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ABSTRACT

Histamine H3 receptor antagonists are being developed to treat a variety of neurological and cognitive disorders that may be ameliorated by enhancement of central neurotransmitter release. Here, we present the in vitro pharmacological and in vivo pharmacokinetic profiles for the nonimidazole, benzofuran ligand ABT-239 [4-(2-{2-[(2R)-2-methylpyrrolidinyl]ethyl}-benzofuran-5-yl)benzonitrile] and compare it with several previously described imidazole- and nonimidazole H3 receptor antagonists. ABT-239 binds to recombinant human and rat H3 receptors with high affinity, with pKi values of 9.4 and 8.9, respectively, and is over 1000-fold selective versus human H1, H2, and H3 histamine receptors. ABT-239 is a potent H3 receptor antagonist at recombinant human and rat receptors, reversing agonist-induced changes in electric field stimulated guinea pig ileal segments (pA2 = 8.7). Additionally, ABT-239 is a potent inverse agonist, inhibiting constitutive [35S]GTPγS binding at both rat and human H3 receptors with respective pEC50 values of 8.9 and 8.2. ABT-239 demonstrates good pharmacokinetic characteristics in rat, dog, and monkey with t1/2 values ranging from 4 to 29 h, corresponding with clearance values and metabolic turnover in liver microsomes from these species, and good oral bioavailability ranging from 52 to 89%. Thus, ABT-239 is a selective, nonimidazole H3 receptor antagonist/inverse agonist with similar high potency in both human and rat and favorable drug-like properties.

Histamine H3 receptors are G protein-coupled receptors that function as central autoreceptors to modulate the release of histamine (Arrang et al., 1983). H3 receptors also function as heteroreceptors, regulating the release of other important neurotransmitters such as acetylcholine, dopamine, norepinephrine, and serotonin, among others (Blandina et al., 1998) that seem to play a role in attention, vigilance, and cognition. H3 receptor antagonists enhance the release of these neurotransmitters and, thus, may be potential human therapeutic agents for the treatment of disorders associated with attentional and cognitive deficits, such as attention deficit/hyperactivity disorder, Alzheimer’s Disease, mild cognitive impairment, and schizophrenia (Leurs et al., 1998).

Since the cloning of the human and rat H3 receptors...
(Lovenberg et al., 1999, 2000), a number of distinctive H₃ receptor properties have been revealed that have influenced H₃ receptor antagonist drug discovery efforts to develop human therapeutics for the treatment of CNS disorders (Hancock et al., 2003; Witkin and Nelson, 2004). Variations of amino acids 119 and 122 within the third transmembrane domain of the H₃ receptor result in marked species differences in the antagonist pharmacology of the rat and human H₃ receptors (Ligneau et al., 2000; Lovenberg et al., 2000; Yao et al., 2003), necessitating the discovery of compounds with similar binding affinities for the two species to meaningfully translate rat behavioral data to potential human clinical utility. Furthermore, multiple H₃ receptor splice isoforms have been identified that exhibit differential expression patterns in human and rat brain (Coge et al., 2001; Drutel et al., 2001). To date, no H₃ receptor antagonists have exhibited any major pharmacological differences in binding to these isoforms although agonists do show increased potencies for the short isoform (Wieland et al., 2001). In addition, H₃ receptors activate a number of signal transduction pathways including the Gₛₒₛ protein-dependent inhibition of adenylate cyclase activity (Lovenberg et al., 1999; Drutel et al., 2001) and the isomform dependent activation of mitogen-activated protein kinase and arachidonic acid release by the rat H₃ receptor (Drutel et al., 2001), perhaps influencing the clinical potential of H₃ receptor antagonists. Native and heterologously expressed cloned H₃ receptors are constitutively active (Morisset et al., 2000; Wieland et al., 2001), and it has been suggested that H₃ receptor inverse agonists may be most desirable as therapeutics because of their ability to reverse constitutive activity (Schwartz et al., 2003).

The first H₃ receptor antagonists were primarily imidazole derivatives such as thioperamide (Arrang et al., 1987), ciproxifan (Ligneau et al., 1998), clobenpropit (Barnes et al., 1993), and cipralisant (Tedford et al., 1998). Although quite useful pharmacological tools, as a class they possess a number of characteristics that hinder their development as human therapeutics. Compounds such as thioperamide, ciproxifan, and cipralisant, although potent at the rat H₃ receptor, have lower affinity at the human receptor (Ligneau et al., 2000; Lovenberg et al., 2000; Ireland-Denny et al., 2001; Wulff et al., 2002; Yao et al., 2003) and, subsequently, as a class, have relatively low selectivity for the human H₃ receptor because of binding to serotonin 5-HT₃, α₂-adrenergic, and/or histamine H₄ receptors (Ebseneshade et al., 2003, 2004). Another limitation of these H₃ receptor antagonists as human therapeutics is the imidazole moiety itself that in other drug molecules has been shown to inhibit cytochrome P₄₅₀ (P₄₅₀) metabolic pathways (Halpert et al., 1994). Indeed, both thioperamide and clobenpropit have been found to interact with cytochrome P₄₅₀ enzymes (Alves-Rodrigues et al., 1996; Harper et al., 1999). Thus, nonimidazole H₃ receptor antagonists may offer greater therapeutic potential in man. Recent reports from a number of industrial and academic laboratories (Apodaca et al., 2003; Chai et al., 2003; Miko et al., 2003; Zaragoza et al., 2004) have described the properties of novel nonimidazole H₃ receptor antagonists (for review, see Cowart et al., 2004b). In addition, we have reported on the pharmacological properties of the nonimidazole, aryloxyalkyl piperazine-based H₃ receptor antagonists, A-304121, A-317920 (Ebseneshade et al., 2003; Fox et al., 2003), and A-349821 (Ebseneshade et al., 2004). However, these compounds exhibit properties that preclude them being human therapeutic agents such as weak human H₃ receptor potency for A-304121 and A-317920 and suboptimal pharmacokinetic parameters for A-349821. Herein, we report the in vitro pharmacological profile and pharmacokinetic properties of the nonimidazole, benzofuran H₃ receptor antagonist ABT-239 (Cowart et al., 2004a; Fig. 1). An accompanying report (Fox et al., 2005) presents the in vivo behavioral characterization of this novel compound.

### Materials and Methods

**Chemicals.** ABT-239 (Cowart et al., 2004a; Fig. 1), A-349821, cipralisant, and ciproxifan were synthesized at Abbott Laboratories (Abbott Park, IL). [³H]N-α-methylhistamine (45–90 Ci/mmol), [³H]pyrilamine (20–30 Ci/mmol), [³H]tiotidine (70–90 Ci/mmol), [³H]prazosin (75–80 Ci/mmol), [³H]rauwolscine (75 Ci/mmol), [³H]histidine (40–60 Ci/mmol), and [³5S]GTPγS (1250 Ci/mmol) were obtained from PerkinElmer Life and Analytical Sciences (Boston, MA), and [³H]histamine (30–60 Ci/mmol) and [³H]LY-275854 (60–85 Ci/mmol) were from Amersham Biosciences Inc. (Piscataway, NJ). Phentolamine was purchased from Novartis (Basel, Switzerland), (R)-α-methylhistamine [(R)-α-MeHA] and clobenpropit were purchased from Tocris Cookson Inc. (Ellisville, MO), and serotonin and thioperamide were purchased from Sigma-Aldrich (St. Louis, MO).

**Animals.** Male Sprague-Dawley rats (weighing 150–200 g upon arrival) and male Hartley guinea pigs (weighing from 150–200 g on arrival) were supplied by Charles River Breeding Laboratories (Portage, MI). Male beagle dogs were obtained from Marshall Farms (North Rose, NY). Male cynomolgus monkeys were obtained from the Abbott Drug Analysis Colony.

Animals for experiments conducted in house were housed in Association for Assessment and Accreditation of Laboratory Animal Care-approved facilities at Abbott Laboratories in a temperature-regulated environment with lights on between 6:00 AM and 6:00 PM. The animals were acclimated to laboratory conditions for at least 1 week before testing. Abbott’s Institutional Animal Care and Use Committee approved all protocols for in-house testing.

**H₃ Receptor Cloning and Cell Membrane Preparation.** The full-length (445 amino acids) human histamine H₃ receptor gene (Lovenberg et al., 1999) was cloned from human thalamus poly-A RNA (BD Biosciences Clontech, Palo Alto, CA), and the full-length rat histamine H₃ receptor cDNA was obtained from Long Evans rat thalamus RNA using reverse transcription-polymerase chain reac-

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**Fig. 1.** Chemical structure of ABT-239.
tion methods as previously described (Esbenshade et al., 2003). Both genes were subcloned into the cDNA expression vector and stably expressed in HEK and C6 cells as described previously (Esbenshade et al., 2003).

Membranes were prepared from cells that were harvested and homogenized in 50 mM Tris/5 mM EDTA buffer, pH 7.4, containing protease inhibitors (1 mM benzamidine, 2 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin; Sigma–Aldrich) using a polytron at 20,000 rpm for 2 × 10–s bursts. Membranes were isolated by centrifugation at 40,000g. The homogenization and centrifugation steps described above were repeated to further purify the membranes. The final pellets were homogenized in 6.25 volumes (w/v) of 50 mM Tris/5 mM EDTA, pH 7.4, buffer, and the membrane preparations were frozen in liquid nitrogen and stored at –70°C until used.

Frozen human (Analytical Biological Services, Wilmington, DE) rat (Pelfreeze, Rogers, AR) brain cerebral cortices or freshly isolated dog or guinea pig brain cerebral cortices were homogenized in cold 50 mM Tris/5 mM EDTA, pH 7.4, buffer containing protease inhibitors, and the homogenate was centrifuged at 40,000g for 20 min at 4°C. This step was repeated, and the resulting pellet was resuspended in 3 to 6.25 volumes (w/v) of 50 mM Tris/5 mM EDTA, pH 7.4. Buffer. Membrane preparations were frozen in liquid nitrogen and stored at –70°C until needed. HEK cells stably expressing the human histamine H₁, H₂, and H₄ receptors were prepared as previously described (Esbenshade and Hancock, 2000; Esbenshade et al., 2003).

**Radioligand Binding Assays.** For H₃ receptor competition binding, membrane preparations were incubated with [³H]N-α-methylhistamine (0.5–1.0 nM) in the presence or absence of increasing concentrations (from 5–11 concentrations over a five-log unit range) of ligands for 30 min at 25°C in a final volume of 0.5 ml of 50 mM Tris/5 mM EDTA, pH 7.4, buffer as previously described (Esbenshade and Hancock, 2000; Esbenshade et al., 2003). Nonspecific binding was defined with 10 μM thioperamide. Cloned human histamine H₁, H₂, and H₄ receptor radioligand binding assays were performed as previously described (Esbenshade and Hancock, 2000; Esbenshade et al., 2003) using [³H]mepyramine, [³H]iotidine, and [³H]histamine, respectively. Radioligand binding assays for the cloned human α2a- and α2c-adrenergic receptors were performed using [³H]rauwolscine, and binding assays for rat frontal cortex 5-HT_{3a} serotonin receptors were conducted using [³H]LY-287854 as previously described (Esbenshade et al., 2003). All binding reactions were terminated by vacuum filtration onto polyethylenimine (0.3%) presoaked Unifilter plates (PerkinElmer Life and Analytical Sciences) or Whatman GF/B filters (for human cortex H₂ receptor, human H₄ receptor, and rat 5-HT₃ receptor; Whatman, Clifton, NJ) followed by three brief washes with 2 ml of ice-cold 50 mM Tris/5 mM EDTA, pH 7.4, buffer. Liquid scintillation counting was used to determine the bound radioactivity.

IC_{50} values and Hill slopes were determined by Hill transformation for all of the radioligand competition binding data as previously described (Esbenshade and Hancock, 2000). pK_{B} values were determined by the generalized Cheng–Prusoff equation (Cheng and Prusoff, 1973), and the data are presented as the mean ± S.E.M. The data were reanalyzed using GraphPad Prism to obtain IC_{50} values and Hill slopes. The potency of the antagonists (pA_{2}) to inhibit the (R)-α-MeHA response was calculated according to the method of Schild (1947). pK_{B} values were determined by the generalized Cheng–Prusoff equation (Cheng and Prusoff, 1973; Leff and Dougall, 1993) and are presented as the mean ± S.E.M.

**Measurement of Intracellular Calcium Levels.** The potency of H₃ receptor antagonists to inhibit 30 nM (R)-α-MeHA-stimulated increases in intracellular calcium levels by the full-length human H₃ receptor was determined in HEK cells stably coexpressing the receptor and Go_{q/11} (Coward et al., 1999) using fluorometric imaging plate reader as previously described (Esbenshade et al., 2003). Peak response values were expressed as a percentage of the reference peak response for 30 nM (R)-α-MeHA in the absence of H₃ receptor antagonists. Experiments were performed in duplicate, and data were analyzed using GraphPad Prism to obtain IC_{50} values and Hill slopes. The generalized Cheng–Prusoff equation (Cheng and Prusoff, 1973; Leff and Dougall, 1993) was used to determine pK_{B} values, which are presented as the mean ± S.E.M.

**Electric Field-Stimulated (EFS) Guinea Pig Ileal Segments.** The reversal of (R)-α-MeHA-mediated inhibition of EFS (test voltage –7–8V, 0.1–Hz frequency, 0.5–ms duration) elicited twitches of guinea pig ileal segments by H₃ receptor antagonists was determined as previously described (Ireland-Denny et al., 2001; Esbenshade et al., 2003). Various concentrations of H₃ receptor antagonists were added to the tissue baths 30 min prior to the generation of (R)-α-MeHA cumulative concentration response curves with no effect noted on the twitch response during the preincubation time. The potency (pA_{2}) of the antagonists to inhibit the (R)-α-MeHA response was calculated according to the method of Schild (1947) as described previously (Ireland-Denny et al., 2001; Esbenshade et al., 2003).

**Rat Cerebral Cortical Histamine Release Assay.** The ability of H₃ receptor antagonists to reverse histamine-mediated inhibition of [³H]histamine release from rat cerebral cortical synaptosomes evoked by potassium (15 mM) was determined as previously described (Esbenshade et al., 2003). Values for the basal release of [³H]histamine (obtained in buffer without additional potassium) were subtracted from each sample, and the data were 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 IC_{50} values and Hill slopes. pK_{B} 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.

**[³S][GTP]Y[S] Binding Assay.** HEK cell membranes expressing the human H₃ receptor or C6 cell membranes expressing the rat H₃ receptor 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 40,000g 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 were added to a final concentration of 10% glycerol and 1% bovine serum albumin prior to freezing the membranes. The inverse agonist activity of H₃ receptor antagonists was determined as described previously (Esbenshade et al., 2003). In brief, membranes were diluted in GTP–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 [³S][GTP]Y[S], and various concentrations of H₃ receptor 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 H₃ receptor), 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 (PerkinElmer Life and Analytical Sciences). After extensive washing, the plates were dried, Microscint 20
was added to the samples, and the amount of bound [\(^{35}\)S]GTP\(_S\) was determined using the Topcount (PerkinElmer Life and Analytical Sciences). The percentage of \([^{35}\text{S}]\text{GTP}\_S\) bound in each sample was calculated as a percentage of that bound to control samples incubated in the absence of histamine H\(_3\) receptor ligands. Triplicate determinations were obtained at each concentration, and the data were analyzed using GraphPad Prism to obtain EC\(_{50}\) or IC\(_{50}\) values and Hill slopes. pK\(_i\) values were calculated using the generalized Cheng-Prusoff equation (Cheng and Prusoff, 1973; Leff and Dougall, 1993). The mean ± S.E.M. of at least three independent experiments is reported.

**ABT-239 Metabolism in Liver Microsomes.** ABT-239 (1 \(\mu\)M) was incubated with liver microsomal protein (0.1–1 mg/ml) from rat (Cedra Corporation, Austin, TX), mouse (In Vitro Technologies, Baltimore, MD), and human, monkey, and dog (BD Gentest, Woburn, MA) in 50 mM potassium phosphate buffer, pH 7.4, in duplicate reaction tubes (Scott, 1999). After a 5-min preincubation at 37°C in a shaking water bath, the enzyme reaction was initiated by the addition of 1 mM NADPH. At \(t = 0\) and at five time points ranging to 30 min, aliquots (200 \(\mu\)l) were removed and added to termination mixtures (100 \(\mu\)l of acetonitrile/methanol, 1:1, v/v). Samples were centrifuged at 14,000 \(g\) for 10 min, then held at 100% acetonitrile for 4 min, and returned to 100% 10 mM ammonium acetate, pH 3.3, and 100% acetonitrile and delivered at a flow rate of 0.2 ml/min. Elution was achieved by a linear gradient of 0 to 100% acetonitrile over 3 min, then held at 100% acetonitrile for 4 min, and returned to 100% 10 mM ammonium acetate, pH 3.3, for 1 min. The column was for 7 min before the next injection. In cytochrome P450 isoform inhibition studies, the inhibitory effect of ABT-239 (2 and 20 \(\mu\)M) on dextromethorphan O-demethylase (CYP2D6), phenacetin O-deethylation (CYP1A2), tolbutamide hydroxylation (CYP2C9), terfenadine hydroxylation (CYP3A4), S-mephenytoin 4'-hydroxylation (CYP2C19), chlorozoxazone 6-hydroxylation (CYP2E1), and coumarin 7-hydroxylation (CYP2B6) activities was investigated in pooled human liver microsomes (BD Gentest) incubated with ABT-239 and isoform-specific substrates for 5 to 60 min at 37°C.

**Plasma Protein Binding.** ABT-239 was incubated with plasma at room temperature for 1 h, and duplicate samples of the drug-spiked plasma from human, monkey, dog, or rat were transferred to 1-ml ultracentrifugation tubes. The samples were centrifuged at 86,000g for 18 h, and four-layer fractions of 0.2 ml each were transferred to 1-ml plastic bullets containing 0.4 ml of acetonitrile. To the remaining 0.2-ml fraction was added 0.8 ml of 0.05 M phosphate-buffered saline, pH 7.4, and 0.2 ml of this diluted fraction was transferred to a 1-ml plastic bullet containing 0.4 ml of acetonitrile. All samples were centrifuged for 5 min at 7000 rpm, and 0.1 ml of supernatant was transferred to a vial containing 0.1 ml of formic acid/water, and 50 \(\mu\)l was analyzed by LC/MS/MS.

**Pharmacokinetic Analysis.** ABT-239 was selectively removed from the blood or brain homogenate from monkey, dog, or rat using liquid-liquid extraction with a mixture of ethyl acetate and hexane (1:1, v/v) at basic pH. The samples were vortexed vigorously followed by centrifugation. The organic layer 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-239 and internal standard were separated from coextracted contaminants on a 5-cm × 3-mm 3-\(\mu\)m Aquasil C18 column with an acetonitrile/0.1% trifluoroacetic acid (60:40, v/v) mobile phase at a flow rate of 0.35 ml/min with a 25-\(\mu\)l injection. ABT-239 was quantified using multiple reaction monitoring detection, m/z 331.3 → 98.0 using a turbo ion spray source on a mass spectrometer (PerkinElmer Sciex Instruments, Boston, MA). The limits of quantitation were approximately 50 pg/ml for blood and 750 pg/g for brain samples.

### Results

**Histamine H\(_3\) Receptor Binding.** H\(_3\) receptor binding affinities for ABT-239, A-349821 (Esbenshade et al., 2004), cipralisant, and ciproxifan (Esbenshade et al., 2003) were determined by displacement of specific \([^{3}\text{H}]\text{N}-\alpha\)-methylhistamine binding from H\(_3\) receptor binding sites in C6 cell membranes expressing the full-length recombinant rat or human H\(_3\) receptor and brain cortical membranes prepared from rat, human, dog, or guinea pig (Table 1). All four compounds bound potently to both the recombinant rat H\(_3\) receptor and rat brain cortical membrane H\(_3\) receptors with pK\(_i\) values

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**TABLE 1**

Comparison of binding affinities of ABT-239, A-349821, cipralisant, and ciproxifan at histamine receptors

<table>
<thead>
<tr>
<th></th>
<th>ABT-239</th>
<th>A-349821(^a)</th>
<th>Cipralisant</th>
<th>Ciproxifan(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human H(_3)</td>
<td>9.35 ± 0.04</td>
<td>9.39 ± 0.08</td>
<td>8.28 ± 0.08</td>
<td>7.20 ± 0.05</td>
</tr>
<tr>
<td>(0.98 ± 0.02)</td>
<td>(0.9 ± 0.03)</td>
<td>(1.05 ± 0.03)</td>
<td>(0.84 ± 0.04)</td>
<td>(0.8 ± 0.05)</td>
</tr>
<tr>
<td>Rat H(_3)</td>
<td>8.91 ± 0.04</td>
<td>8.78 ± 0.12</td>
<td>9.95 ± 0.10</td>
<td>9.29 ± 0.09</td>
</tr>
<tr>
<td>(0.87 ± 0.02)</td>
<td>(0.93 ± 0.06)</td>
<td>(1.02 ± 0.05)</td>
<td>(0.88 ± 0.02)</td>
<td>(1.02 ± 0.05)</td>
</tr>
<tr>
<td>Human Brain Cortex H(_3)</td>
<td>8.34 ± 0.06</td>
<td>9.37 ± 0.08</td>
<td>7.91 ± 0.12</td>
<td>7.05 ± 0.06</td>
</tr>
<tr>
<td>(0.78 ± 0.04(^b))</td>
<td>(0.77 ± 0.06(^b))</td>
<td>(1.16 ± 0.10)</td>
<td>(1.06 ± 0.07)</td>
<td>(1.06 ± 0.07)</td>
</tr>
<tr>
<td>Rat Brain Cortex H(_3)</td>
<td>8.49 ± 0.04</td>
<td>8.84 ± 0.07</td>
<td>9.60 ± 0.07</td>
<td>9.20 ± 0.04</td>
</tr>
<tr>
<td>(0.81 ± 0.02)</td>
<td>(0.87 ± 0.04)</td>
<td>(1.05 ± 0.03)</td>
<td>(0.90 ± 0.03)</td>
<td>(0.90 ± 0.03)</td>
</tr>
<tr>
<td>Guinea Pig Brain Cortex H(_3)</td>
<td>8.59 ± 0.12</td>
<td>9.26 ± 0.13</td>
<td>9.20 ± 0.04</td>
<td>8.76 ± 0.07</td>
</tr>
<tr>
<td>(0.86 ± 0.05)</td>
<td>(0.71 ± 0.07(^b))</td>
<td>(1.01 ± 0.05)</td>
<td>(0.89 ± 0.05)</td>
<td>(0.89 ± 0.05)</td>
</tr>
<tr>
<td>Dog Brain Cortex H(_3)</td>
<td>8.41 ± 0.07</td>
<td>8.92 ± 0.10</td>
<td>8.88 ± 0.05</td>
<td>8.24 ± 0.06</td>
</tr>
<tr>
<td>(0.84 ± 0.09)</td>
<td>(0.83 ± 0.07)</td>
<td>(1.16 ± 0.13)</td>
<td>(0.88 ± 0.02)</td>
<td>(0.88 ± 0.02)</td>
</tr>
<tr>
<td>Human H(_1)</td>
<td>5.79 ± 0.04</td>
<td>5.63 ± 0.04</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>(0.96 ± 0.06)</td>
<td>(0.85 ± 0.12)</td>
<td>(0.85 ± 0.12)</td>
<td>(0.85 ± 0.12)</td>
<td>(0.85 ± 0.12)</td>
</tr>
<tr>
<td>Human H(_4)</td>
<td>5.17 ± 0.04</td>
<td>&lt;5</td>
<td>7.29 ± 0.19</td>
<td>5.73 ± 0.09</td>
</tr>
<tr>
<td>(0.83 ± 0.11)</td>
<td>(&lt;5)</td>
<td>(0.68 ± 0.05)</td>
<td>(0.81 ± 0.19)</td>
<td>(0.81 ± 0.19)</td>
</tr>
</tbody>
</table>

\(^a\) Data are from Esbenshade et al. (2004) and Esbenshade et al. (2003).

\(^b\) Inhibition curve best fit to one-site model when compared with two-site model (GraphPad Prism).
greater than 8.0 with ABT-239 demonstrating a slightly higher affinity at the rat recombinant (pKᵢ = 8.9) versus native (pKᵢ = 8.5) H₃ receptors. However, the tested compounds displayed a much larger range of binding affinities for the human H₃ receptors with a similar rank order of potency (A-349821 > ABT-239 > cipraлизант > ciproфиан) at both the cloned and native human H₃ receptors. ABT-239 displayed about a 10-fold higher affinity (pKᵢ = 9.4) at the recombinant human H₃ receptor when compared with the native human brain H₃ receptors (pKᵢ = 8.3). This affinity difference is likely attributable to the high plasma protein binding (see below) and lipophilicity (cLogP = 5.15) of the compound that may contribute to the loss of free ABT-239 to nonspecific binding that becomes more apparent with the large amount of tissue homogenate used for the human brain H₃ receptor binding compared with the recombinant receptor preparation. Indeed, the difference in affinities between the recombinant and native rat H₃ receptor preparations is less, probably reflecting the lesser amount of native rat brain membrane used in the assay compared with the human brain cortex membrane preparation. The affinity of ABT-239 for native H₃ receptor expressed in rat, human, dog, and guinea pig was approximately equivalent (pKᵢ ~ 8.5) in contrast to the more rat selective H₃ receptor antagonists such as cipraлизант and ciproфиан. The Hill slopes of the ABT-239 displacement curves approached unity for all of the H₃ receptor preparations indicating the recognition of a single H₃ receptor binding site in each membrane preparation with the exception of the human brain H₃ receptor for which a lower Hill slope of 0.78 was obtained. However, nonlinear regression curve fitting revealed that, also in this case, the data were best fit by a one-binding site model.

H₃ Receptor Binding Selectivity Profile. The binding affinities of ABT-239 at the three other histamine receptor subtypes (H₁, H₂, and H₄) were determined and compared with A-349821 (Esbenshade et al., 2004), cipraлизант, and ciproфиан (Esbenshade et al., 2003) to determine H₃ receptor selectivity versus other histaminergic receptors (Table 1). ABT-239 exhibited no binding affinity at concentrations up to 10 μM (pKᵢ < 5) for the H₁ receptor and low affinity for the H₂ receptor (pKᵢ = 5.8) and H₄ receptor (pKᵢ = 5.2), providing a greater than 1000-fold H₃ receptor selectivity versus other histaminergic receptors. Conversely, the imidazoles cipraлизант and ciproфиан are only 10- and 30-fold selective for the human H₃ receptor compared with the human H₂ receptor (pKᵢ values = 7.3 and 5.7, respectively). Additionally, like the nonimidazole antagonist A-349821, ABT-239 has low affinity for the α₂A-, and α₂C-adrenergic receptor binding sites (pKᵢ values = 6.6 and 7.2, respectively) and the serotonin 5-HT₃ receptor (pKᵢ = 6.0), demonstrating over 100-fold selectivity for the human H₃ receptor (data not shown). The imidazole compounds cipraлизант and ciproфиан show lower levels of H₃ receptor selectivity, exhibiting appreciable binding to the α₂-adrenergic (pKᵢ values = 8.2 and 7.4, respectively) and α₂C-adrenergic (pKᵢ values = 8.0 and 7.2, respectively) receptors and serotonin 5-HT₃ receptors (pKᵢ value = 6.5 for ciproфиан) (Esbenshade et al., 2003).

**Functional Antagonism at Recombinant H₃ Receptors.** In C6 cells expressing the human and rat H₃ receptors, ABT-239 potently inhibited (R)-α-MeHA-mediated reversal of forskolin-stimulated cAMP accumulation in a concentration-dependent manner with pKᵢ values of 7.9 and 7.6 (Table 2), similar to the profile seen with A-349821 (Esbenshade et al., 2004). In contrast, ciproфиан exhibited more potent inhibition of rat H₃ receptor-mediated adenylate cyclase responses, consistent with its binding affinity, as previously reported (Esbenshade et al., 2003), whereas cipraлизант exhibited agonist activity at both the human and rat H₃ receptors (pEC₅₀ values = 6.7 and 8.7, respectively) in this assay, consistent with previous reports of the agonist activity of this compound (Wulff et al., 2002). In Schild experiments, ABT-239 demonstrated competitive H₃ receptor antagonism, shifting the concentration response curves for (R)-α-MeHA-activated GTPγS binding at both the human and rat H₃ receptors (pKᵢ values = 9.0 and 8.3, respectively, Table 2), similar again to the profile for A-349821 (Esbenshade et al., 2004), with ciproфиан again exhibiting lower potencies for the human H₃ receptor (Esbenshade et al., 2003) and cipraлизант demonstrating full agonist activity at both the human and rat H₃ receptors (pEC₅₀ = 7.4 and 8.6, respectively), with maximal increases in GTPγS binding over basal similar to the 240 and 220% increases, respectively, seen with R-α-MeHA. In HEK cells coexpressing the human H₃ receptor with the chimeric Grα₁/δ protein, ABT-239 also inhibited (R)-α-MeHA-stimulated increases in intracellular calcium with a pKᵢ value of 7.9 (Fig. 3), similar in potency to A-349821 and

**Table 2**

<table>
<thead>
<tr>
<th>Functional Assay</th>
<th>Mean pKᵢ (± S.E.M.)</th>
<th>Cipraлизант</th>
<th>Ciproфиан</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human H₃ cyclase</td>
<td>7.89 ± 0.04</td>
<td>8.24 ± 0.10</td>
<td>6.68 ± 0.14</td>
</tr>
<tr>
<td>Rat H₃ cyclase</td>
<td>7.60 ± 0.05</td>
<td>8.08 ± 0.10</td>
<td>8.72 ± 0.06</td>
</tr>
<tr>
<td>Human H₃ GTPγS</td>
<td>9.04 ± 0.04</td>
<td>9.26 ± 0.02</td>
<td>7.40 ± 0.04</td>
</tr>
<tr>
<td>Rat H₃ GTPγS</td>
<td>8.34 ± 0.14</td>
<td>8.62 ± 0.11</td>
<td>8.60 ± 0.08</td>
</tr>
<tr>
<td>Human H₃ FLIPR</td>
<td>7.87 ± 0.15</td>
<td>8.27 ± 0.12</td>
<td>5.82 ± 0.16</td>
</tr>
<tr>
<td>EFS guinea pig ileum</td>
<td>8.74 ± 0.44</td>
<td>9.47 ± 0.56</td>
<td>7.83 ± 0.27</td>
</tr>
<tr>
<td>Rat synaptosomes</td>
<td>7.73 ± 0.07</td>
<td>9.24 ± 0.16</td>
<td>7.31 ± 0.29</td>
</tr>
</tbody>
</table>

* Data are from Esbenshade et al. (2004).

* Data represent agonist pEC₅₀ values. Cipraлизант was a full agonist in all assays except the rat synaptosome [³H]histamine release (partial agonist with an intrinsic activity of 0.5) and the EFS guinea pig ileum (antagonist) assays.
Cells were incubated with increasing concentrations of the H3 receptor antagonist again exhibiting full agonist activity (pEC50 over 10-fold more potent than ciproxifan (Table 2) with cipralisant demonstrated weak partial agonist activity (pEC50 = 7.3) in this assay.

**Inverse Agonism: [35S]GTPγS Binding.** ABT-239 reduced basal [35S]GTPγS binding in membranes from HEK cells expressing the human H3 receptor in a concentration-dependent manner (Fig. 6, top) with a pEC50 value of 8.9, equipotent with A-349821 (pEC50 = 9.1), and a maximal inhibition of 26% from basal, comparable with that for A-349821 (23%). Ciproxifan demonstrated lower potency (pEC50 = 7.0) and efficacy (8%) in reversing basal GTPγS binding levels. ABT-239, A-349821, and ciproxifan were all equally efficacious inverse agonists at the rat H3 receptor, inhibiting basal [35S]GTPγS binding in a concentration-dependent manner (Fig. 6, bottom) with respective pEC50 values of 8.2, 8.5, and 9.2 and a similar maximal inhibition of 15% from basal for all three compounds.

**In Vitro Metabolism of ABT-239.** ABT-239 does not inhibit human CYP1A2, 2A6, 2C19, 3A4, and 2E1 isoforms at concentrations up to 20 μM and showed less than 25% inhibition of CYP2C9 and 2D6 at 20 μM in assays measuring the metabolism of isofrom-specific substrates in human liver microsomes (Fig. 7, top). Incubation of ABT-239 for 30 min with liver microsomes prepared from human, monkey, dog, rat, and mouse results in the time-dependent loss of parent ABT-239 (Fig. 7, bottom) with the fastest rate of disappearance in rat and slowest in human liver microsomes.

**Pharmacokinetic Properties of ABT-239.** The pharmacokinetics of ABT-239 were evaluated in Sprague-Dawley rat, beagle dog, and cynomolgus monkey. ABT-239 concentrations declined slowly after i.v. dosing in all species, with blood clearance values ranging from 1.5 l/h/kg in rat to 0.3 l/h/kg in the monkey (Table 3). ABT-239 exhibited high volumes of distribution in all species, with Vβ values >6 l/kg that, in combination with the low clearance values, provide elimination half-lives averaging 5.3, 8.3, and 29.2 h in rat, dog, and monkey, respectively (Table 3). Rats demonstrated the highest clearance values and correspondingly the lowest bioavailability at approximately 52% following a 1 mg/kg dose in the rat. Dogs and monkeys exhibited higher bioavailability values, averaging 74 and 89%, respectively (Table 3).
ABT-239 also exhibits a high degree of plasma protein binding in rat (94%), dog (91%), monkey (94%), and human (97%).

Blood and brain levels of ABT-239 were compared with those for A-349821 at 1, 5, and 24 h following a 5 mg/kg i.v. dose (Table 4). At 1 h, the blood levels of ABT-239 and A-349821 were comparable (685 and 677 ng/ml, respectively), but at 5 h, the level of ABT-239 was higher (204 ng/ml) than A-349821 (42 ng/ml) with both compounds having levels below the limit of detection at 24 h. The brain levels of ABT-239 were 21.6- and 15.8-fold higher than the corresponding blood levels at 1 and 5 h, respectively, in contrast to A-349821, where brain levels were only one-half of those in the blood at these time points. Moreover, brain levels of ABT-239 were detectable at 24 h after dosing, although they were less than 1% of the 1-h values (Table 4).

**Discussion**

ABT-239 potently binds both the rat and human H₃ receptor, unlike many imidazole H₃ receptor compounds such as cipralisant and ciproxifan as well as our previously described nonimidazole H₃ receptor antagonists A-304121 and A-317920 (Esbenshade et al., 2003) that demonstrate high affinity for only the rat receptor. ABT-239 is over 10-fold more potent than cipralisant and 100-fold more potent than ciproxifan at the human H₃ receptor, much like the previously reported A-349821 (Esbenshade et al., 2004), demonstrating subnanomolar affinity at the human receptor and low nanomolar affinity for the rat H₃ receptor, allowing accurate assessment of the H₃ receptor-mediated cognitive effects of ABT-239 in rodent behavioral models (see Fox et al., 2005). Among the four histaminergic receptor subtypes, ABT-239 is over 1000-fold selective for the human histamine H₃ receptor, in marked contrast to the 10- to 30-fold selectivity of the imidazoles cipralisant and ciproxifan for the human H₃ receptor compared with the human H₄ receptor. ABT-239 is also over 100-fold selective for the human H₃ receptor versus the biogenic amine α₂-adrenergic and serotonin 5-HT₃ receptors that generally bind imidazole H₃ receptor antagonists with relatively high affinity (Esbenshade et al., 2003) as well as over 80 other rodent and human G
protein-coupled receptors, including muscarinic receptors, and ligand-activated ion channels (data not shown).

ABT-239 is a H₃ receptor competitive antagonist, inhibiting recombinant rat and human H₃ receptor-mediated signaling pathways as well as reversing neurotransmitter release in well-established native H₃ receptor models. Schild analysis of the inhibition of (R)-α-MeHA-induced reversal of forskolin-stimulated cAMP accumulation in C6 cells expressing the human H₃ receptor clearly demonstrates the competitive antagonist properties of ABT-239, revealing a pA₂ value equivalent to the pKᵦ value obtained from the concentration-dependent inhibition of a maximally stimulating concentration of (R)-α-MeHA, demonstrating the validity of this approach to estimate antagonist potencies. ABT-239 was approximately equipotent with A-349821 at both the human and rat H₃ receptors in competitively inhibiting the (R)-α-MeHA-stimulated response in adenylate cyclase assays and was over 20-fold more potent than the imidazole H₃ antagonist ciproxifan in inhibiting the response in the human H₃ receptor cyclase assay but 40-fold less potent than ciproxifan in the rat H₃ receptor cyclase assay. A similar pharmacological profile across both rat and human H₃ receptors was seen for ABT-239-mediated inhibition of (R)-α-MeHA-stimulated GTPγS binding, although greater potencies are generally observed in this assay compared with the cyclase assay. Again, ABT-239 is equipotent with A-349821 but is over 100-fold more potent than ciproxifan in inhibiting (R)-α-MeHA-stimulated GTPγS binding mediated by the human H₃ receptor and equipotent with ciproxifan in inhibiting
versed H3 receptor agonist-mediated inhibition of the release, respectively, ABT-239 potently and competitively re-
H3 receptor regulation of acetylcholine and histamine re-
and rat brain synaptosome assays that model native tissue
isant (Fox et al., 2002). In the EFS guinea pig ileal segment
account for the distinct in vivo behavioral effects of cipral-
zole-based compound (Wulff et al., 2002) and may perhaps
ously reported complex pharmacological profile of this imida-
receptors, ligand affinities, conformational changes
induced by the ligands, or other factors. Interestingly, H3
receptor inverse agonists can also enhance neurotransmitter
release in vitro and in vivo via constitutively active natively
ized as H3 receptor inverse agonists, effectively reversing
basal H3 receptor constitutive activity, in addition to their H3
receptor antagonist activity. Likewise, ABT-239 is an inverse
agonist at the human H3 receptor, equipotent with A-349821
and over 100-fold more potent than ciproxifan, reflecting
their binding potencies. ABT-239 seemed to be a more effi-
cacious inverse agonist than ciproxifan and is equally efficacious
between species-dependent differences in inverse agonist potency and/or
efficacy result from differences in the constitutive activities
of the H3 receptors, ligand affinities, conformational changes
induced by the ligands, or other factors. Interestingly, H3
receptor inverse agonists can also enhance neurotransmitter
release in vitro and in vivo via constitutively active natively
expressed H3 receptors (Morisset et al., 2000). Although no
clinical data as yet have demonstrated the superiority of H3
receptor inverse agonists versus antagonists blocking an ag-
onist response, it may be important to design H3 receptor
inverse agonists with both high potency and efficacy to in-
hibit the constitutive activity of native H3 receptors and
potentially obtain a highly effective drug with therapeutic
utility for cognitive disorders.

ABT-239 exhibited a number of drug-like attributes includ-
ing favorable metabolism and pharmacokinetic properties.
Unlike imidazole-containing drugs (Halpert et al., 1994), the
nonimidazole ABT-239 does not appreciably inhibit P450
enzymes at concentrations up to 20 μM in human liver mi-
crosomes. However, ABT-239 was metabolized, albeit at dif-
ferent rates, by liver microsomes from human, monkey, dog,
rat, and mouse, with rat exhibiting the fastest rate of ABT-
239 metabolism and human the slowest. In vivo, ABT-239
metabolism and human the slowest. In vivo, ABT-239
pharmacokinetics. ABT-239 demonstrated high volumes of distribution in all species ex-
examined, perhaps reflecting the high degree of plasma protein
binding or CNS access and relatively low clearance values,
with elimination half-lives ranging from 5 (rat) to 29 (mon-
ABT-239 quickly accesses the brain following i.v. administration and achieves levels in the brain that exceed blood levels by over 20-fold at 1 h, perhaps accounting for the relatively lower potency of ABT-239 (0.1 mg/kg) in the five-trial inhibitory avoidance test (Esbenshade et al., 2004) compared with the potency for ABT-239 (1 mg/kg) (see Fox et al., 2005) or may result from lower brain levels of A-349821 despite similar H₃ receptor binding potencies for the two compounds. Thus, higher brain exposures of H₃ receptor antagonists may offer more favorable therapeutic effects. As in blood, ABT-239 is also cleared from the brain despite the relatively higher levels, with greater than 99% clearance from the brain within 24 h after an i.v. dose.

We have identified ABT-239 as a novel, nonimidazole, highly selective H₃ receptor competitive antagonist/inverse agonist with balanced high potency at both rat and human H₃ receptors. In addition, this compound modulates neurotransmitter release in several in vitro models, possesses favorable metabolic and pharmacokinetic properties, and exhibits cognitive enhancing effects in a variety of behavioral models (see Fox et al., 2005). To date, no selective, potent, and safe H₃ receptor antagonist has been tested for human efficacy despite numerous proposals that H₃ receptor antagonists may offer therapeutic utility for the treatment of a variety of cognitive disorders including attention deficit/hyperactivity disorder, Alzheimer’s disease, and schizophrenia. Therefore, although H₃ receptor antagonists are clearly efficacious in many animal models of neurological disorders, it still remains to be determined whether such an approach can be translated to treatment in humans. ABT-239 exemplifies H₃ receptor ligands identified through our drug discovery efforts that have focused on the synthesis of highly potent, selective, and efficacious nonimidazole H₃ receptor antagonists/inverse agonists that exhibit cognition-enhancing effects with an acceptable CNS therapeutic window (see accompanying report by Fox et al., 2005) and favorable drug-like properties. We believe that potent, selective, and efficacious nonimidazole H₃ receptor antagonist/inverse agonists such as ABT-239 will facilitate validation of the hypothesis that such drugs will be important therapeutic agents in the treatment of a variety of neuropsychiatric disorders in humans.

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