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RESEARCH PAPER

Use of the H_3 receptor antagonist radioligand [^{3}H]-A-349821 to reveal *in vivo* receptor occupancy of cognition enhancing H_3 receptor antagonists

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Background and purpose: The histamine H_3 receptor antagonist radioligand [^{3}H]-A-349821 was characterized as a radiotracer for assessing *in vivo* receptor occupancy by H_3 receptor antagonists that affect behaviour. This model was established as an alternative to *ex vivo* binding methods, for relating antagonist H_3 receptor occupancy to blood levels and efficacy in preclinical models.
Experimental approach: *In vivo* cerebral cortical H_3 receptor occupancy by [^{3}H]-A-349821 was determined in rats from differences in [^{3}H]-A-349821 levels in the isolated cortex and cerebellum, a brain region with low levels of H_3 receptors. Comparisons were made to relate antagonist H_3 receptor occupancy to blood levels and efficacy in a preclinical model of cognition, the five-trial inhibitory avoidance response in rat pups.
Key results: In adult rats, [^{3}H]-A-349821, 1.5 $\mu g \cdot kg^{-1}$, penetrated into the brain and cleared more rapidly from cerebellum than cortex; optimally, [^{3}H]-A-349821 levels were twofold higher in the latter. With increasing [^{3}H]-A-349821 doses, cortical H_3 receptor occupancy was saturable with a binding capacity consistent with *in vitro* binding in cortex membranes. In studies using tracer [^{3}H]-A-349821 doses, ABT-239 and other H_3 receptor antagonists inhibited H_3 receptor occupancy by [^{3}H]-A-349821 in a dose-dependent manner. Blood levels of the antagonists corresponding to H_3 receptor occupancy were consistent with blood levels associated with efficacy in the five-trial inhibitory avoidance response.
Conclusions and implications: When employed as an occupancy radiotracer, [^{3}H]-A-349821 provided valid measurements of *in vivo* H_3 receptor occupancy, which may be helpful in guiding and interpreting clinical studies of H_3 receptor antagonists.
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Keywords: ABT-239; histamine; H_3 receptor; H_3 receptor antagonist; radiotracer; H_3 receptor antagonist radioligand; [^{3}H]-A-349821; receptor occupancy; five-trial inhibitory avoidance

Abbreviations: [^{3}H]-NAMH, [^{3}H]-N-α-methylhistamine; DPM, disintegrations per min; K_i, equilibrium inhibition constant; PET, positron emission tomography; RAMH, R-α-methylhistamine; SHR, spontaneously hypertensive rat; SPECT, single photon emission computed tomography

Introduction

The histamine H_3 receptor, a G protein-coupled receptor localized primarily in the central nervous system (CNS), modulates the release of multiple neurotransmitters that play important roles in homeostatic mechanisms and cognition (Esbenshade *et al.*, 2008; Panula *et al.*, 2009; Selbach and Haas, 2009). Antagonist ligands of the H_3 receptor enhance the release of these neurotransmitters, and H_3 antagonists are being pursued as potential drugs for the treatment of the cognitive deficits associated with attention-deficit hyperactivity disorder, Alzheimer's disease and schizophrenia (Wijtmans *et al.*, 2007; Passani *et al.*, 2009). In preclinical research H_3 receptor occupancy studies are conducted to assess *in vivo* binding of novel H_3 receptor antagonists; importantly, these studies can establish the relationships of drug dose, blood exposure level and efficacy to H_3 receptor occupancy.

To date, studies of receptor occupancy by H_3 receptor ligands have employed *ex vivo* binding approaches, where *in vivo* treatments with the test compound are subsequently followed by *in vitro* radioligand binding analysis of either sections or homogenates of excised brain tissue. H_3 receptor occupancy by the compound is then quantified as the

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reduction in H_3 receptor radioligand binding in comparison with vehicle-treated controls. The *ex vivo* binding method has been used extensively by academic and industrial investigators to assess the relationship between dose and blood levels of various H_3 receptor ligands and receptor occupancy (Taylor *et al.*, 1992; Barnes *et al.*, 1993; Tedford *et al.*, 1995; Mochizuki *et al.*, 1996; Yates *et al.*, 1999; Barbier *et al.*, 2004; 2007; Vacondio *et al.*, 2004; Medhurst *et al.*, 2007a,b; Le *et al.*, 2008). More broadly, the method has been utilized to assess receptor occupancy by ligands of various other CNS targets, such as dopamine D2 receptors, the 5-hydroxytryptamine transporter and the benzodiazepine binding site of $GABA_A$ receptors (Paul, 1979; Schotte *et al.*, 1989; Barbier *et al.*, 2007). The *ex vivo* approach may offer certain advantages such as the opportunity for measuring receptor occupancy in discrete brain regions by using tissue section autoradiography. However, studies based on the *ex vivo* method may be confounded by dissociation of the compound administered from the target receptor during *ex vivo* tissue processing and/or radioligand binding assays. Significant dissociation of the compound may occur, depending upon the dissociation rate of the particular compound and the incubation time of the *in vitro* binding assay. In order to reduce compound dissociation during the *ex vivo* procedures, some investigators have employed shorter incubation times (Kapur *et al.*, 2001; Li *et al.*, 2003) and used brain sections (Schotte *et al.*, 1989; 1993), rather than membrane preparations, for the *in vitro* binding assays. However, *in vitro* binding assay incubation times must be long enough to yield adequate radioligand binding signal-to-noise, and therefore incubation times are somewhat limited by the association rate of the specific radioligand and the density of the target receptor.

An alternative to the *ex vivo* binding method is herein referred to as the *in vivo* method. The *in vivo* approach is akin to positron emission tomography (PET) and single photon emission computed tomography (SPECT) imaging, in that both the test compound and an appropriate radiotracer are administered systemically and compete for target receptor occupancy *in vivo*. Following the *in vivo* treatments, radiotracer levels in the isolated brain region of interest are determined by scintillation counting. Similar to PET/SPECT imaging, receptor occupancy by the test compound is quantified as the reduction in radiotracer levels in this region, in comparison with vehicle-treated controls. This method has been employed to assess receptor occupancy by ligands of various CNS targets, including those mentioned previously with respect to the *ex vivo* method (Stockmeier *et al.*, 1993; Scheffel *et al.*, 1994; Li *et al.*, 2006). However, due to the lack of a suitable H_3 receptor occupancy radiotracer, receptor occupancy studies for H_3 receptor ligands have not been conducted in this manner. In preclinical evaluations, previously described H_3 receptor radiotracers, such as [^{123}I]-iodoproxyfan, [^{18}F]-fluoroproxyfan, [^{18}F]-VUF 5000 and [^{11}C]-JNJ-10181457 (Windhorst *et al.*, 1999a,b; Airaksinen *et al.*, 2006; Funaki *et al.*, 2007), were found to be unsuitable candidates due to inadequate brain penetration or high levels of non-specific binding, which precluded a specific *in vivo* H_3 receptor occupancy signal.

The objectives of the present study were to evaluate the H_3 receptor antagonist radioligand [^{3}H]-A-349821 as an *in vivo* radiotracer for preclinical H_3 receptor occupancy studies. As described previously, A-349821 is a highly potent and selective H_3 receptor antagonist/inverse agonist, with favourable pharmacokinetic properties, that penetrates the brain *in vivo* to elicit pharmacological and behavioural responses, including procognitive effects in the five-trial inhibitory avoidance response in spontaneously hypertensive rat (SHR) pups (Esbenshade *et al.*, 2004; 2005). Further, radiolabelled A-349821 ([^{3}H]-A-349821, 22–36 Ci·mmol^{-1}) is a useful radioligand for *in vitro* studies of H_3 receptor pharmacology (Witte *et al.*, 2006). Because of these favourable pharmacological properties, [^{3}H]-A-349821 seemed a suitable candidate as a radiotracer for *in vivo* H_3 receptor occupancy studies. In initial *in vivo* studies with rats, [^{3}H]-A-349821 did indeed exhibit specific H_3 receptor occupancy in the cerebral cortex. To evaluate [^{3}H]-A-349821 further as an *in vivo* radiotracer, we used it to determine the fraction of H_3 receptors occupied by unlabelled H_3 receptor antagonists, so that this could be related to their blood levels and efficacy in the five-trial inhibitory avoidance response model. [^{3}H]-A-349821 proved to be a suitable *in vivo* radiotracer in a model that provided valid measurements of receptor occupancy by procognitive H_3 receptor antagonists.

Methods

Animals

Adult male Sprague-Dawley rats for receptor occupancy studies were obtained from Charles River Laboratories (Wilmington, MA, USA) and housed at Abbott Laboratories until use approximately 1 week later (at approximately 230–250 g). Male SHR pups for repeated acquisition avoidance studies were obtained from Harlan (Indianapolis, IN, USA) 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 2 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). All rats were housed under conditions of 12 h lights on/12 h lights off (on at 06 h 00 min), with food and water available *ad libitum*; experiments were carried out during the light phase. 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; facilities were accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care.

[^{3}H]-A-349821 autoradiography

In vitro autoradiography of [^{3}H]-A-349821 binding was carried out with sagittal sections of the rat brain. Whole brains from male Sprague-Dawley rats were frozen in 2-methylbutane at –35 to –40°C, and 20 µm sections were cut sagittally with a Jung Frigocut 2800N cryostat and thaw-mounted onto Superfrost Plus glass microscope slides. For [^{3}H]-A-349821 binding assays, the brain sections were incubated with approximately 1 nmol·L^{-1} [^{3}H]-A-349821 for 60 min at room temperature

(22°C), in assay buffer comprising 50 mmol·L^{-1} Tris-HCl, pH 7.4, 5 mmol·L^{-1} EDTA and 0.1% bovine serum albumin (BSA). Non-specific [^{3}H]-A-349821 binding was determined with adjacent brain sections, in the presence of 10 μmol·L^{-1} thioperamide. Following incubations, the assay buffers were aspirated, and the slides were rinsed three times for 20 s in cold 50 mmol·L^{-1} Tris-HCl, pH 7.4. Finally, the slides were quickly rinsed once in cold distilled water and subsequently dried. For autoradiography, Fugi Phosphor Imaging Plates were exposed to the slides for approximately 6 weeks, and images were acquired using the Storm PhosphorImaging system.

Cell membrane preparations

Cell membranes were prepared from Sprague-Dawley rat brain tissues as described previously (Witte *et al.*, 2006). Tissues were homogenized, using an Ultra-Turrax T25 homogenizer, in 50 mmol·L^{-1} Tris-HCl, pH 7.4, containing 5 mmol·L^{-1} EDTA (TE buffer) and protease inhibitors (1 mmol·L^{-1} benzamidine, 2 μg·mL^{-1} aprotinin, 1 μg·mL^{-1} leupeptin and 1 μg·mL^{-1} pepstatin). Homogenates were centrifuged at 41 000× *g* for 20 min at 4°C, and the resulting pellets were similarly homogenized and centrifuged once again. The final pellets were suspended in 6.25 mL TE buffer·g^{-1} wet weight pellet. Membrane suspensions were aliquoted, frozen by liquid nitrogen and stored at –80°C until use in radioligand binding assays.

Radioligand binding assays – competition and saturation studies

Competition binding assays with the H_3 receptor radioligand [^{3}H]-N-α-methylhistamine ([^{3}H]-NAMH) were performed as previously described (Esbenshade and Hancock, 2000), using cell membranes prepared from brain tissues of Sprague-Dawley rats. Stored membranes were thawed, diluted and re-homogenized with 50 mmol·L^{-1} Tris-HCl, pH 7.4, containing 5 mmol·L^{-1} EDTA (assay buffer). Membranes, equivalent to ~100 μg membrane protein per microplate well, were incubated with approximately 0.6 nmol·L^{-1} [^{3}H]-NAMH and various concentrations of H_3 receptor antagonists in a total volume of 0.5 mL. Non-specific [^{3}H]-NAMH binding was defined with 10 μmol·L^{-1} thioperamide. Assay incubations were 30 min at 25°C, and were terminated by vacuum filtration through polyethylenimine (0.3%) pre-soaked MultiScreen FB Harvest Plates, followed by extensive washing with cold 50 mmol·L^{-1} Tris-HCl, pH 7.4. The amount of bound [^{3}H]-NAMH was determined by the TopCount liquid scintillation analyser. Competition binding data were analysed by non-linear regression to determine the IC_{50} values for inhibition of specific [^{3}H]-NAMH binding, from which equilibrium inhibition constant (K_i) values were calculated using the Cheng-Prusoff equation.

The methods utilized for [^{3}H]-A-349821 saturation binding assays were similar, with the exceptions that the assay buffer contained 0.1% BSA and the incubation time was 60 min, as described previously (Witte *et al.*, 2006). Saturation binding assay incubations contained a range of [^{3}H]-A-349821 concentrations, and the [^{3}H]-A-349821 K_D and binding capacity (fmol·mg^{-1} protein) were determined from non-linear regression with GraphPad Prism (San Diego, CA, USA) software. Membrane protein concentrations were determined using the bicinchoninic method and BSA as standard (Smith *et al.*, 1985).

Receptor occupancy studies

In these studies, *in vivo* cerebral cortical H_3 receptor occupancy by [^{3}H]-A-349821 was reflected by the differences in [^{3}H]-A-349821 levels in cerebral cortex and cerebellum, a brain region with comparatively low levels of H_3 receptor. The indicated doses of [^{3}H]-A-349821 were administered by tail vein injection, in a volume of 1 mL·kg^{-1}, to male Sprague-Dawley rats weighing approximately 230–250 g. At the times indicated post-dosing, the rats were anaesthetized with CO_2 and decapitated; cerebral cortex and cerebellum tissues were isolated and frozen separately on dry ice. Later, the brain tissues were thawed, weighed and dissolved in Solvable for liquid scintillation counting to determine [^{3}H]-A-349821 levels (mg^{-1} tissue) in the cortex and cerebellum. Average [^{3}H]-A-349821 tissue levels were expressed primarily as disintegrations per min (DPM) ·mg^{-1} tissue (±SEM). For the saturation study of *in vivo* [^{3}H]-A-349821 H_3 receptor occupancy, [^{3}H]A-349821 was isotopically diluted with unlabelled A-349821 in order to achieve the highest doses used. In the inhibition studies, various doses of unlabelled H_3 receptor antagonists were administered i.p. in a volume of 1 mL·kg^{-1}, and [^{3}H]-A-349821 (1.5 μg·kg^{-1}) was subsequently administered 30 min later. After an additional 30 min post-dosing, blood and brain tissues were collected for quantification of unlabelled test compound and [^{3}H]-A-349821 levels respectively. Details of the data analysis are described in a separate section below.

Pharmacokinetic analysis

Terminal blood samples were collected in the behavioural and receptor occupancy studies to determine antagonist exposure levels. For the five-trial inhibitory avoidance studies, blood samples were obtained via cardiac puncture immediately after behavioural testing, approximately 40 min post-dosing. For the receptor occupancy studies, blood samples were obtained via cardiac puncture immediately prior to isolating brain tissues, 60 min post-dosing. The compounds were selectively removed from blood samples using protein precipitation with acetonitrile at neutral pH. The samples were vortexed vigorously followed by centrifugation. The resulting supernatants were 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, compounds 1 and 2, and their internal standards were separated from coextracted contaminants on a 50 mm × 3 mm × 5 mm 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^{-1} with a 25 μL injection. Compounds were quantified using MRM detection with a turbo ionspray source on a mass spectrometer. The limits of measurement were approximately 0.5, 0.05 and 0.5 ng·mL^{-1} for ABT-239, compound 1 and compound 2 respectively.

Five-trial inhibitory avoidance studies

Spontaneously hypertensive rat pups were trained in a five-trial, repeated acquisition avoidance response with compo-

nents of attention and impulsiveness as previously described (Fox *et al.*, 2002a). Briefly, pups were trained to avoid a mild foot shock (0.1 mA, 1 s duration) delivered upon transfer from a brightly illuminated to a darkened compartment (which they normally prefer) of a computer-controlled Gemini inhibitory avoidance system. After the first trial, the pup was removed and returned to its home cage and the transfer latency was noted. After approximately 60 s, the same pup was again placed in the brightly illuminated compartment, and the training process was repeated; a total of five trials were conducted in this manner. A criterion time of 60 and 180 s applied for the first and second through fifth trials respectively. An H_3 receptor antagonist or saline vehicle was injected s.c. 30 min prior to the first trial. A V-212 oscilloscope (20 MHz) and a 100-kOhm resistor were used frequently to ensure correct calibration of the equipment in producing this relatively mild foot shock. Pups were not habituated to the avoidance apparatus before the first trial to avoid potentially confounding latent inhibitory effects. Each experimental group was equally represented among each litter ($n = 12$ per group), and the experimenter was blinded to treatment. Non-parametric Kruskal–Wallis and individual Mann–Whitney *U* tests were used to compare performance; $P < 0.05$ was considered significant. All analyses were performed using Statview 5.0 or JMP 5.01 for Windows (both from SAS Institute, Cary, NC, USA). Control experiments with SHR pups, described by Fox *et al.* (2005), were conducted to demonstrate that treatments with the H_3 receptor antagonists have no effect on foot shock sensitivity threshold.

Data analysis – receptor occupancy studies

In these studies, *in vivo* cerebral cortical H_3 receptor occupancy by [^{3}H]-A-349821 was reflected by the differences in [^{3}H]-A-349821 levels in cerebral cortex and cerebellum, a brain region with comparatively low levels of H_3 receptors. For the saturation study of *in vivo* [^{3}H]-A-349821 H_3 receptor occupancy, differences between cortical and cerebellar [^{3}H]-A-349821 levels were determined for each individual rat, and the cortical [^{3}H]-A-349821 H_3 receptor occupancy thus determined was expressed as fmol·mg^{-1} tissue. The average [^{3}H]-A-349821 H_3 receptor occupancy values (±SEM) were plotted versus [^{3}H]-A-349821 dose, and the data were analysed using the equation for one site binding to determine the maximum capacity and potency of [^{3}H]-A-349821 for occupying cortical H_3 receptors. For inhibition studies, H_3 receptor occupancy by unlabelled antagonists was reflected as a decrease in the cortex : cerebellum distribution ratio of [^{3}H]-A-349821, relative to control; % H_3 receptor occupancy was calculated from the distribution ratio of [^{3}H]-A-349821 for each individual rat using the following formula, where cortex and cerebellum are abbreviated ctx and cb respectively.

$$\%\ \text{receptor occupancy} = 100 - \frac{\text{ctx:cb ratio} - 1}{\text{average control ctx:cb ratio} - 1} \times 100$$

The average % receptor occupancy values (±SEM) were plotted versus the antagonist dose, and an ED_{50} for receptor occupancy was determined by non-linear regression using the equation for sigmoidal dose–response curves. Similarly, the % receptor occupancy values from individual rats were plotted versus the corresponding antagonist blood levels, and an ED_{50} was determined in terms of ng·mL^{-1} blood. In all cases, GraphPad Prism software was used for non-linear regression analysis.

Chemicals and materials

[^{3}H]-A-349821 ([dimethylpyrrolidinyl-3,4-$^3H_2$]{4′-[3-(2*R*,5*R*-dimethyl-pyrrolidin-l-yl)-propoxy]-biphenyl-4-yl}-morpholin-4-yl-methanone); 24–36 Ci·mmol^{-1}) was synthesized at Abbott Laboratories as described previously (Witte *et al.*, 2006). The Abbott H_3 receptor antagonists ABT-239 (Esbenshade *et al.*, 2005) and compounds 1 and 2 were synthesized at Abbott Laboratories. Compound 1 is 2-{6-[2-((R))-2-methyl-pyrrolidin-1-yl)-ethyl]-naphthalen-2-yl}-2*H*-pyridazin-3-one (Black *et al.*, 2008) and compound 2 is 2-(2,7-dimethyl-pyrazolo[1,5-a]pyrimidin-6-yl)-6-[2-((R)-2-methyl-pyrrolidin-1-yl)-ethyl]-quinoline (Altenbach *et al.*, 2007). The typical H_3 receptor antagonist thioperamide was purchased from Tocris-Cookson (Ellisville, MO, USA). [^{3}H]-NAMH (~80 Ci·mmol^{-1}) was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA, USA). Other chemicals and reagents were from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise indicated.

The Jung Frigocut 2800N cryostat was obtained from Leica (Bannockburn, IL, USA); Superfrost Plus glass microscope slides, VWR International (West Chester, PA, USA); Fugi Phosphor Imaging Plates, Fugi (Tokyo, Japan); Storm PhosphorImaging system, Molecular Dynamics (Sunnyvale, CA, USA); Ultra-Turrax T25 Homogenizer, IKA (Wilmington, NC, USA); MultiScreen FB Harvest Plates, Millipore (Billerica, MA, USA); TopCount liquid scintillation analyser and Solvable, PerkinElmer; mass spectrometer, PE Sciex Applied Biosystems (Foster City, CA, USA); V-212 oscilloscope, Hitachi (San Jose, CA, USA); Gemini inhibitory avoidance system, San Diego Instruments (San Diego, CA, USA).

Results

In vitro *[3H]-A-349821 binding: comparisons of rat cerebellum and cerebral cortex*

In vitro [^{3}H]-A-349821 binding to sites in rat cerebellum was very low in comparison with cerebral cortex. As assessed by autoradiography with sagittal rat brain sections, [^{3}H]-A-349821 binding in cerebellum was very low and indistinguishable from non-specific radioligand binding determined in the presence of 10 μmol·L^{-1} thioperamide, whereas specific [^{3}H]-A-349821 binding to H_3 receptors in cortex and certain other brain regions was comparatively high (Figure 1). The regional distribution of specific [^{3}H]-A-349821 binding was consistent with H_3 receptor localization in rat brain (Pollard *et al.*, 1993; Pillot *et al.*, 2002). In saturation binding assays with membranes prepared from rat cerebellum and cortex, the average [^{3}H]-A-349821 binding capacities (±SEM) were 16.5 ± 2.4 and 269 ± 25.1 fmol·mg^{-1} protein ($n = 3$), respectively, indicating that H_3 receptor expression in cerebellum was approximately 5% of that in cerebral cortex. The average

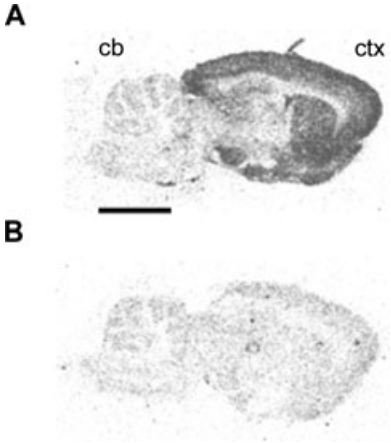

Figure 1 *In vitro* autoradiography of [^{3}H]-A-349821 binding to sagittal sections of rat brain. Brain sections were incubated with [^{3}H]-A-349821 (1 nmol·L^{-1}) for 1 h and subsequently washed. The images shown are from phosphor imaging plates exposed to brain sections for approximately 6 weeks and are representative of total [^{3}H]-A-349821 binding (A), and non-specific [^{3}H]-A-349821 binding determined in the presence of 10 µmol·L^{-1} thioperamide (B). Cortex (ctx) and cerebellum (cb) are indicated. The scale bar represents 5 mm.

negative logarithm of the dissociation constant values (±SEM) for [^{3}H]-A-349821 were 9.66 ± 0.15 and 9.29 ± 0.02 (n = 3).

Biodistribution of [^{3}H]-A-349821: comparisons of rat cerebellum and cerebral cortex

[^{3}H]-A-349821, 1.5 µg·kg^{-1}, given to male rats, penetrated into the brain and subsequently cleared more rapidly from the cerebellum than from the cerebral cortex. Fifteen minutes after the i.v. dose of 1.5 µg·kg^{-1} (approximately 21 µCi per 250 g rat), average cortical [^{3}H]-A-349821 levels (±SEM) were 21.1 ± 0.8 DPM·mg^{-1} tissue (n = 4), equivalent to 0.046 ± 0.003% of the dose·g^{-1} cortex tissue. To compare tissue clearances, cerebellar and cortical [^{3}H]-A-349821 levels were determined 15, 30, 45, 60 and 90 min post-dosing. [^{3}H]-A-349821 levels in cerebellum declined rapidly during the initial 30 min after dosing, while [^{3}H]-A-349821 levels in cortex were sustained for 30 min and then declined rapidly thereafter (Figure 2A). The regional differences in [^{3}H]-A-349821 levels were greatest 30–45 min after dosing when [^{3}H]-A-349821 levels were approximately twofold greater in cortex than cerebellum (Figure 2A,B).

In vivo *[^{3}H]-A-349821 receptor occupancy: saturation study*

In order to characterize *in vivo* [^{3}H]-A-349821 H_3 receptor binding, various amounts, ranging from 0.5 to 120 µg·kg^{-1}, of [^{3}H]-A-349821 were given, and differences between cortical and cerebellar [^{3}H]-A-349821 levels were used to define cortical H_3 receptor occupancies by the radiotracer. With increasing [^{3}H]-A-349821 dose, cerebellar [^{3}H]-A-349821 levels increased linearly and were consistently less than the respective cortical [^{3}H]-A-349821 levels (Figure 3A). To obtain values for specific [^{3}H]-A-349821 H_3 receptor binding, cerebellar levels of the radiotracer were subtracted from the respective

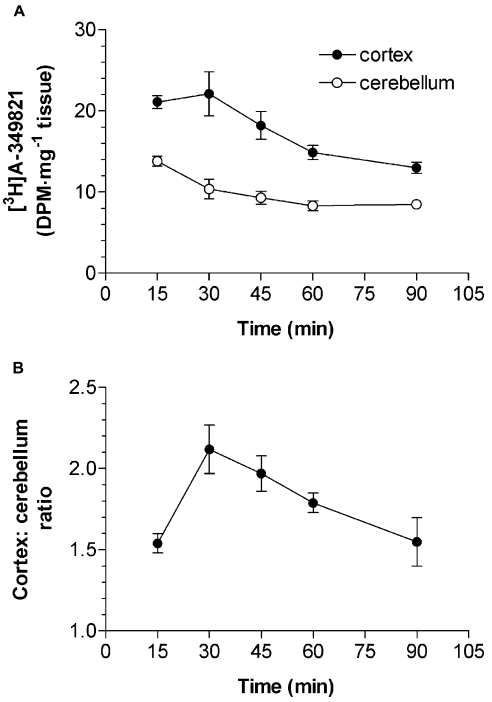

Figure 2 Biodistribution of [^{3}H]-A-349821 in rat cerebral cortex and cerebellum. [^{3}H]-A-349821 (1.5 µg·kg^{-1}) was administered i.v., and brain tissues were collected 15, 30, 45, 60 or 90 min post-dosing. Cortical and cerebellar [^{3}H]-A-349821 levels were determined by scintillation counting as described in Methods. The biodistribution of [^{3}H]-A-349821 was plotted as DPM·mg^{-1} tissue (A), and as [^{3}H]-A-349821 cortex : cerebellum distribution ratios (B). Data shown are the average from four rats per experimental group (±SEM).

cortical levels. With increasing doses of [^{3}H]-A-349821, cortical [^{3}H]-A-349821 H_3 receptor binding as thus defined increased hyperbolically, approaching a maximum at the highest doses (Figure 3A,B). From analysis with the equation for one site binding, the [^{3}H]-A-349821 H_3 receptor binding capacity was 11.5 fmol·mg^{-1} cortex tissue, and the ED_{50} for H_3 receptor occupancy was 66 µg·kg^{-1}. The binding capacity of [^{3}H]-A-349821 *in vivo* was consistent with that determined *in vitro* in rat cortical membrane determinations, which was equivalent to 10.2 ± 1.0 fmol·mg^{-1} cortex tissue (n = 3).

In vivo *[^{3}H]-A-349821 receptor occupancy: inhibition studies*

Cortical [^{3}H]-A-349821 H_3 receptor occupancy was inhibited by thioperamide, ABT-239 and other structurally diverse H_3 receptor antagonists (see Figure 4 for chemical structures). In vehicle-treated control rats, cortical [^{3}H]-A-349821 H_3 receptor occupancy was 0.15 fmol·mg^{-1} tissue, which was equivalent to approximately 1.5% of the total number of cortical H_3 receptors. In rats pretreated with maximally effective antagonist doses, [^{3}H]-A-349821 levels in cortex and cerebellum were indistinguishable, indicating that the antagonists completely inhibited [^{3}H]-A-349821 H_3 receptor occupancy. At these antagonist doses, the average cortical and cerebellar levels (±SEM) were 14.9 ± 0.7 and 14.0 ± 0.7 DPM·mg^{-1} tissue (n = 23) respectively. Also, cerebellar [^{3}H]-A-349821 levels were unaffected by antagonist treatments; for example, the cerebellar [^{3}H]-A-349821 levels in rats treated with vehicle and the

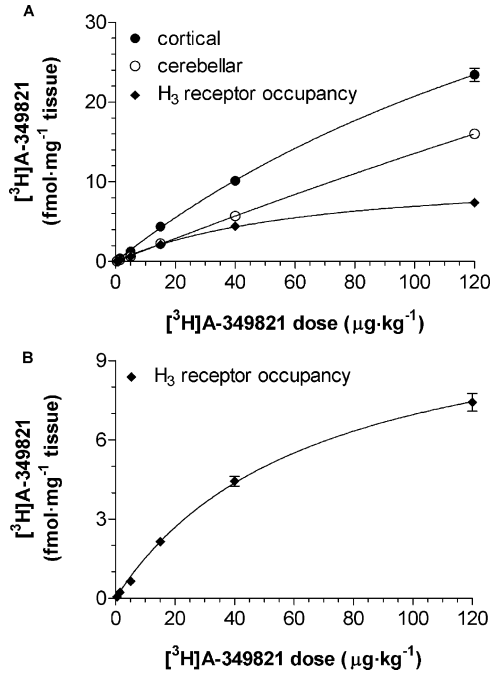

Figure 3 *In vivo* H_3 receptor occupancy by [^{3}H]-A-349821 in rat cerebral cortex. Various doses of [^{3}H]-A-349821, ranging from 0.5 to 120 μg·kg^{-1}, were administered i.v., and brain tissues were collected 30 min post-dosing. Cortical and cerebellar [^{3}H]-A-349821 levels were determined by scintillation counting as described in Methods. Regional [^{3}H]-A-349821 levels and [^{3}H]-A-349821 H_3 receptor occupancies, defined as the difference between cortical and cerebellar [^{3}H]-A-349821 levels, were plotted as fmol·mg^{-1} tissue (A). The [^{3}H]-A-349821 H_3 receptor occupancy plot is also shown rescaled, by itself (B). Data shown are the average from three rats per experimental group (±SEM), and were analysed using the equation for one site binding.

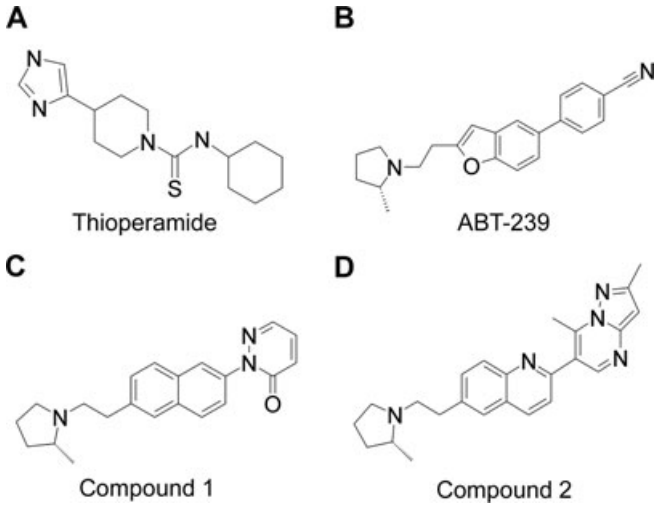

Figure 4 Structures of the H_3 receptor antagonists tested for *in vivo* H_3 receptor occupancy: thioperamide (A), ABT-239 (B), compound 1 (C) and compound 2 (D).

highest antagonist doses were 13.7 ± 0.8 and 14.0 ± 0.7 DPM·mg^{-1} tissue (n = 23) respectively. With the low levels of radiotracer receptor occupancy employed here, the degree of inhibition of radiotracer receptor occupancy by the unlabelled ligand linearly reflects the degree of receptor occupancy by that ligand (Farde *et al.*, 1992; Hume *et al.*, 1998).

Thioperamide, ABT-239 and compounds 1 and 2 occupied cortical H_3 receptors in a dose-dependent manner, and antagonist potencies were determined from dose-occupancy studies (Figure 5A–D). ED_{50} values (doses producing 50% cortical H_3 receptor occupancy) ranged from 0.15 to 2.7 mg·kg^{-1} (Table 1), and doses producing greater than 90% occupancy ranged from 1.0 to 10 mg·kg^{-1}. In these studies, H_3 receptor antagonist levels in terminal blood samples were determined in order to relate compound exposure levels and H_3 receptor occupancy. As anticipated, the amounts of antagonist in the blood and H_3 receptor occupancies were well correlated (Figure 5E–H). For ABT-239, compound 1, and compound 2, the ED_{50} values derived from concentration in blood-occupancy plots ranged from 14 to 24 ng·mL^{-1}; for thioperamide the ED_{50} value was 230 ng·mL^{-1} (Table 1). Factoring in the plasma protein binding (% bound) of ABT-239 (94%), compound 1 (29%), and compound 2 (70%), the ED_{50} values in terms of unbound free blood concentrations ranged from approximately 3 to 50 nmol·L^{-1} and differed by no more than ~10-fold from the respective antagonist K_i values for the rat cortical H_3 receptor, which spanned from 1.6 to 8.1 nmol·L^{-1} (Table 1). In a limited comparison of antagonist H_3 receptor occupancy in distinct brain regions, the ABT-239 potencies for occupancy in hippocampal and cortical regions were similar (data not shown).

H_3 receptor antagonist receptor occupancy: relationships with behavioural efficacy

The H_3 receptor antagonist blood levels associated with cortical H_3 receptor occupancy were compared with the respective blood levels associated with procognitive efficacy in a behavioural model of attention deficits and impulsiveness, the five-trial inhibitory avoidance response by SHR rat pups (Fox *et al.*, 2002a). These comparisons were made for ABT-239, compound 1 and compound 2, each of which is at least 100-fold selective for the H_3 receptor versus the other histamine receptor subtypes and several other off-target receptors and sites (Esbenshade *et al.*, 2005; Altenbach *et al.*, 2007; T. A. Esbenshade *et al.*, unpubl. obs.) In the five-trial inhibitory avoidance response, each antagonist was fully effective in comparison with ciproxifan, a reference H_3 receptor antagonist (Fox *et al.*, 2005; Altenbach *et al.*, 2007; K. E. Browman *et al.*, unpubl. obs.); at maximally effective doses for each antagonist, the average improvement over control for cumulative transfer latencies was approximately 200%. Table 2 shows the antagonist blood levels observed within the range of behaviourally effective doses and the antagonist blood levels at the ED_{25}, ED_{50} and ED_{75} for receptor occupancy, as deduced from the blood level-occupancy curves. Effective blood levels in the SHR rat pups corresponded to varying ranges of cortical H_3 receptor occupancy that differed from antagonist to antagonist. For compound 1, blood levels in SHR rat pups at the lowest effective doses corresponded to <10% cortical H_3 receptor occupancy; while for ABT-239 and compound 2, blood levels at the lowest effective doses corresponded to approximately 25 and 50% H_3 receptor occupancy respectively. At the highest effective doses, the blood levels of

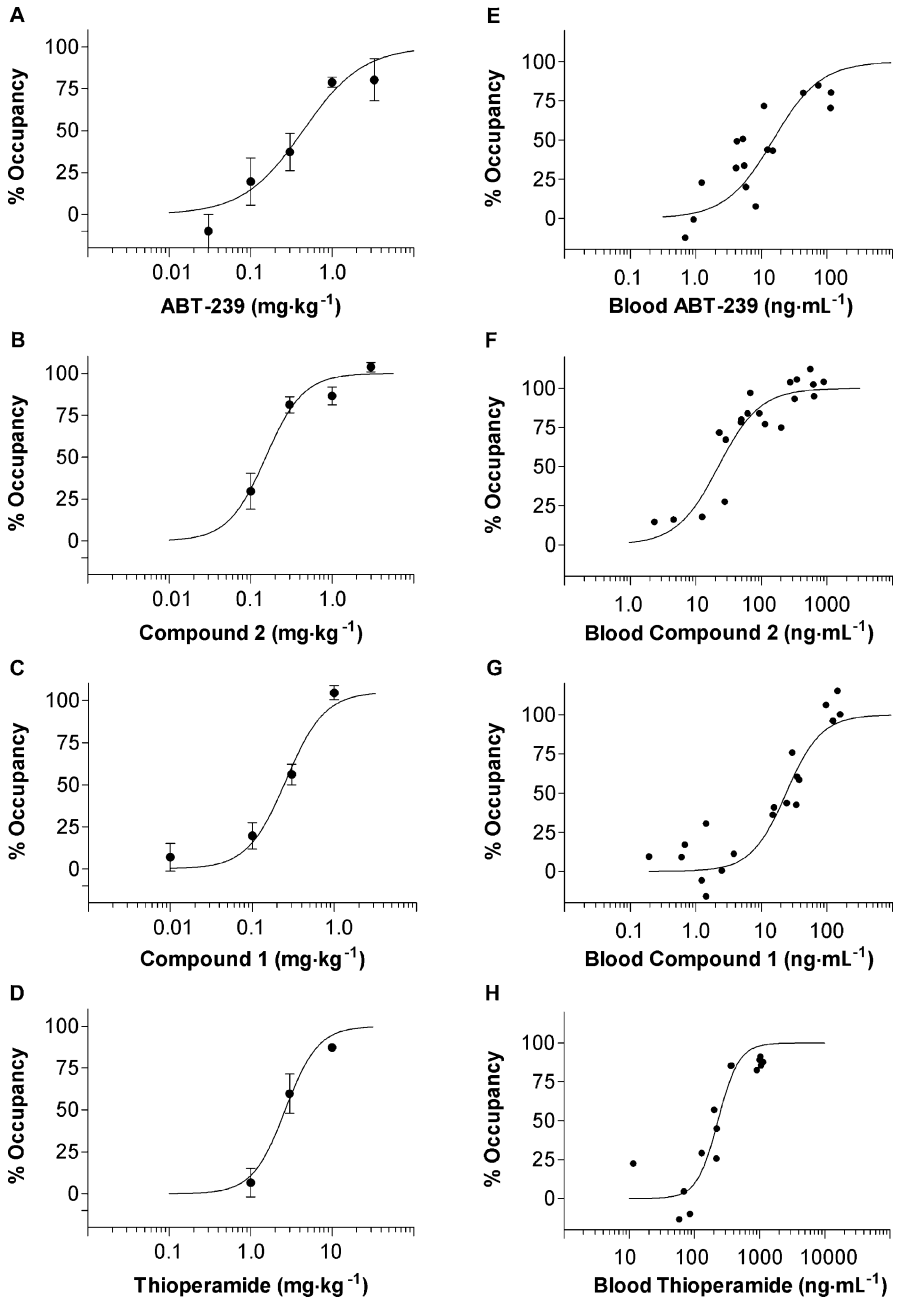

Figure 5 *In vivo* H_3 receptor occupancies by antagonists ABT-239 (A,E), compound 2 (B,F), compound 1 (C,G) and thioperamide (D,H). Various doses of the H_3 receptor antagonists were administered i.p. After 30 min pretreatment, [^{3}H]-A-349821 (1.5 μg·kg^{-1}) was administered i.v., and brain tissues were collected 30 min post-dosing. Cortical and cerebellar [^{3}H]-A-349821 levels were determined, and the degree of H_3 receptor occupancy by antagonist was calculated from [^{3}H]-A-349821 cortex : cerebellum distribution ratios as described in Methods. The % H_3 receptor occupancy data were plotted versus antagonist dose (A–D) and antagonist levels in terminal blood samples (E–H). The data shown in the dose-occupancy plots are the average from five rats per treatment group (±SEM), whereas the data shown in the blood level-occupancy plots are from each individual rat. Data were analysed using the equation for sigmoidal dose–response curves.

compound 1 corresponded to approximately 15% H_3 receptor occupancy; whereas the blood levels of ABT-239 and compound 2 corresponded to approximately 80% occupancy. Thioperamide blood levels were not determined for efficacy in the five-trial inhibitory avoidance studies.

Discussion

The results from these studies indicate that [^{3}H]-A-349821 is a valid *in vivo* radiotracer for preclincical H_3 receptor occupancy studies. In these studies, cerebellar [^{3}H]-A-349821 levels could serve as an internal measure of free and non-specifically bound radiotracer, as H_3 receptor levels are very low in cerebellum, and as [^{3}H]-A-349821 did indeed clear more rapidly from cerebellum than the cerebral cortex. Using the difference between cortical and cerebellar [^{3}H]-A-349821 levels to define [^{3}H]-A-349821 H_3 receptor occupancy in cerebral cortex, we demonstrated that the *in vivo* H_3 receptor binding by this radiotracer was saturable, with a binding capacity consistent with the [^{3}H]-A-349821 binding capacity deter-

Table 1 H_3 receptor antagonist potencies from *in vivo* receptor occupancy and *in vitro* competition binding assays

Compound	*Rat cortical H_3 receptor occupancy*			*Rat cortical H_3 receptor binding*
	ED_{50} (mg·kg^{-1})	*ED_{50} (ng·mL^{-1})*	*ED_{50} (nmol·L^{-1})*	*K_i (nmol·L^{-1}) (95% CL)*
Compound 2	0.15	22	17	1.58 (1.01–2.46)
Compound 1	0.26	24	51	8.07 (6.55–10.0)
ABT-239	0.44	14	2.7	3.30 (2.83–3.83)
Thioperamide	2.7	230	NA[a]	6.31 (4.66–8.57)

Antagonist ED_{50} values in terms of mg·kg^{-1} and total ng·mL^{-1} blood were determined by non-linear regression analysis of antagonist dose-occupancy and blood level-occupancy data respectively. Antagonist ED_{50} values were also expressed as nmol·L^{-1} concentrations of free antagonist in blood, accounting for plasma protein binding by these compounds. Antagonist K_i values obtained from competition binding assays are the geometric mean from at least three independent assays and are shown with 95% CL.
[a]Data not available because plasma protein binding unknown.
CL, confidence limits; NA, not available.

Table 2 H_3 receptor antagonist blood levels corresponding to behavioural efficacy and rat cortical H_3 receptor occupancy

Compound	*Five-trial inhibitory avoidance*	*Rat cortical H_3 receptor occupancy*		
	Efficacy range (ng·mL^{-1})	*ED_{25} (ng·mL^{-1})*	*ED_{50} (ng·mL^{-1})*	*ED_{75} (ng·mL^{-1})*
Compound 2	19–51[a]	9.9	22	50
Compound 1	0.73–7.9	12	24	47
ABT-239	4.7–56[b]	4.2	14	48

Antagonist efficacy ranges were determined as the blood levels at the lowest and highest antagonist doses effective in the five-trial inhibitory avoidance test; the values are the average from at least nine rats per experimental group. The antagonist ED_{25}, ED_{50} and ED_{75} values for rat cortical H_3 receptor occupancy were derived from the respective blood level-occupancy curve.
[a]From Altenbach *et al.* (2007).
[b]From Fox *et al.* (2005).

mined *in vitro* with rat cerebral cortical membranes. Further, upon pretreatment with unlabelled H_3 receptor antagonists, cortical [^{3}H]-A-349821 binding was specifically inhibited. Antagonist potencies for inhibition of *in vivo* [^{3}H]-A-349821 H_3 receptor binding were consistent with the respective antagonist potencies in an *in vivo* H_3 receptor pharmacological model, blockade of R-α-methylhistamine (RAMH)-induced mouse dipsogenia (Fox *et al.*, 2002b; 2005). The RAMH-induced dipsogenic response is mediated by H_3 receptors located in the CNS (Lecklin *et al.*, 1998; Fox *et al.*, 2002b); hence, cerebral cortical H_3 receptor occupancy by these antagonists was observed at centrally effective doses. Further, cortical H_3 receptor occupancy was closely related to procognitive efficacy in the five-trial inhibitory avoidance model, as discussed below.

To our knowledge, [^{3}H]-A-349821 is the first H_3 receptor radioligand successfully employed as an *in vivo* radiotracer for receptor occupancy studies. Successful *in vivo* radiotracers satisfy several criteria that generally include high affinity and displaceable binding at the target receptor, high selectivity for the target receptor versus off-target sites, and low binding to other non-specific sites, for example membrane lipids. Additionally, successful *in vivo* radiotracers are resistant to metabolism that produces confounding metabolites and, for the H_3 receptor and other CNS target receptors, penetrate into the brain at low doses. While *in vitro* pharmacological studies of [^{3}H]-A-349821 and the unlabelled ligand have been described previously (Esbenshade *et al.*, 2004; Witte *et al.*, 2006), and the [^{3}H]-A-349821 binding specificity in rat brain sections has been shown here, the metabolism of this compound has not yet been characterized. Pharmacokinetic studies with rat, by Esbenshade *et al.* (2005), indicated that less than 30% of A-349821 was cleared from the blood by either elimination or metabolism within the first 30 min following i.v. dosing. Drug metabolites are generally more polar than the parent compound and thus are less likely to penetrate into the brain. Consequently, metabolites of radiolabelled [^{3}H]-A-349821 may comprise a small fraction, if any, of the total radioactivity recovered from brain tissues in these receptor occupancy studies. In contrast to [^{3}H]-A-349821, previously examined H_3 receptor radioligands such as [^{123}I]-iodoproxyfan, [^{18}F]-fluoroproxyfan, [^{18}F]-VUF 5000 and [^{11}C]-JNJ-10181457 were found to be unsuitable as *in vivo* radiotracers (Windhorst *et al.*, 1999a,b; Airaksinen *et al.*, 2006; Funaki *et al.*, 2007). In biodistribution studies with rats, these unsuccessful H_3 receptor radiotracer candidates exhibited low brain penetration and/or high non-specific binding which precluded a specific *in vivo* H_3 receptor occupancy signal.

The *in vivo* H_3 receptor occupancy method employing [^{3}H]-A-349821 described in the present study enhances the preclinical characterization of potentially therapeutic H_3 receptor antagonists. Previously, receptor occupancy studies within the field of H_3 receptor research have utilized the *ex vivo* binding method, in which receptor occupancy by a particular compound is measured by *in vitro* radioligand binding assays. Should dissociation of the test compound during *ex vivo* procedures be significant, the results would underestimate the degree of *in vivo* H_3 receptor occupancy by this compound. For example, in a direct comparison of *in vivo* and *ex vivo* methods, the *ex vivo* method was much less

sensitive for measuring striatal dopamine D2 receptor occupancy by raclopride, owing to dissociation of raclopride from receptors during the *in vitro* binding assay (Kapur *et al.*, 2001). Similarly, the *ex vivo* binding method apparently underestimated the potency of ABT-239 for H_3 receptor occupancy (Le *et al.*, 2008); the ABT-239 ED_{50} was more than 10-fold higher compared with the ED_{50} obtained here. This discrepancy is possibly due to dissociation of ABT-239 from H_3 receptors during the *ex vivo* procedures, which included a 60 min incubation for [^{3}H]-NAMH binding. In contrast, the two H_3 receptor occupancy methods yielded very similar ED_{50} values for thioperamide (Taylor *et al.*, 1992; Yates *et al.*, 1999; Vacondio *et al.*, 2004), suggesting that thioperamide does not appreciably dissociate from H_3 receptors during *ex vivo* processing. Even though the confounding effects of test compound dissociation might be reduced by utilizing shorter binding assay incubations and brain sections rather than tissue homogenates (Schotte *et al.*, 1989; 1993; Kapur *et al.*, 2001; Li *et al.*, 2003), the *in vivo* [^{3}H]-A-349821 method remains an attractive approach for determining H_3 receptor occupancy. As this method is analogous to PET/SPECT imaging studies, the results obtained for potentially therapeutic H_3 receptor antagonists may be more applicable and beneficial to clinical development.

In our dose-occupancy studies of unlabelled H_3 receptor antagonists, free blood concentrations of the antagonist corresponding to 50% H_3 receptor occupancy did not differ greatly from the respective antagonist K_i values for the rat H_3 receptor, indicating that the free antagonist concentrations in blood were somewhat similar to free concentrations at cortical H_3 receptors. Likewise, for low affinity H_3 receptor antagonists not included in this report, correspondingly higher free blood concentrations were required for H_3 receptor occupancy. Similar relationships between free blood concentrations, binding affinities and CNS receptor occupancy have been obtained for other compounds, for instance selective CRF_1 antagonists (Li *et al.*, 2003).

The H_3 receptor occupancy observed for these antagonists was closely related to procognitive efficacy in the five-trial inhibitory avoidance response by SHR rat pups, a preclinical model of attention and impulsivity. The antagonist blood concentrations that corresponded to efficacy in the behavioural model also corresponded to cortical H_3 receptor occupancy, although the antagonists exhibited differing relationships between behavioural efficacy and levels of H_3 receptor occupancy. That is, at the lowest effective blood levels of the H_3 receptor antagonists, the corresponding H_3 receptor occupancy varied from compound to compound and ranged from less than 10% to approximately 50%. Likewise, at the highest effictive blood levels, the corresponding H_3 receptor occupancy also varied from compound to compound, ranging from approximately 15% to 80%. Medhurst *et al.* (2007a,b) have reported that, for various H_3 receptor antagonists including GSK-189254, efficacy in preclinical cognition models corresponded to 50% or more H_3 receptor occupancy. Our comparisons between H_3 receptor antagonist efficacy in five-trial inhibitory avoidance and H_3 receptor occupancy may be confounded by pharmacokinetic, pharmacodynamic or regional brain differences between the two models. A more rigorous examination of H_3 receptor occupancy, with respect to behavioural efficacy in the five-trial inhibitory avoidance model, would utilize SHR rat pups as subjects for occupancy studies along with various antagonist pretreatment times. However, our present results and interpretations were apparently not confounded by disparities in antagonist brain penetration in the behavioural and receptor occupancy models, as indicated by comparisons of antagonist brain levels (data not shown). Further, cerebral cortical H_3 receptor densities are similar in the SHR and Sprague-Dawley rats utilized in the respective models (Witte *et al.*, 2006; Shaw *et al.*, 2007).

In summary, these studies have conclusively demonstrated that the H_3 receptor antagonist radioligand [^{3}H]-A-349821 is useful as an *in vivo* radiotracer for determining receptor occupancy by unlabelled H_3 receptor antagonists. The observed H_3 receptor occupancy by these antagonists was related to the behavioural efficacy of these compounds in the five-trial inhibitory avoidance model. [^{3}H]-A-349821 is the first reported *in vivo* radiotracer successfully employed for H_3 receptor occupancy studies and will augment our preclinical characterization of H_3 receptor antagonists. Information gained from such receptor occupancy studies will importantly assist in planning and interpreting clinical studies of H_3 receptor antagonists and will facilitate the development of these compounds as potential drugs for treating cognitive deficits of neurological diseases. Additionally, these positive results with [^{3}H]-A-349821 demonstrate that radiotracer biodistribution studies in rat can be used to assess prospective H_3 receptor PET/SPECT ligands.

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Conflict of interest

We are all current employees of Abbott Laboratories, and all Abbott-related research presented in this research paper was conducted during our employment at Abbott Laboratories.

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