 40-60 min after dosing with an elevation in acetylcholine levels that lasted for up to 2 hr seen at the higher doses. A similar profile for acetylcholine release was seen in the hippocampus although the magnitude of the increase in acetylcholine levels was smaller (data not shown). Higher doses of ABT-288 (3.0 and 10.0 mg/kg) were required to increase dopamine levels that also peaked at around 40 min after dosing with a sustained elevation seen for up to 2 hr. One-way ANOVA for the area under the curve from time 0 to 120 min revealed a significant treatment effect for microdialysate acetylcholine [$F(5,49) = 6.41$, $p < 0.0002$] and dopamine [$F(2,27) = 18.84$, $p < 0.0001$] levels in the medial prefrontal cortex. Subsequent Dunnett’s pair wise comparisons revealed a significant increase of microdialysate acetylcholine ($p < 0.05$ at 0.3, 1.0, 3.0 mg/kg) and dopamine ($p < 0.01$ at 3.0 and 10 mg/kg) levels compared with vehicle treated animals in the medial prefrontal cortex following administration of ABT-288. Interestingly, dopamine levels were not increased in the striatum (Fig. 3, middle panel, inset). ABT-288 (0.3 and 1 mg/kg) showed a trend in increasing histamine levels in the
medial prefrontal cortex that peaked at 20 min after dosing and remained elevated for up to 2 hr after dosing (Fig. 3, bottom panel); however, one-way ANOVA for the area under the curve from time 0 to 120 min did not reveal a significant treatment effect for microdialysate histamine levels.

Effects of ABT-288 in Rodent Models of Cognition

Five-Trial Inhibitory Avoidance in SHR pups. ABT-288 treatment 30 min prior to training produced a significant improved performance when compared to vehicle-treated rats (Fig 4). Plasma samples collected from a separate set of pups revealed efficacious plasma concentrations of 0.1-2.0 ng/mL. Plasma levels of 1 ng/ml (at 0.01 mg/kg) were considered an indicator of preclinical cognitive efficacy to establish the safety index for this compound. To assess efficacy with repeated dosing, SHR pups were dosed for five days with the maximally-eficacious dose of ABT-288, 0.01 mg/kg. After five days of repeated dosing, the efficacy of ABT-288 was maintained with observed mean crossover latencies of 309 ± 38 secs (± SEM) compared to acute treatment (342 ± 42 secs) and vehicle control (175 ± 46 secs), suggesting that the development of tolerance is unlikely. In addition, no diminution of the 0.01 mg/kg response was seen when animals are repeatedly dosed at the higher efficacious dose of 0.03 mg/kg for four days, then acutely tested on Day 5 with the lower effective dose of 0.01 mg/kg (data not shown).
Social Recognition. Overall differences in the ratios of the ABT-288 treatment groups compared to the vehicle were evaluated by applying a one-way ANOVA [F(9,83 = 4.574, p<0.05] with significance assessed by a subsequent Dunnett’s post hoc analysis. ABT-288 produced significant increases in recall performance (expressed as investigation ratio) ratio in the social recognition assay at doses of 0.03 and 0.1 mg/kg i.p., equivalent to that seen with ABT-239 (Fig. 5). Lower (0.01 mg/kg) and higher (0.3 mg/kg) doses of ABT-288 did not improve performance. To assess efficacy with repeated dosing in this model, rats were dosed for five days with 0.03 mg/kg ABT-288.

There was no significant difference between repeatedly dosed animals and acutely dosed animals with observed investigation ratios of 0.71 ± 0.06 (mean ± SEM) for the repeatedly dosed treatment and acute treatment (0.62 ± 0.06), compared to vehicle control (0.91 ± 0.06). ABT-288 did not exhibit any nonspecific effects in this task, as evidenced by a lack of an effect on the investigation of a novel juvenile (data not shown), indicating the effects in this model are not due to general changes in investigational behavior.

Scopolamine Induced Deficit in Rat 2 Platform Discrimination Water Maze. Vehicle treated rats normally learned the task over five days of testing, while rats treated with scopolamine (0.3 mg/kg, i.p.) exhibited impaired discrimination learning in this assay as demonstrated by a consistent number of errors (contacts with the incorrect, floating platform) across test days (Fig. 6). Overall differences in errors were evaluated by applying a repeated measures ANOVA, indicating an overall effect for day [F(4,41) = 6.446, p<0.0001], treatment [F(5,41) = 6.268, p = 0.0002] and a significant day x
treatment interaction \( [F(20,41) = 1.753, p = 0.03] \). For each day, significance was assessed by ANOVA with subsequent post hoc analysis using Bonferroni’s method. ABT-288, administered each day of testing, significantly attenuated the scopolamine deficit at doses of 0.1 and 1 mg/kg (Fig. 6). This response is comparable to the positive control group dosed with ABT-239 (1 mg/kg), which also significantly attenuated the scopolamine deficit.

**Methamphetamine – Induced Hyperactivity.** Clinically effective antipsychotic agents can block the long lasting hyperactivity induced by methamphetamine in rodents. ABT-288 attenuated methamphetamine induced hyperactivity in a dose related manner, reaching significance following the administration of 1.0 mg/kg (data collapsed over 90 min following methamphetamine administration, Fig. 7). Antipsychotics that are efficacious in this assay, such as risperidone, significantly attenuated methamphetamine induced hyperactivity.

**EEG Slow Wave Activity.** ABT-288 at doses of 0.01 and 0.1 mg/kg i.p. had no significant effect on slow-wave EEG activity for up to 2 h after administration (Fig. 8). At the highest dose tested (1.0 mg/kg), ABT-288 significantly decreased the amplitude of the 1-4 Hz slow wave (\( p<0.01 \), Neuman-Keuls multiple comparison test) (Fig. 8). Corresponding plasma levels of ABT-288 at the two lower doses were 0.9 and 11.2 ng/ml, whereas the plasma level at 1 mg/kg corresponded to 165 ng/ml. In this model, the histamine H\(_1\) antagonist diphenhydramine increased slow wave activity (Fig. 8), consistent with its sedative effects in humans.
Receptor Occupancy. ABT-288 inhibited cortical \(^3\text{H}\)A-349821 H\(_3\)R occupancy as shown in Fig. 9. At the dose of \(^3\text{H}\)A-349821 used, cortical H\(_3\)R occupancy was 0.15 fmol/mg tissue, which was equivalent to approximately 1.5\% of the total cortical H\(_3\)Rs. The levels of \(^3\text{H}\)A-349821 were approximately two-fold greater in cortexes than cerebellum. In rats pretreated with maximal doses of ABT-288, \(^3\text{H}\)A-349821 levels in cortex and cerebellum were indistinguishable, indicating that ABT-288 completely inhibited \(^3\text{H}\)A-349821 H\(_3\)R occupancy. ABT-288 occupied cortical H\(_3\)Rs in a dose-dependent manner with an ED\(_{50}\) = 0.1 mg/kg, and at the dose of 1.0 mg/kg it produced greater than 90\% occupancy. ABT-288 plasma levels were determined in these studies and an EC\(_{50}\) value of 3.17±0.7 ng/ml was calculated from a plasma level-occupancy plot (Fig. 9).

Cardiovascular Effects. ABT-288 exhibited low affinity for \(^3\text{H}\)-dofetilide binding to the hERG channel with a \(K_i\) value of 8,830 nM. Blockade of hERG current by ABT-288 in a stably transfected cell line \textit{in vitro} was characterized with an IC\(_{50}\) value of 3,760 ± 600 ng/ml, more than 3,000-fold the preclinical \(C_{\text{max}}\) of 1 ng/ml. These results contrast with those for ABT-239 which blocked \(^3\text{H}\)-dofetilide binding and the hERG current more potently (\(K_i = 250\) nM and IC\(_{50} = 56\) ng/ml, respectively).

Detailed cardiovascular studies were conducted in anesthetized dogs where peak blood concentrations of 570 ± 40, 1,870 ± 120, and 6,300 ± 330 ng/mL for ABT-288 were achieved at the end of each infusion period corresponding to 570- to 6,300-fold above
the preclinical efficacious level (1 ng/mL). At the highest concentration tested, ABT-288 produced no physiologically relevant effects on any hemodynamic parameter measured including mean arterial pressure (Figure 10, top panel) as well as systolic arterial pressure, diastolic arterial pressure, heart rate, contractility, cardiac output, pulmonary arterial pressure, pulmonary vascular resistance, and systemic vascular resistance, central venous pressure, left ventricular end-diastolic pressure, or hematocrit. At all doses tested and at the highest blood concentration tested (6,300 ± 330 ng/mL) and relative to vehicle controls, ABT-288 produced no statistically significant changes in the QT-interval corrected for heart rate (Figure 10, lower panel).

**Pharmacokinetic properties of ABT-288.** The pharmacokinetics of ABT-288 were evaluated in Sprague-Dawley rat and CD-1 mouse. ABT-288 exhibited blood clearance (CLβ) values of 1.3 L/hr•kg in mouse and 2.7 L/hr•kg in the rat. ABT-288 exhibited high volumes of distribution, with Vss values of 1.7 L/kg in mouse and 5.3 L/kg in rat. In vitro plasma protein binding of ABT-288 was 86% and 88% respectively in mouse and rat. The elimination half-lives for ABT-288 were 1.0 and 1.3 hours in mouse and rat respectively. Rats demonstrated the highest clearance values and correspondingly the lowest bioavailability at approximately 37% following a 1 mg/kg dose in the rat. Mice exhibited higher bioavailability at 66%.
DISCUSSION

Our findings show that ABT-288 is an H₃R competitive antagonist that potently and selectively binds rat and human H₃Rs, exhibiting slightly higher affinity towards the human receptor. The high degree of selectivity of ABT-288 is illustrated by its over 5,000-fold greater affinity for the human histamine H₃R versus other histamine receptor subtypes and its good selectivity against other GPCR and ion channel targets. Functionally, ABT-288 is a competitive antagonist, inhibiting agonist-stimulated H₃R GTPγS binding by both rat and human receptors and reversing H₃R agonist-induced inhibition of neurotransmitter release in in vitro native H₃R models. Schild analysis of the inhibition of (R)-α-MeHA-induced GTPγS binding mediated by H₃Rs clearly demonstrates the competitive antagonist properties of ABT-288, revealing pA₂ values that approximate the binding affinities. Although ABT-239 binding affinities were somewhat greater than ABT-288, the antagonist potencies of ABT-288 and ABT-239 were similar. Like almost all H₃R antagonists described to date, ABT-288 is also an H₃R inverse agonist, potently reversing basal human and rat H₃R constitutive GTPγS binding activity. Although the inverse agonist effects of ABT-288 described here are in an in vitro recombinant system, it should be noted that H₃R inverse agonists have also been shown to reduce the activity of native, constitutively active expressed H₃Rs, decreasing basal GTPγS binding (Arrang et al., 2007) and enhancing neurotransmitter release in vitro and in vivo (Morisset et al., 2000).
ABT-288 enhanced the in vitro release of histamine and acetylcholine, two neurotransmitters important for cognition and vigilance (Esbenshade et al., 2008; Haas et al., 2008; Passani et al., 2009), by potently and competitively reversing H₃R agonist-mediated inhibition of their release in the rat brain synaptosome and EFS guinea pig ileal segment assays, respectively. This work was extended in vivo, where ABT-288 significantly enhanced acetylcholine and dopamine levels in rat prefrontal cortex. ABT-288 was most potent and efficacious in enhancing acetylcholine release in prefrontal cortex in comparison to hippocampus, where higher doses were required and the magnitude of the effect was lower, and in comparison to histamine and dopamine in the prefrontal cortex where higher doses were required to enhance release. Interestingly, GSK189254 enhanced acetylcholine and dopamine levels at equivalent doses in the rat interior cingulate cortex (Medhurst et al., 2007), perhaps a brain region specific event. In addition, ABT-288 only increased dopamine release in the prefrontal cortex and not striatum. While ABT-288 did not significantly increase histamine release in the prefrontal cortex, there was a trend toward enhanced release, consistent with the brain region specific enhancement of histamine release by the H₃ antagonist GSK189254 (Giannoni et al., 2010). These findings suggest that the effects of H₃ antagonists on the release of neurotransmitter release are likely not due to general brain activation but instead are likely more regionally and functionally discrete, dependent upon histaminergic innervation and localization of H₃Rs.

Preclinical evidence suggests that histamine H₃R blockade can decrease impulsivity, improve attention, and enhance learning and memory, and much of the research in
these areas has focused on cognition assays that measure the different learning and memory domains thought to be most affected in CNS diseases. ABT-288 was efficacious in three different cognitive tests designed to assess attention, short-term social memory, and spatial cognition. As is often seen with H₃ antagonists, the potency and efficacy of ABT-288 varied dependent upon the cognitive domain measured. ABT-288 produced a dose-dependent improvement of acquisition in a five-trial inhibitory avoidance task in SHR pups previously linked to attention/impulsivity deficits (Fox et al., 2002). Full efficacy was achieved at 0.001 mg/kg and maintained over a broad dose range up to 0.03 mg/kg with corresponding plasma exposure levels of 0.1-2.0 ng/ml. Interestingly, these exposure levels correspond to an H₃R occupancy (EC₅₀ = 3.17 ng/ml) of 10-40%, indicating that only a small proportion of H₃Rs needed to be blocked to achieve efficacy in this model. Although slightly less potent in vitro than ABT-239 at the rat H₃R, ABT-288 is 100-fold more potent on a dosage basis than ABT-239 in this model, which is reflective of the higher volume of distribution and higher plasma protein binding of ABT-239. It has been reported for GSK189254 and other GSK compounds that over 60% H₃R occupancy is required for efficacy, but it must be recognized that these agents were tested in different behavioral models (passive avoidance, novel object recognition).

ABT-288 was less potent and exhibited a narrower range of efficacious doses in a model of short-term social memory in adult rats than in the 5-trial inhibitory avoidance model, with full efficacy achieved at 0.03 to 0.1 mg/kg i.p.. Another aspect of cognition that is impaired in patients with AD and schizophrenia is working memory that can be
measured with a spatial navigation test in the water maze. ABT-288 reversed scopolamine-induced deficits in this task at 0.1 and 1.0 mg/kg, i.p., similar to the doses required for efficacy in the social recognition assay. Interestingly, efficacy across the cognitive models is first seen at lower doses (0.001-0.1 mg/kg) of ABT-288 than the dose (1.0 mg/kg) that causes a decrease in the amplitude of 1-4 Hz slow waves. This suggests that improvements in cognitive performance occurs at lower levels of H3R occupancy than is required for wakefulness, in agreement with an earlier report showing that a high degree of receptor occupancy was required to increased wakefulness by H3R antagonists when sleep parameters were measured (Le et al., 2008). Additional studies appear warranted to determine whether differential neurotransmitter release, different sites or signaling events, or other reasons account for the differences in doses of H3R antagonists required for cognitive effects and those that cause EEG activation.

Acetylcholine release in the frontal cortex was the most robust and sensitive (0.3-3 mg/kg) of the three neurotransmitters measured but dopamine levels were also increased at higher doses in the prefrontal cortex, perhaps implicating these neurotransmitters in the cognitive effects of ABT-288. While the doses required for stimulating release of the neurotransmitters were higher than those required for cognitive effects, probably reflecting the limitations in sensitivity (spatial, temporal resolution) of the microdialysis approach, it is likely that more discreet changes in local levels of these neurotransmitters in general occur following administration of ABT-288. On the other hand, all microdialysis studies reported here have been performed under so called resting conditions: no behavioral /cognitive challenges or environmental stimuli
(Pepeu and Giovannini 2004), which may result in limited sensitivity of neurotransmitter release towards H3 blockade by ABT-288. Social memory is impaired in Alzheimer’s disease and schizophrenia (Pinkham et al., 2003) and both acetylcholine (Winslow and Camacho, 1995) and histamine (Prast et al., 1996) have been implicated as being involved in the social recognition assay. Cholinergic neurotransmission in the hippocampus is integral to spatial memory, and ABT-288 was able to overcome the deficit produced by scopolamine in the water maze task. It is possible that the positive effects of ABT-288 observed across the cognitive domains examined in this study are mediated at least in part by acetylcholine with contributions from histaminergic and dopaminergic systems dependent upon the doses and brain regions involved in the processes.

The effect of ABT-288 in a model related to schizophrenia was also assessed. Current antipsychotics are believed to work through blockade of dopamine hyperactivity in mesolimbic and mesocortical regions which is hypothesized to underlie the positive symptoms in schizophrenia. Positive symptoms of schizophrenia are thought to be modeled in the methamphetamine-induced psychomotor hyperactivity rodent test, where antipsychotic drugs have been shown efficacious to block the methamphetamine-induced hyperlocomotion. In these studies, ABT-288 (1.0 mg/kg) blocked the psychostimulant effects of methamphetamine. The efficacious dose of ABT-288 in this model was higher than that required for efficacy in the cognitive models, an effect also seen with ABT-239 (Fox et al., 2005). Interestingly, GSK207040 did not reverse
hyperlocomotion induced by methamphetamine (Southam et al., 2009) and pitolisant was only partially effective (Ligneau et al., 2007a).

ABT-288 exhibits good drug-like attributes including favorable pharmacokinetic properties in mouse and rat characterized by moderate to high plasma clearance values and moderate to high volumes of distribution. The plasma elimination half-life was short in rodents (< 1.5 hours), but still more than adequate for testing in rodent cognition models. The oral bioavailability values ranged from 37% in rat to 67% in mouse. The brain to plasma ratios were greater than 1.5:1 in both mouse and rat, indicating ABT-288 readily partitioned into the brain. In addition, ABT-288 exhibited no appreciable effects in in vitro hERG models or in vivo canine cardiovascular studies at dose multiples of up to 6,300-fold over the behaviorally efficacious level in rodents, a margin superior to that previously seen with ABT-239.

ABT-288 is a highly selective H₃R competitive antagonist with high potency at both rat and human H₃Rs. ABT-288 modulates neurotransmitter release in vitro, enhances the release of acetylcholine and dopamine in vivo, and exhibits preclinical efficacy in rodent behavioral tests across multiple cognitive domains affected in disorders of cognition such as AD, ADHD, and CDS. ABT-288 exhibits a safe preclinical profile in rodent and dog studies, and has advanced to the clinical stage to determine its PK and tolerability in healthy volunteers. ABT-288 is currently in Phase 2 trials to evaluate its therapeutic benefit for the treatment of cognitive deficits in Alzheimer’s disease and schizophrenia.
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antagonist 6-[(3-cyclobutyl-2,3,4,5-tetrahydro-1H-3-benzazepin-7-yl)oxy]-N-methyl-3-pyridinecarboxamide hydrochloride (GSK189254) on histamine release in the central nervous system of freely moving rats. J Pharmacol Exp Ther. 332:164-172.


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FIGURE LEGENDS.

Figure 1. Chemical structure of ABT-288 (2-[4'-(3aR,6aR)-5-Methyl-hexahydropyrrolo[3,4-b]pyrrol-1-yl)-biphenyl-4-yl]-2H-pyridazin-3-one).

Figure 2. Schild analysis of ABT-288 competitive antagonism of (R)-α-MeHA-mediated stimulation of [35S]-GTPγS binding in C6 cell membranes stably expressing the full-length human histamine H3R (panel A). Membranes were incubated with varying concentrations of ABT-288 prior to the addition of increasing concentrations of (R)-α-MeHA and levels of [35S]-GTPγS binding were determined as described in Methods. Inset panel shows the Schild transformation of the shifts in (R)-α-MeHA concentration response curves by ABT-288. Data are expressed as a percentage of the basal [35S]-GTPγS binding in the absence of H3R antagonist and (R)-α-MeHA. Results represent the mean ± S.E.M. of 3 experiments performed in triplicate. Panel B: ABT-288 blocks histamine-mediated reversal of potassium-stimulated [3H]-histamine release in rat brain cortical synaptosomes. Synaptosomes were incubated with varying concentrations of ABT-288 and 1 μM histamine prior to the addition of 15 mM potassium. [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 H3R antagonist and histamine. Results represent the mean ± S.E.M. of 3 concentration response curves performed in triplicate.
Figure 3. ABT-288 effects on the release of acetylcholine (top panel), dopamine (middle panel), and histamine (bottom panel), in the medial prefrontal cortex of freely moving rats. ABT-288 (10 mg/kg) did not enhance the cumulative release of dopamine over time 0 to 120 min from rat striatum (bottom panel, inset). Data represent the mean ± S.E.M. of baseline neurotransmitter levels from 5-9 animals per group as described in the Methods.

Figure 4. ABT-288 improves acquisition of a 5-trial, repeated acquisition test in SHR pups. Data are presented as cumulative inhibitory avoidance response in SHR pups compared to vehicle (improvement over control) for the 4 learning trials as described in Methods. ABT-288 or vehicle was dosed 30 min prior to the first training trials; the range of vehicle control response is depicted by the bar. Data are expressed as mean ± S.E.M. * indicates a significant (p<0.05) effect with respect to vehicle-treated controls.

Figure 5. ABT-288 improves social memory in adult rats. Rats were dosed with vehicle or ABT-288 immediately after a 5-min exposure to an unfamiliar juvenile. After a period of 120 min, the same juvenile was reintroduced for a second 5-min period and the ratio of investigation between the second and first exposure periods was determined. Lower ratios indicate improved social memory. Data are expressed as the mean ± SEM (n = 10/group). ** indicates a significant (p<0.01) effect with respect to vehicle-treated controls.
Figure 6. ABT-288 reverses scopolamine-induced deficits in a rat 2-platform discrimination water maze assay. The vehicle or ABT-288 were administered i.p. 15 minutes prior to an i.p. injection of scopolamine hydrobromide on each test day. Rats were tested 15 minutes after scopolamine treatment. Data are expressed as mean errors (± SEM) n = 7-8/group. *p<0.05 versus saline vehicle animals; †p<0.05 indicates a significant difference from the scopolamine treated group.

Figure 7. ABT-288 dose-dependently attenuated methamphetamine (METH)-induced hyperactivity in mice. METH was administered 1.0 mg/kg i.p. to mice after 60 min of baseline activity recording and total distance traveled was recorded over a further 90 min in mice pretreated with vehicle (Veh) or ABT-288 at the indicated doses (n = 12 per group). The data represent the AUCs for each of the treatment group time courses for total distance traveled in 90 min. *p<0.05 compared to vehicle group, as indicated by a one-way ANOVA [F(4,57) = 5.700, p = 0.001] followed by Dunnett’s post-hoc comparison. #p<0.05 compared to vehicle / methamphetamine 1 mg/kg group.

Figure 8. Slow-wave EEG amplitude in conscious freely moving adult rats. Vehicle, diphenhydramine, or ABT-288 were administered using a within-subjects design, and EEG recordings were commenced 5 min after injection. Data are represented as the percentage change from vehicle in 1-4 Hz slow wave amplitude over 120 min (mean ± S.E.M). ABT-288 significantly decreased the amplitude of the 1-4 Hz slow waves at the highest dose tested of 1 mg/kg whereas diphenhydramine significantly increased amplitude at 10 mg/kg (p<0.01, Neuman-Keuls multiple comparison test).
Figure 9. ABT-288 occupies rat brain cortical H₃Rs in a dose-dependent and saturable manner as determined by displacement of systemically administered [³H]-A-349821 as described in Methods. ABT-288 inhibits specific [³H]-A-349821 binding in vivo in a plasma concentration-dependent fashion with an EC₅₀ = 3.17 ng/ml. Results represent the mean ± S.E.M. of specific cortical binding, performed in triplicate, from each dose group, 5-6 rats per group.

Figure 10. Mean arterial pressure (mm Hg; top panel) and QT-interval (bottom panel), corrected for change in heart rate with Van de Water's formula, in dogs administered escalating intravenous infusions of ABT-288; vehicle shown in open symbols.
Table 1. Comparison of binding affinities of ABT-288, ABT-239, and ciproxifan at histamine receptors. Data are expressed as the mean $K_i$ (nM) with the associated 95% confidence limit in parentheses.

<table>
<thead>
<tr>
<th></th>
<th>ABT-288</th>
<th>ABT-239</th>
<th>Ciproxifan</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(1.07-3.36)</td>
<td>(0.37-0.54)</td>
<td>(50.6-77.2)</td>
</tr>
<tr>
<td>Human H$_3^a$</td>
<td>1.90</td>
<td>0.45</td>
<td>62.5</td>
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<tr>
<td>Human Brain Cortex H$_3$</td>
<td>0.64</td>
<td>4.61</td>
<td>89.4</td>
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<tr>
<td>Rat H$_3$</td>
<td>8.15</td>
<td>1.24</td>
<td>0.42</td>
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<tr>
<td>Rat Brain Cortex H$_3$</td>
<td>8.09</td>
<td>3.23</td>
<td>0.59</td>
</tr>
<tr>
<td>Mouse Brain Cortex H$_3$</td>
<td>10.0</td>
<td>3.59</td>
<td>0.48</td>
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<tr>
<td>Human H$_1$</td>
<td>&gt;10,000</td>
<td>1,640</td>
<td>&gt;10,000</td>
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<tr>
<td>Human H$_2$</td>
<td>&gt;10,000</td>
<td>6,720</td>
<td>&gt;10,000</td>
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<td>Human H$_4$</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>1,250</td>
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</tbody>
</table>

$n = 3-36$ independent experiments performed in duplicate.

$^a$H$_3$ receptors were stably expressed in C6 cells; H$_1$, H$_2$, and H$_4$ receptors were stably expressed in HEK cells.
Table 2. Comparison of potencies of ABT-288, ABT-239, and ciproxifan in histamine H₃R functional assays.

<table>
<thead>
<tr>
<th>Antagonist Assay</th>
<th>ABT-288 (pmol/L)</th>
<th>ABT-239 (pmol/L)</th>
<th>Ciproxifan (pmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human H₃ GTPγS</td>
<td>8.70 ± 0.05</td>
<td>8.84 ± 0.07</td>
<td>6.82 ± 0.02</td>
</tr>
<tr>
<td>Rat H₃ GTPγS</td>
<td>8.21 ± 0.02</td>
<td>8.43 ± 0.05</td>
<td>8.90 ± 0.07</td>
</tr>
<tr>
<td>EFS Guinea Pig Ileum</td>
<td>9.1 ± 0.96</td>
<td>8.74 ± 0.44</td>
<td>8.12 ± 0.56</td>
</tr>
<tr>
<td>Rat Synaptosome [³H]-Histamine Release (Kᵦ)</td>
<td>3.23 (0.67-15.6)</td>
<td>18.5 (9.2-36.9)</td>
<td>0.73 (0.53-1.0)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Inverse Agonist Assay</th>
<th>ABT-288 (nM)</th>
<th>ABT-239 (nM)</th>
<th>Ciproxifan (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human H₃ GTPγS</td>
<td>3.78 (1.82-7.86)</td>
<td>1.94 (1.17-3.24)</td>
<td>90.2 (25.4-319.5)</td>
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<tr>
<td>Rat H₃ GTPγS</td>
<td>7.2 (0.6-93.7)</td>
<td>17.4 (5.2-58.5)</td>
<td>1.34 (0.47-3.82)</td>
</tr>
</tbody>
</table>

aAll antagonist potencies shown represent pA₂ values (mean ± S.E.M.) for each of the assays except for the rat synaptosome [³H]-histamine release assay where the values represent the mean Kᵦ values (nM, 95% C.I.).

bFor the GTPγS binding assays, human and rat H₃ receptors were stably expressed in HEK and C6 cells, respectively.

cAll inverse agonist potencies shown represent EC₅₀ values (nM, 95% C.I.) for each of the assays.

n = 3-37 independent experiments performed in duplicate or triplicate.
Figure 1

ABT-288
Figure 2

A
- Control
- ABT-288 (3.2 nM)
- ABT-288 (10 nM)
- ABT-288 (32 nM)
- ABT-288 (100 nM)
- ABT-288 (320 nM)

% Basal GTPγS Binding

R-α-MeHA (log M)

B

% Maximum Histamine Release

ABT-288 (log M)
Figure 3

**Acetylcholine**

![Graph showing the effect of ABT-288 on Acetylcholine levels.](image)

**Dopamine**

![Graph showing the effect of ABT-288 on Dopamine levels.](image)

**Histamine**

![Graph showing the effect of ABT-288 on Histamine levels.](image)
Figure 4

![Graph showing the effect of ABT-288 (mg/kg, s.c.) on Improvement Over Control (sec). The x-axis represents different concentrations of ABT-288, and the y-axis represents the improvement over control in seconds. The graph includes error bars and asterisks indicating statistical significance.]
Figure 5

Investigation Ratio vs. ABT-288 (mg/kg, i.p.)

- Veh
- 0.01
- 0.03
- 0.1
- 0.3

* indicates a significant difference.
Figure 8

The graph shows the percentage change from vehicle for two substances: ABT-288 and Diphenhydramine, measured in milligrams per kilogram administered intraperitoneally (mg/kg i.p.). The x-axis represents the dose levels of the substances, ranging from 0.01 to 10 mg/kg i.p., while the y-axis indicates the percentage change from the vehicle. The data points for each dose are represented by error bars, and an asterisk (*) indicates statistical significance.

* This article has not been copyedited and formatted. The final version may differ from this version.
Figure 9

![Graph showing the relationship between Total Plasma ABT-288 (ng/ml) and % Receptor Occupancy. The graph displays a curve that suggests a dosedependent increase in receptor occupancy as the plasma levels of ABT-288 increase.](image-url)
Figure 10

Mean Arterial Pressure

- ABT-288 (n=6)
- Vehicle (n=6)

Corrected QT Interval (Van de Water)

- ABT-288 (n=5)
- Vehicle (n=6)