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European Journal of Pharmacology 487 (2004) 183–197
www.elsevier.com/locate/ejphar

Antiobesity effects of A-331440, a novel non-imidazole histamine H_3 receptor antagonist

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Received 4 September 2003; received in revised form 5 January 2004; accepted 13 January 2004

Abstract

Histamine affects homeostatic mechanisms, including food and water consumption, by acting on central nervous system (CNS) receptors. Presynaptic histamine H_3 receptors regulate release of histamine and other neurotransmitters, and histamine H_3 receptor antagonists enhance neurotransmitter release. A-331440 {4′ -[3-(3(*R*)-(dimethylamino)-pyrrolidin-1-yl)-propoxy]-biphenyl-4-carbonitrile} is a histamine H_3 receptor antagonist which binds potently and selectively to both human and rat histamine H_3 receptors ($K_i \leq 25$ nM). Mice were stabilized on a high-fat diet (45 kcal % lard) prior to 28-day oral b.i.d. dosing for measurement of obesity-related parameters. A-331440 administered at 0.5 mg/kg had no significant effect on weight, whereas 5 mg/kg decreased weight comparably to dexfenfluramine (10 mg/kg). A-331440 administered at 15 mg/kg reduced weight to a level comparable to mice on the low-fat diet. The two higher doses reduced body fat and the highest dose also normalized an insulin tolerance test. These data show that the histamine H_3 receptor antagonist, A-331440, has potential as an antiobesity agent.

Keywords: Antiobesity; Histamine H_3 receptor antagonist; Magnetic resonance imaging

1. Introduction

A large percentage of people worldwide (in excess of 50% in the US and Russia, for example) are overweight (Bonetta, 2001), with a body mass index (BMI) between 25 and 29.9 kg m^{-2} (Gumbiner and Battiwalla, 2002). Excess body weight has been linked to adverse cardiovascular events, diabetes and premature mortality (Leonhardt et al., 1999). Of greater concern are more severe cases of excess weight, manifested as frank obesity (BMI>30 kg m^{-2}), which is especially prevalent in the United States (over 90% of type 2 diabetics, for example; Gumbiner and Battiwalla, 2002). Estimates suggest that one quarter to one-third of the population is obese (Kuczmarski et al., 1994). Moreover, the disease incidence is increasing throughout the world (Martin et al., 2000).

Historically, antiobesity treatments have included aminergic stimulant drugs, like amphetamine, although efficacy may diminish over time and abuse liabilities have been recognized (Leonhardt et al., 1999). More recently, drugs acting through the serotonin system have been employed. The combination of dexfenfluramine and phentermine was highly efficacious in reducing body weight when part of a diet and weight control regimen (Clapham et al., 2001), but the combination was linked to adverse cardiovascular effects resulting in clinical abandonment of the fen–phen combination (Leonhardt et al., 1999; Clapham et al., 2001). Currently available therapeutic modalities include the lipase inhibitor, orlistat and the centrally acting inhibitor of serotonin and norepinephrine uptake, sibutramine (Hauner, 2001). However, the increasing prevalence of obesity has been predicted to lead to increasing drug therapy based on greater understanding of novel pharmacological principles (Hauner, 2001). As a result, there is a medical need for more efficacious treatment modalities for obesity (Clapham et al., 2001).

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doi:10.1016/j.ejphar.2004.01.015

Several recent animal studies have indicated potential for a novel molecular approach via modulation of activity of histaminergic systems in the central nervous system (CNS). For example, it is known that blockade of postsynaptic histamine H_1 receptors induces weight gain (Morimoto et al., 2001). Similarly, knockout mice lacking the histamine H_1 receptor tend to exhibit a phenotype of inadequate modulation by the leptin system of appetite and of increased food consumption (Morimoto et al., 1999). Blockade of histamine synthesis has also been shown to cause increased weight gain (Orthen-Gambill and Salomon, 1992), and obese Zucker rats have been shown to have low concentrations of hypothalamic histamine (Sakata et al., 1997). These results share the common theme that insufficient stimulation of postsynaptic CNS histamine H_1 receptors causes increased food consumption and/or weight gain. Conversely, several studies have shown the ability of histamine H_3 receptor antagonists to promote weight loss or prevent weight gain. The presumed mechanism of action would be the enhanced release of histamine from histaminergic terminals leading to stimulation of postsynaptic histamine H_1 receptors to reduce appetite. Thus, a presumed CNS mode of action is likely, although findings from recent in situ hybridization indicating the prominent expression of histamine H_3 receptor mRNA in brown adipose tissue (Karlstedt et al., 2001) would also support a potential non-CNS role in regulating thermogenesis through peripheral sites of action. The compounds used in the aforementioned studies include the histamine H_3 receptor antagonists thioperamide (Fig. 1, Itoh et al., 1999) and ciproxifan (Fig. 1, Bjenning et al., 2000a,b), as well as several compounds from Gliatech (Leonhardt et al., 1999; Yates et al., 2000). These compounds share a common imidazole substituent and may be viewed as antagonist analogs of the naturally occurring neurotransmitter, histamine or 4-imidazole ethylamine.

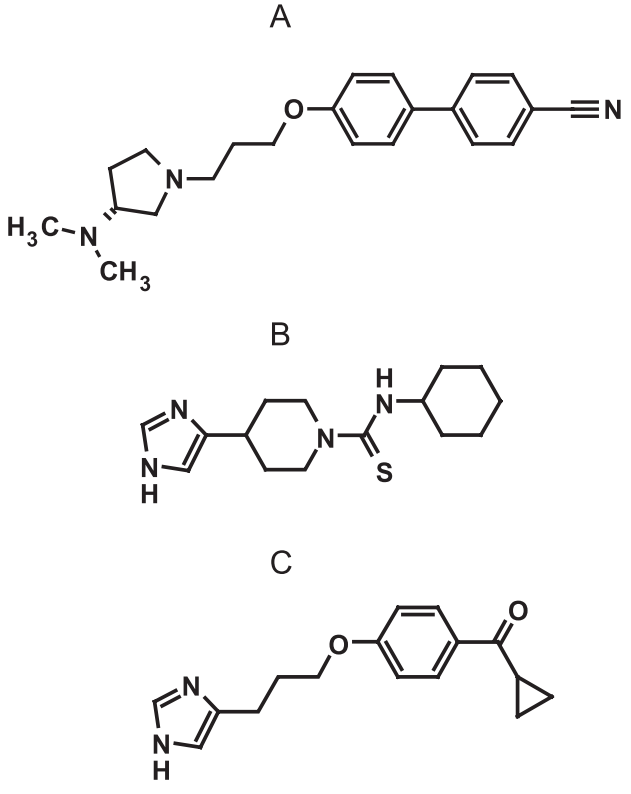

Fig. 1. Chemical structures of (A) A-331440 {4′ -[3-(3(*R*)-(dimethylamino)-pyrrolidin-1-yl)-propoxy]-biphenyl-4-carbonitrile}, (B) thioperamide and (C) ciproxifan.

In contrast, we have been studying the pharmacological effects of several novel series of non-imidazole-based histamine H_3 receptor antagonists that possess high potency for and selectivity toward the histamine H_3 receptor (Faghih et al., 2002a,b). Among these novel compounds, A-331440 {4′ -[3-(3(*R*)-(dimethylamino)-pyrrolidin-1-yl)-propoxy]-biphenyl-4-carbonitrile} (Fig. 1) emerged as having characteristics favorable for a potential antiobesity agent. The pharmacological properties of this compound and its effects in a model of obesity are herein described.

2. Methods

2.1. Radioligand binding assays

Radioligand binding assays for cloned human histamine H_1 and histamine H_2 receptors stably expressed in human embryonic kidney (HEK) 293 cells (approximately 10 pmol/mg protein) were performed as described (Esbenshade and Hancock, 2000) using [^{3}H]mepyramine and [^{3}H]tiotidine as radioligands, respectively. [^{3}H]*N*-α-methylhistamine binding to histamine H_3 receptors in rat cortical homogenates (receptor density ~ 120 fmol/mg protein) was also performed as described at a concentration of 6 mg/ml (original wet weight). Assays for human cortical histamine H_3 receptor binding assays were performed as described for rat with minor modifications. Human cortical tissue homogenates (receptor density ~ 10 fmol/mg protein) were prepared as described for the rat by ABS (Analytical Biological Services, Wilmington, DE 19801) and used at a concentration of 40 mg/ml. Alternatively, binding assays to cloned rat or human histamine H_3 receptors (the 445-amino acid containing form of each receptor, histamine $H_{3(445)}$) were performed as described (Lovenberg et al., 1999; Yao et al., 2003) using HEK cells expressing approximately 2 and 1 pmol/mg protein for rat histamine $H_{3(445)}$ and human histamine $H_{3(445)}$ receptors, respectively. Binding to human histamine H_4 receptors transiently expressed in HEK cells was performed essentially as described (Oda et al., 2000). Briefly, HEK 293 cells were seeded 24 h before transfection in Dulbecco's modified eagle's medium with 10% fetal bovine serum at 7 million cells per T75 flask. The HEK 293 cells were transiently transfected with pCIneo-hH_4 plasmid using LipofectAMINE 2000 reagent. The cells were harvested 48 h post-transfection, membranes were prepared and competition binding assays were performed (Oda et al.,

2000) using approximately 10 nM [^{3}H]histamine and 75 μg protein, and incubated at 25 °C for 1 h in a total volume of 0.5 ml 50 mM Tris, 5 mM EDTA, pH = 7.4. Nonspecific binding (typically <25%) was defined with 0.5 μM clobenpropit. The receptor density averaged 300 fmol/mg protein and the K_d of the radioligand was approximately 20 nM. All radioligand binding data were analyzed as previously described (Esbenshade and Hancock, 2000).

2.2. Functional blockade at cloned histamine H_3 receptors determined by calcium imaging

Histamine H_3 receptor antagonist activity was determined as previously described (Esbenshade et al., 2003) in a stable HEK 293 cell line co-expressing either the human or rat histamine H_3 receptor and the promiscuous G-protein, $G\alpha_{q/i5}$, by measuring agonist-evoked increases in intracellular calcium (Coward et al., 1999). Fluo-4, a calcium-sensitive fluorescent dye, was used to indicate intracellular calcium levels. Relative fluorescence was measured in a 96-well format Fluorometric Imaging Plate Reader (FLIPR, Molecular Devices, Sunnyvale, CA). Confluent cells grown in 96-well black-walled tissue culture plates treated with polyethelyenimine were loaded at room temperature with 8 μM Fluo-4-AM (acetoxymethylester) in Dulbecco's phosphate-buffered saline for 1–2 h. Before measuring fluorescence, each plate was washed three times. Increasing concentrations of histamine H_3 receptor antagonists were added at 10 s followed by addition of 30 nM (*R*)-α-methylhistamine 5 min later. Basal fluorescence values (prior to compound addition) were subtracted from raw fluorescence data at all time points. Peak fluorescence values were determined within the period of drug exposure. Peak response values were then expressed as a percentage of the reference peak response for 30 nM (*R*)-α-methylhistamine in the absence of histamine H_3 receptor antagonists. Experiments were performed in duplicate, and data were analyzed using GraphPad Prism (San Diego, CA) to obtain IC_{50} values and Hill slopes. pK_b values were determined by the generalized Cheng–Prusoff equation (Cheng and Prusoff, 1973) and are presented as the mean ± S.E.M.

2.3. Functional blockade and/or inverse agonist activity at cloned histamine H_3 receptors determined by GTPγS binding

Membranes from HEK cells expressing the human histamine H_3 receptor or from C6 cells expressing the rat histamine H_3 receptor were prepared by homogenization in cold buffer containing 50 mM Tris–HCl (pH 7.4), 5 mM EDTA, 10 mM $MgCl_2$, 1 mM benzamidine, 2 μg/ml aprotinin, 1 μg/ml leupeptin and 1 μg/ml pepstatin. The homogenate was centrifuged at 40,000 × *g* for 20 min at 4 °C, and the resulting pellet was resuspended in 50 mM Tris–HCl (pH 7.4), 5 mM EDTA, 10 mM $MgCl_2$ and homogenized. Glycerol and bovine serum albumin were added to a final concentration of 10% glycerol and 1% bovine serum albumin. For assays to determine inverse agonist activity, membranes were diluted in GTPγS assay buffer (25 mM HEPES, 2.5 mM $MgCl_2$ and 75 mM NaCl, pH 7.4) and 10 μg of membrane protein was incubated in a 96-well deep-well block in the presence of 5.0 μM unlabeled GDP, approximately 0.5 nM of [^{35}S]-GTPγS and various concentrations of test compounds. Samples were subsequently incubated at 37 °C for 20 min. For assays to determine antagonist activity, (*R*)-α-methylhistamine was added at a final concentration of either 30 nM (human histamine H_3 receptor) or 300 nM (rat histamine H_3 receptor) prior to incubating the samples 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 $MgCl_2$, pH 7.6) and subsequent harvesting onto a Packard Unifilter 96-well GF/B plate (Perkin Elmer Life Sciences, Boston, MA) followed by extensive washing. Microscint 20 (Perkin Elmer Life Sciences) was added to the samples and the amount of bound [^{35}S]-GTPγS was determined utilizing the Topcount (Perkin Elmer Life Sciences). The percentage of [^{35}S]-GTPγS bound in each sample was calculated as a percentage of that bound to control samples incubated in the absence of the histamine H_3 receptor ligands (basal). A minimum of three independent experiments were performed in triplicate, the data were analyzed using GraphPad Prism and pEC_{50} values for the inverse agonist assays and pK_b values for the antagonist assays were obtained.

2.4. Gross behavioral and antiobesity effects in high-fat diet-fed mice

All in vivo experiments were conducted in accordance with guidelines established by Abbott Laboratories' Animal Care and Use Committee and National Institutes of Health Guide for Care and Use of Laboratory Animals. C57BL/6J mice (age 5–6 weeks) were obtained from Jackson Labs (Bar Harbor, ME) and individually housed at Abbott facilities under conditions of 12 h lights on, 12 h lights off (on at 22:00), with food and water available ad libitum. At the beginning of the study, mice were administered a standard diet (D12450B, 3.8 kcal/g) or a high-fat content diet (D12451 containing 45 kcal % lard, 4.7 kcal/g), both obtained from Research Diets (New Brunswick, NJ) for approximately 14 weeks. Mice were examined using a modified behavioral rating scale (Irwin, 1968) to validate that doses selected for obesity studies were devoid of overt confounding behavioral effects. Therefore, obese mice were administered A-331440 at doses of 5, 15, 50 and 100 mg/kg p.o., b.i.d., for 3 days and observed following the first and fifth doses. Core (rectal) temperature was recorded for each animal, and observations of generalized behavior that deviated from vehicle-treated animals were recorded at discrete time periods of 0.5, 1, 2, 4 and 8 h on both days 1 and 3.

For obesity studies, mice were weighed and food consumption monitoring initiated 3 weeks prior to commence-

Table 1
Potencies of histamine H_3 ligands at histamine receptors in radioligand binding assays

Compound	Potency to inhibit radioligand binding [K_i, nM (95% CL)]						
	Rat cortex histamine H_3	Human cortex histamine H_3	Rat histamine H_{3L}	Human histamine H_{3L}	Human H_1	Human H_2	Human H_4
A-331440	21.7 (18.1–25.8)	22.7 (19.4–26.5)	6.28 (4.86–8.11)	3.24 (2.68–3.91)	2940 (2440–3530)	14,400 (10,000–20,800)	>10,000 –
Thioperamide	7.50 (6.56–8.57)	61.3 (49.4–75.8)	3.21 (2.16–4.77)	72.6 (53.9–97.9)	>10,000 –	>10,000 –	47.8 (26.9–85.1)
Ciproxifan	0.654 (0.587–0.729)	89.4 (76.9–104)	0.427 (0.281–0.648)	62.5 (49.3–79.3)	>10,000 –	>10,000 –	1880 (1510–2320)
GT-2016	25.8 (21.6–30.9)	499 (389–642)	ND	136 (109–171)	2560 (2250–2900)	7200 (6400–8070)	734 (667–809)
Histamine	4.90 (4.60–5.23)	2.32 (2.02–2.66)	2.66 (2.08–3.40)	2.61 (2.42–2.82)	7010 (6730–7310)	>10,000 –	39.8 (29.8–53.2)

Compounds were tested as described under Methods at cortical rat and human histamine H_3 receptors, cloned rat and human histamine $H_{3(445)}$ receptors and cloned human H_1, histamine H_2 and H_4 receptors.
Data are the geometric means and 95% confidence limits for experiments performed in duplicate with $N \geq 3$.

ment of drug administration. Nine days prior to drug treatment, baseline leptin levels were determined from 100 μl of whole blood obtained via tail-snip and collected in microhematocrit tubes, and postprandial blood glucose was determined via a Medisense-G glucometer (Abbott Laboratories Medisense Division, Bedford, MA). This was repeated at days 14 and 26 of drug treatment. On day 21, animals were fasted for 3 h and fasting blood glucose determined in a blood sample obtained by tail snip. Insulin tolerance was determined by administering insulin (Lilly Humulin-R, 0.25 U/kg, i.p. Eli Lilly and Company, Indianapolis, IN) with blood glucose measured at 0, 30, 60, 90 and 120 min using the glucometer. Pharmacological treatments were administered daily at 09:00 and 16:00 and food weights were determined at 09:00, 12:00 and 15:00 on the first day and periodically thereafter for up to 28 days. A-331440 was administered p.o. at doses of 0.5, 5 and 15 mg/kg b.i.d. and dexfenfluramine at a dose of 10 mg/kg p.o., b.i.d. On day 29, mice were humanely euthanized under CO_2–O_2 anesthesia. For a subset of animals, epididymal fat, liver or kidneys were removed to obtain tissue weights. The remaining animals were evaluated using magnetic resonance imaging (MRI) techniques (see below) to determine changes in the amount of adipose tissue.

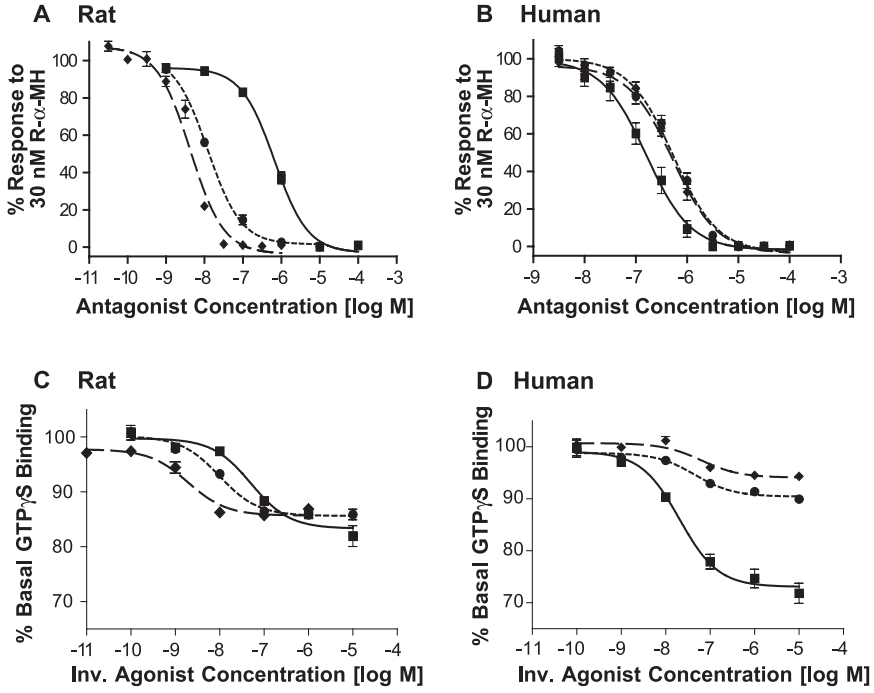

Fig. 2. Antagonistic and inverse agonist effects of A-331440, ciproxifan and thioperamide at rat and human histamine H_3 receptors. The effects of ciproxifan (♦), thioperamide (●) or A-331440 (■) to block calcium mobilization in FLIPR assays in rat (panel A) or human (panel B) or to inhibit basal binding of GTPγS to rat (panel C) or human (panel D) histamine H_3 receptors, respectively, were determined as described under Methods. A-331440 was a competitive antagonist and a potent and efficacious inverse agonist in these assays.

Statistical analyses were performed on weekly body weight data in order to investigate the differences between treatment groups, as well as the differences in body weight change over time between groups. Two sets of comparisons were made: first, the comparison between the positive control group (dexfenfluramine) and the vehicle group, and second, the comparison between the vehicle group and the A-331440-treated groups. Because the repeated measurements of body weight on each animal were correlated in nature (covariance), a mixed model approach was used to analyze the 5-week body weight data. The interaction term between day and treatment group was tested at the 0.25 level of significance. If the interaction was significant, comparisons were made between the drug-treated groups and the vehicle group for each week. Otherwise, treatment group comparisons were made on the average weight over the 5-week period. Also, the changes from baseline for each drug-treated group were compared to the changes from baseline of the vehicle group for any week after week 0. Bonferroni adjustment for *p*-values (at a 0.05 level of significance) was applied to comparisons between A-331440-treated groups and the vehicle group to account for multiplicity of tests. Other obesity study data (not subject to repeated measures) were analyzed using GraphPad InStat (San Diego, CA) using a one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test.

2.5. MRI evaluation of body fat

Magnetic resonance images (MRI) of mice treated with A-331440, dexfenfluramine or vehicle and fed with a high-fat diet were collected on a small animal imaging system, consisting of an Inova console from Varian NMR Instruments (Palo Alto, CA) interfaced to a 4.7-T–40-cm bore magnet-gradient coil from Magnex Scientific (Abingdon, England). Euthanized mice were placed in a quadrature rf coil (40-mm diameter) in the magnet, and 11 scout sagittal images, 1 mm each, were collected through the abdominal region of the mouse. The sagittal slices in which the kidneys were viewed were used to index the positions of all transverse slices through the abdominal region of the mice.

Table 3
Antagonistic potencies of histamine H_3 ligands at cloned rat and human histamine H_3 receptors in vitro

Compound	Potency to block [^{35}S]GTPγS binding (p$K_b \pm$ S.E.M.)	
	Rat histamine H_3	Human histamine H_3
A-331440	7.60 ± 0.12	8.16 ± 0.04
Thioperamide	8.13 ± 0.14	7.39 ± 0.04
Ciproxifan	8.78 ± 0.12	7.03 ± 0.13

Compounds were tested as described under Methods for their ability to antagonize (*R*)-α-methylhistamine-stimulated [^{35}S]GTPγS binding to membranes from HEK 293 or C6 cells stably transfected with human or rat histamine H_3 receptors, respectively.
Data are the mean and S.E.M. for experiments performed in triplicate with $N \geq 4$.

The transverse slices through the abdominal region of the mice were collected using the SATSEMS imaging acquisition program from Varian Instruments. Briefly, the water resonance is saturated using an rf frequency selective pulse (Gaussian) before the imaging sequence was started. During the course of image acquisition, 36 contiguous transverse slices, each 1 mm thick, were collected from the middle of the most caudal kidney to the inguinal region of each mouse. Of the 36 acquired images for each mouse, only every other image was analyzed (i.e., only 1/2 of the total abdominal region captured in the 18 selected images). The transverse slice with the caudal tip of the most caudal kidney was identified and designated as the 15th slice. The fat in the images was also enhanced by collecting the images with a short repetition time (TR = 0.7 s) and a short echo time (20 ms). The data matrix collection for each slice was 256×128 which was zero-filled to 256×256. The field of view was 4×4 cm, which gives an in-slice pixel resolution of 156×156 μm. There were four acquisitions for each image, so the total imaging time for each mouse was approximately 6 min.

The imaging protocol generated fat-enhanced (i.e., water-suppressed) images, which were analyzed for the fat content and distribution in each slice. Image analysis software was written in Abbott's MRI lab using IDL (RSINC, Boulder, CO) for determining the amount of fat in each slice. First, the total amount of fat was determined

Table 2
Antagonistic potencies of histamine H_3 ligands at cloned rat and human histamine H_3 receptors in vitro

Compound	Potency to block (*R*)-α-methylhistamine-induced calcium mobilization (FLIPR responses) (p$K_b \pm$ S.E.M.)	
	Rat histamine H_3	Human histamine H_3
A-331440	7.38 ± 0.10	7.37 ± 0.29
Thioperamide	9.11 ± 0.14	6.82 ± 0.05
Ciproxifan	9.42 ± 0.11	6.86 ± 0.07

Compounds were tested as described under Methods for their ability to antagonize the increase in calcium mobilization in response to (*R*)-α-methylhistamine in HEK 293 cells stably transfected with human or rat histamine H_3 receptors.
Data are the mean and S.E.M. for experiments performed in triplicate with $N \geq 4$.

Table 4
Inverse agonistic potencies of histamine H_3 ligands at cloned rat and human histamine H_3 receptors in vitro

Compound	Potency to reduce basal [^{35}S]GTPγS binding (p$EC_{50} \pm$ S.E.M.)	
	Rat histamine H_3	Human histamine H_3
A-331440	7.07 ± 0.24	7.70 ± 0.08
Thioperamide	8.06 ± 0.10	7.41 ± 0.11
Ciproxifan	8.87 ± 0.16	7.04 ± 0.17

Compounds were tested as described under Methods for their ability to reduce basal binding of [^{35}S]GTPγS to membranes from HEK 293 or C6 cells stably transfected with human or rat histamine H_3 receptors, respectively.
Data are the mean and S.E.M. for experiments performed in triplicate with $N \geq 4$.

in each slice in each mouse by setting a threshold in each image (slice) consisting of fat only. This procedure was then repeated for each mouse, this time circumscribing only the fat within the abdominal cavity in each slice by manual image highlighting. The abdominal fat in each slice was then subtracted from the corresponding total fat in each slice to determine the subcutaneous fat for that slice. From the above procedure, the total, abdominal and subcutaneous fat were determined in each slice for each animal. Since the slices were registered across animals based on the position of the kidney for each mouse, the fat (total, abdominal and subcutaneous) for each mouse could be combined within groups, allowing statistical analysis on the resulting grouped data. Data were analyzed using GraphPad InStat using a one-way ANOVA followed by Dunnett's post hoc test (Statistica version 5.5).

2.6. Materials

Radioligands [^{3}H]mepyramine, [^{3}H]tiotidine, [^{3}H]*N*-α-methylhistamine and [^{35}S]-GTPγS were purchased from Perkin Elmer Life Sciences, while [^{3}H]histamine was obtained from Amersham Pharmacia Biotech (Piscataway, NJ). A-331440, GT-2016 (5-cyclohexyl-1-[4-(3*H*-imidazol-4-yl)-piperidin-1-yl]-pentan-1-one, L-tartaric acid salt) and ciproxifan were synthesized at Abbott Laboratories. (*R*)-α-methylhistamine, thioperamide and clobenpropit were purchased from Tocris Cookson (Ballwin, MO), dexfenfluramine, benzamidine, aprotinin, leupeptin and pepstatin from Sigma (St. Louis, MO) and LipofectAMINE 2000 reagent from Invitrogen Life Technologies (Rockville, MD).

3. Results

3.1. Radioligand binding assays

A-331440 bound with high affinity to both human and rat native histamine H_3 receptors, with K_i values (the inhibition constant) of approximately 20–25 nM (Table 1). In experiments using the cloned human and rat histamine $H_{3(445)}$ receptors, A-331440 also exhibited high affinity for these sites, approximately 3–6 nM, respectively (Table 1). The lower affinity of A-331440 for native receptors is believed to result from physicochemical properties of the compound that allow sequestration in lipid-rich homogenates, like those used for native tissue assays. A-331440 had very low affinity for histamine H_1, H_2 and H_4 receptors (Table 1), in contrast to high affinity at both native and cloned histamine H_3 receptors. Notably, A-331440 had similar affinity for both the rat and the human receptor. This is in marked contrast to a number of histamine H_3 receptor antagonist compounds (Ligneau et al., 2000; Lovenberg et al., 2000; Yao et al., 2003), including thioperamide, ciproxifan and GT-2016 which are 8–130-fold less potent at the human histamine H_3 receptor compared to the rat histamine H_3 receptor (Table 1). Unlike thioperamide, which has comparable affinity for the human histamine H_3 and histamine H_4 sites (Table 1) (Liu et al., 2001), A-331440 lacks affinity for histamine H_4 receptors. A-331440 also demonstrates very low affinity for more than 75 other receptors (K_i>400 nM) except for human clonal α_{2C}-adrenoceptors and muscarinic M_3 receptors (K_i = 200 and 300 nM, respectively; Krueger et al., 2002).

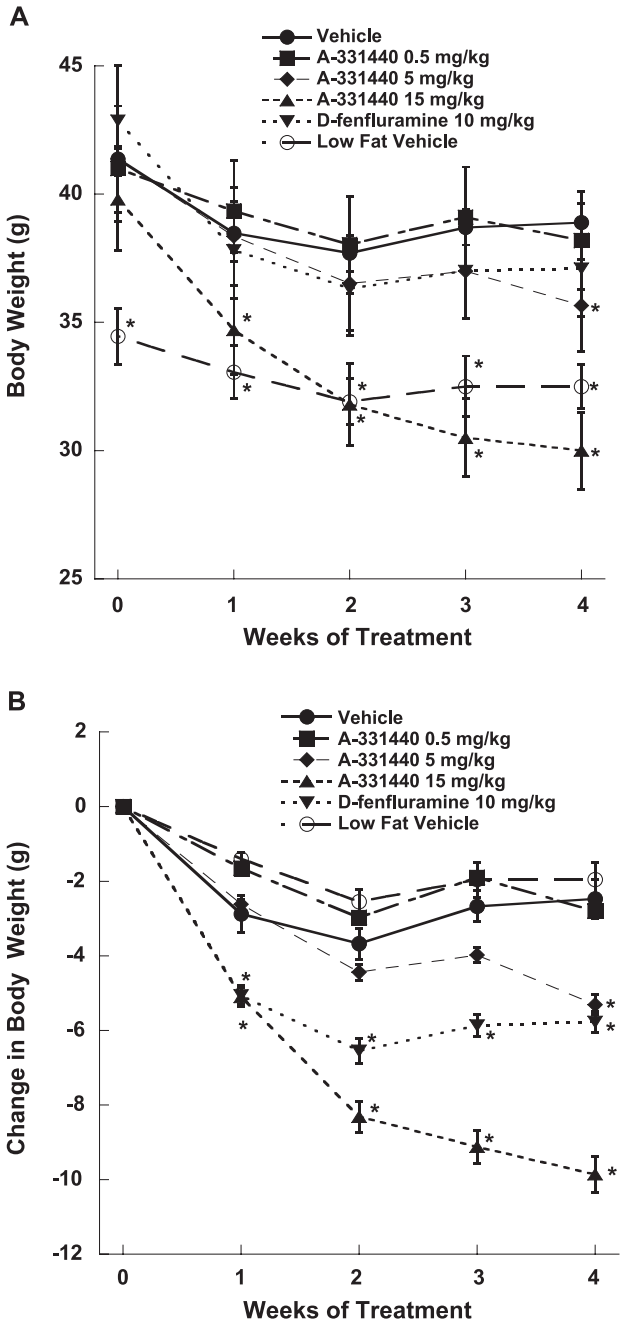

Fig. 3. Effect of A-331440 on body weight and weight loss in mice. (A) The absolute body weight in grams and (B) the cumulative weight loss in grams occurring over 4 weeks of compound administration. Asterisks indicate statistically significant differences from vehicle treatment (P<0.05 after Bonferroni adjustment).

3.2. Functional blockade of cloned histamine H_3 receptors

Fig. 2 (panels A and B) shows the potency of several histamine H_3 receptor antagonists to attenuate (*R*)-α-methylhistamine-mediated stimulation of calcium mobilization in cells expressing both histamine H_3 receptors and $G_{\alpha q/i5}$. The potency of A-331440 was approximately equipotent at both rat and human histamine H_3 receptors, whereas both thioperamide and ciproxifan were slightly less potent at the human receptor than A-331440 and considerably less potent at the human receptor than at the rat receptor (Table 2). Likewise, Table 3 shows the ability of these antagonists to attenuate (*R*)-α-methylhistamine-mediated stimulation of binding of [^{35}S]-GTPγS to both human and rat histamine H_3 receptors. In this assay, there was a smaller species difference comparing the potencies of thioperamide and ciproxifan, and A-331440 was approximately 3-fold more potent at the human histamine H_3 receptor.

3.3. Inverse agonist effects determined by GTPγS binding

The histamine H_3 receptor has been shown to be constitutively active (Morisset et al., 2000; Wieland et al., 2001). Therefore, the possibility exists that antagonists may also have inverse agonist properties. Fig. 2 illustrates the effects of ciproxifan, thioperamide and A-331440 on basal levels of binding of [^{35}S]-GTPγS to either rat (panel C) or human (panel D) histamine H_3 receptors, respectively. Potency values of these compounds as inverse agonists are listed in Table 4. Each compound behaved as an inverse agonist at both human and rat histamine H_3 receptors, with potencies very similar to those observed as antagonists in the [^{35}S]-GTPγS binding assay (Table 3). At human histamine H_3 receptors, A-331440 had higher efficacy as an inverse agonist than either of the imidazole compounds (Fig. 2).

3.4. Gross behavioral and antiobesity effects in high-fat diet-fed mice

Based on behavioral evaluation (Irwin, 1968), there was no apparent change in overall behavior for vehicle-treated obese mice. Core rectal temperature of the vehicle controls remained stable (between 36.5 and 37.5 °C) over the 24-h test period. Mice treated with A-331440 (up to 50 mg/kg, p.o.) did not differ from control mice. At a dose of 100 mg/kg, piloerection and hypoactivity were observed for the first 2 h, but diminished thereafter. Similar data were observed in naive male CD-1 mice administered with comparable doses of A-331440 i.p. (data not shown). At the doses administered in the 28-day feeding study, no gross behavioral changes that might have confounded the results of the feeding study were observed. Thus, the mice were not hyperactive and had no change in locomotor activity that would have contributed to increased caloric expenditure and hence to loss of body weight.

Mice administered with a high-fat diet gained weight to levels significantly greater than their littermates maintained on a normal diet (Fig. 3A). Thus, at the initiation of compound administration, mice on the normal (12450B) diet had an average weight of 34.5 ± 1.09 g (N = 10), while the average for the high-fat (12451) diet-fed mice was 40.5 ± 0.33 g (N = 55). These latter mice were apportioned to groups of 10 each for the remainder of the study (15 for the vehicle group) and administered either vehicle, dexfenfluramine or one of three doses of A-331440 for 28 days as described under Methods. As shown in Fig. 3, each group of animals, including vehicle controls, lost weight during the study with a portion of the weight loss probably attributable to the stresses involved in twice-daily p.o. dosing. As the study progressed and dosing continued, weights stabilized and or increased slightly above their maximal declines of −3.67 ± 0.41 and −2.47 ± 0.52 g in the low- and high-fat vehicle-treated groups, respectively (Fig. 3A). Therefore, low-fat diet vehicle-treated mice ended the study at an average weight of 32.5 ± 0.86 g, and the high-fat vehicle-treated mice averaged 38.9 ± 0.72 g on the last day of the study, so that in each case, the loss of weight attributable to nonpharmacological intervention was approximately 2 g.

Dexfenfluramine (10 mg/kg p.o., b.i.d.) caused a statistically significant loss of weight in high-fat diet-fed mice from an initial 42.9 ± 0.89 g to an average of 37.1 ± 0.79 g, although at earlier time points in the study, there appeared to be a more substantial reduction in weight (Fig. 3A). How-

Table 5
Effect of A-331440 and dexfenfluramine treatment on weekly body weight

Compound	Week 0[a]	Week 1	Week 2	Week 3	Week 4
T0: High-fat vehicle	41.37 ± 0.49	38.48 ± 0.76	37.70 ± 0.69	38.70 ± 0.70	38.90 ± 0.72
T1: A-331440, 0.5 mg/kg	41.00 ± 0.64	39.35 ± 0.69	38.03 ± 0.66	39.10 ± 0.74	38.20 ± 0.81
T2: A-331440, 5 mg/kg	41.38 ± 0.64	38.36 ± 0.51 (9)	36.53 ± 0.58 (9)	37.00 ± 0.75 (9)	35.67 ± 0.93[b] (9)
T3: A-331440, 15 mg/kg	39.80 ± 0.97	34.70 ± 0.88[b]	31.80 ± 0.87[b] (9)	30.50 ± 1.02[b] (8)	30.00 ± 0.69[b] (7)
T4: Dexfenfluramine, 10 mg/kg	42.87 ± 0.89	37.81 ± 0.68[b]	36.32 ± 0.66[b]	37.00 ± 0.84[b]	37.10 ± 0.79[b]
T0 vs. T1, T2 and T3	NS[c]	T3 < T0	T3 < T0	T3 < T0	T3, T2 < T0
T0 vs. T4	NS	NS	NS	NS	NS

[a] Data are expressed as mean ± S.E.M., N = 10/group (except as noted in parenthesis).
[b] Statistically significant differences of change from baseline between a treatment group and the control group using a $P < 0.05$ level of significance.
[c] NS denotes no significant differences between A-331440-treated groups (or positive control group) and the vehicle-treated group. If there are significant differences, the relevant groups are listed.

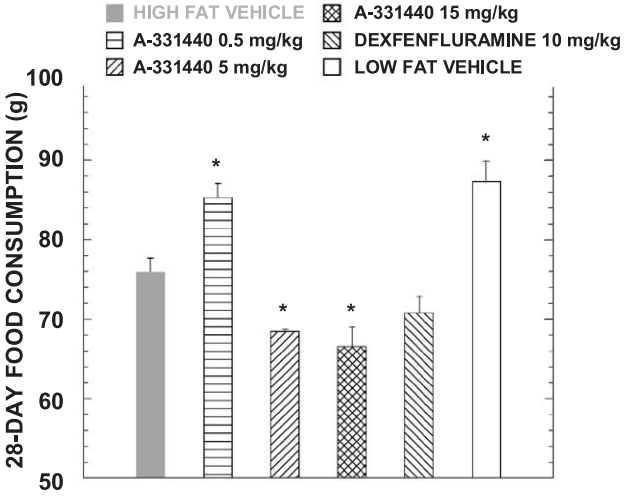

Fig. 4. Effect of A-331440 on food consumption. This figure illustrates cumulative food consumption (in g) over the course of the 28-day study. Asterisks indicate statistically significant differences from high-fat vehicle control food consumption ($P<0.05$) based on ANOVA followed by Dunnett's multiple comparison test. Food consumption (but not caloric intake, data not shown) was statistically significantly greater in the low-fat diet-fed mice compared to the vehicle-treated high-fat diet-fed mice.

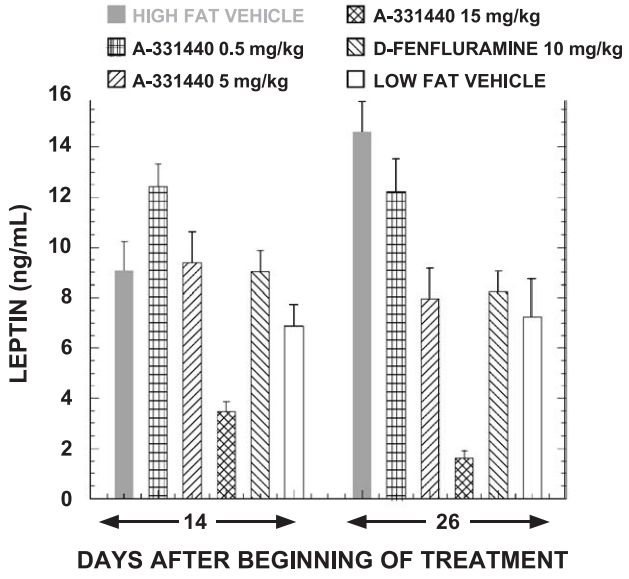

Fig. 6. Effect of A-331440 and dexfenfluramine on plasma leptin levels. Plasma leptin levels were evaluated on days 14 and 26 of the study as described under Methods. Asterisks indicate statistically significant differences (compared to high-fat vehicle control plasma leptin levels, $P<0.01$, ANOVA followed by Dunnett's multiple range test).

ever, the maximal weight loss to 36.3 ± 0.66 g (at week 2) coincided with the point of peak weight loss in vehicle controls, and therefore also may have had a component related to the dosing stress. The effects of dexfenfluramine were more apparent when data were expressed on the basis of cumulative change in body weight (Fig. 3B).

A-331440 has a half-life of approximately 4 h and bioavailability of 35% following oral administration to rats (Hancock et al., unpublished observations) and was therefore administered twice daily. At the lowest tested dose (0.5 mg/kg p.o., b.i.d.), A-331440 elicited no appreciable effect on weight in the high-fat diet-fed mice, compared to the

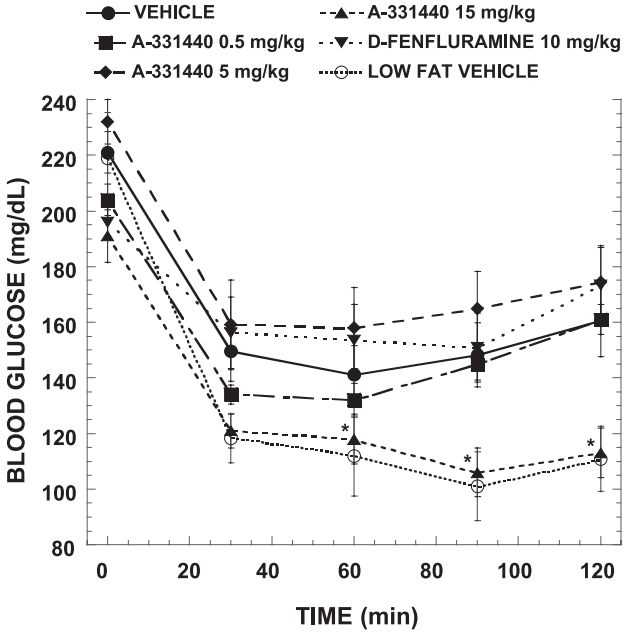

Fig. 5. Effect of A-331440 on insulin tolerance in high-fat diet-fed mice. Blood glucose levels were determined on day 21 of treatment following insulin injection as described under Methods. The high dose of A-331440 (15 mg/kg p.o., b.i.d.) normalized the glucose levels to those seen in low-fat diet-fed mice. Asterisks indicate statistically significant differences compared to the high-fat fed vehicle group ($P<0.05$, ANOVA followed by Dunnett's test).

Table 6
Effect of A-331440 and dexfenfluramine treatment on total body weight, liver, kidney and epididymal fat pad weights following 28 days administration

Compound	Effect of A-331440 and dexfenfluramine on total body, liver, kidney and epididymal fat pad weights [weight (g) ± S.E.M.][a]			
	Body weight	Liver	Kidney	Epididymal fat pad
High-fat vehicle	38.4 ± 0.62	1.43 ± 0.06	0.43 ± 0.02	2.23 ± 0.10
A-331440, 0.5 mg/kg p.o. b.i.d.	38.2 ± 0.96	1.37 ± 0.16	0.42 ± 0.01	1.92 ± 0.08
A-331440, 5.0 mg/kg p.o. b.i.d.	35.7 ± 0.64	1.28 ± 0.14	0.40 ± 0.01	1.62 ± 0.10[b]
A-331440, 15 mg/kg p.o. b.i.d.	30.0 ± 0.69[b]	1.66 ± 0.09	0.43 ± 0.02	0.60 ± 0.08[b]
Dexfenfluramine	37.5 ± 0.82	1.27 ± 0.14	0.41 ± 0.01	1.73 ± 0.07[b]
Low-fat vehicle	32.5 ± 0.86	1.19 ± 0.14	0.38 ± 0.01[b]	1.14 ± 0.14[b]

[a] Tissue weights were determined at the end of the 28-day study. Groups of 10 mice were evaluated.

[b] Significantly different ($P<0.05$, ANOVA followed by Dunnett's multiple range test) from the high-fat vehicle group.

vehicle-control cohort (Fig. 3). By the end of the study, the average weight of mice receiving the low dose of A-331440 was 38.2 ± 0.81 g, an average weight loss of 2.8 ± 0.32 g. At a dose of 5.0 mg/kg (p.o., b.i.d.), there was an initial weight loss in the high-fat diet group similar to that observed with dexfenfluramine (Fig. 3). In contrast to dexfenfluramine, the trend to weight loss continued to the end of the study, such that by day 28, mice treated with the intermediate dose of A-331440 weighed 35.7 ± 0.93 g, an average weight loss of 5.3 ± 0.99 g (Fig. 3B). The high dose of A-331440 (15 mg/kg, p.o., b.i.d.) elicited a marked and sustained weight loss resulting in animals that weighed less at the end of the study than their low-fat diet-fed littermates. These mice in the high dose group completed the study at an average weight of 30.0 ± 0.69 g, a net weight loss of approximately 9.8 g, or 25% of their initial body weight. Statistical evaluation of weight loss was determined comparing treated groups to the vehicle-treated high-fat diet-fed group of mice (Table 5). At the intermediate dose of A-331440, weight loss was significantly reduced by the fourth week of treatment, while the high dose of the compound caused significant weight loss after only 1 week of dosing (Table 5).

Effects on food intake were monitored in each treatment group. Vehicle-treated mice on either diet consumed approximately 2.8 g food per day, although food consumption was variable across treatment groups (data not shown). Food consumption was dramatically suppressed during early treatment with dexfenfluramine, coinciding temporally with the peak weight loss in this group. Food intake returned to normal after about day 7 of the study (data not shown) and remained relatively constant for the remaining 21 days

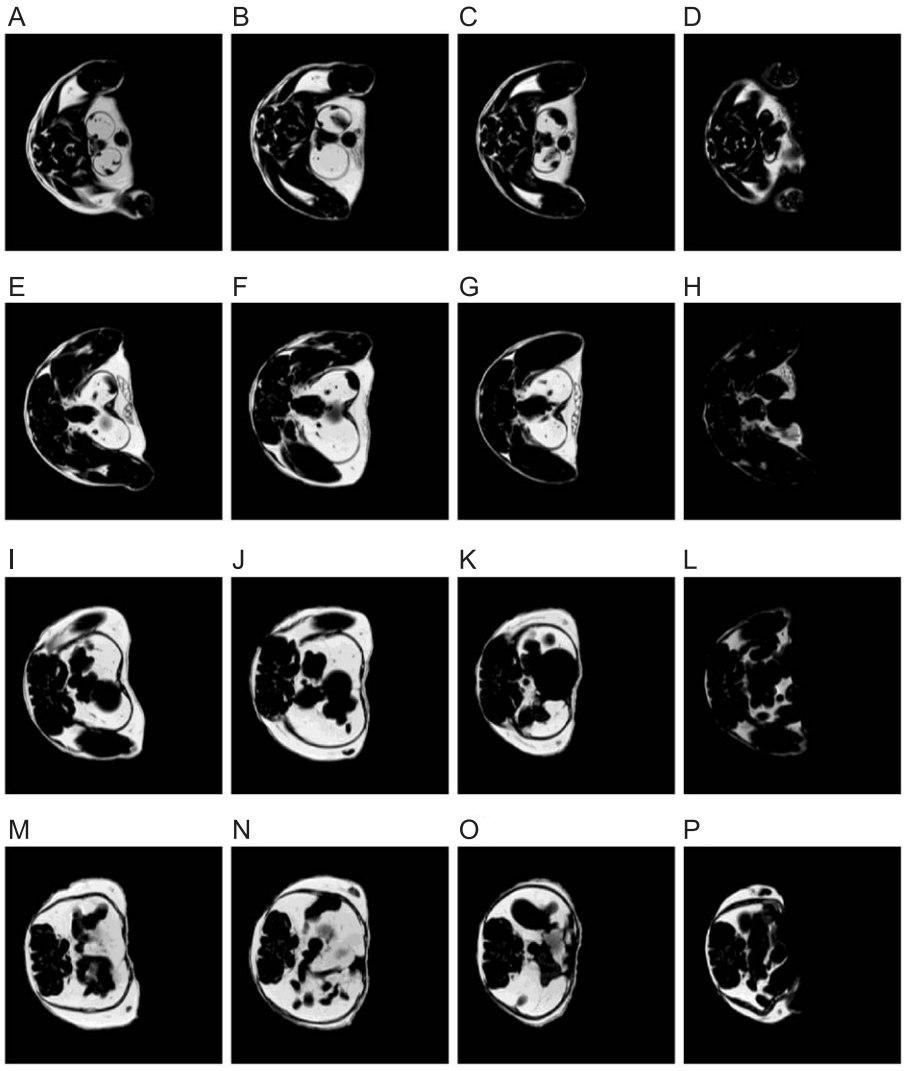

Fig. 7. Effects of A-331440 on abdominal fat assessed by MR imaging. Mice, on either a low- or high-fat diet and administered either A-331440 or vehicle, were treated and MR images were collected as described under Methods. Panels A through D are images at "slice 6," panels E through H are images from "slice 9," panels I through L are images from "slice 12" and panels M through P are images from "slice 15" from low-fat vehicle-treated (A, E, I, M), high-fat vehicle-treated (B, F, J, N), high-fat treated with A-331440 5 mg/kg p.o., b.i.d. (C, G, K, O) and high-fat treated with A-331440 15 mg/kg p.o., b.i.d. (D, H, L, P), respectively. White areas represent fat content. Mice treated with either dose of A-331440 show reduced accumulation of abdominal and subcutaneous fat.

despite continuing dexfenfluramine treatment, such that 28-day cumulative food consumption was not statistically significantly different from control (Fig. 4).

For the low dose of A-331440, there was no suppression of food intake at any time during the course of the study (data not shown). Indeed, cumulative food consumption was statistically significantly greater than vehicle control by the conclusion of the study (Fig. 4). A-331440 (5 mg/kg p.o., b.i.d.) did not reduce daily food intake significantly compared to control treatment (data not shown), although there was a significant effect on cumulative food consumption over the course of the study (Fig. 4). Similarly, a high dose of A-331440 (15 mg/kg, p.o., b.i.d.) also significantly reduced 28-day food consumption (Fig. 4), although daily food consumption levels were only irregularly reduced by significant amounts (data not shown).

Fig. 5 illustrates the effects of the various treatments on an insulin tolerance test. Only the high dose of A-331440 (15 mg/kg, p.o., b.i.d.) normalized the test toward blood glucose levels observed in the low-fat diet control mice. When data were grouped for comparison of area under the curve analysis over the 120-min period of the test, the high-dose A-331440 group failed to reach statistical significance (data not shown), although at each of the 60-, 90- and 120-min time points, the blood glucose levels in the A-331440 high-dose and low-fat diet vehicle group were significantly lower than the high-fat diet vehicle group (Fig. 5).

Treatment with either dexfenfluramine or A-331440 had no significant effects on postprandial blood glucose levels measured at either day 14 or 26 (data not shown), nor were significant effects on plasma insulin levels observed in any treatment group (data not shown). However, treatment of mice on the high-fat diet (initial leptin levels of 14.1 ± 0.74 ng/ml at day 0) did lower plasma leptin levels in animals receiving A-331440 (15 mg/kg p.o., b.i.d.) on days 14 and 26 (Fig. 6) as well as dexfenfluramine and A-331440 (5 mg/

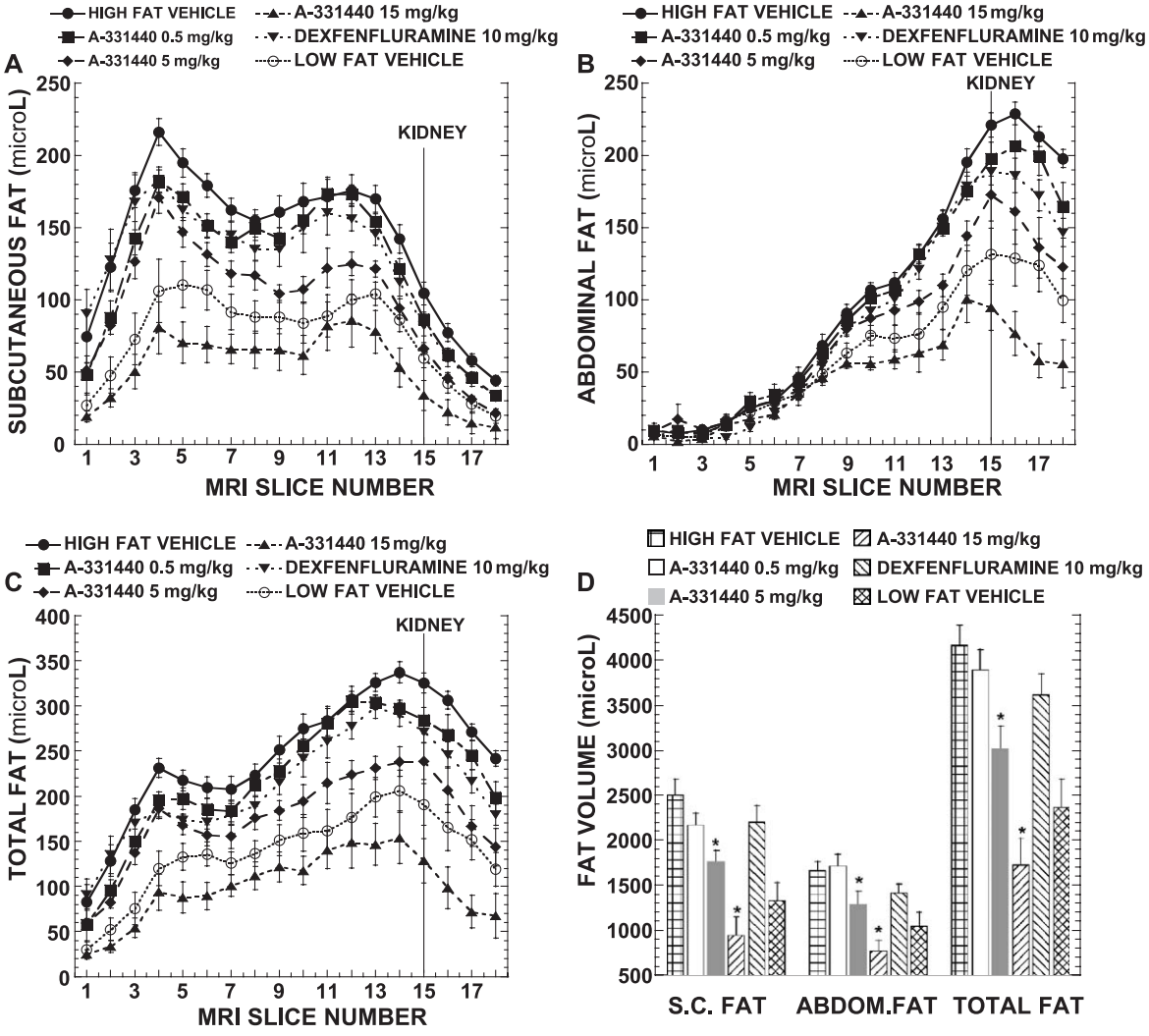

Fig. 8. Effect of A-331440 on body fat determined by MRI. Transverse MR images were analyzed as described under Methods using the caudal tip of the most caudal kidney as a reference point. Panel A illustrates subcutaneous fat distribution across each MR image, grouped by treatment. Panels B and C show abdominal and total fat, respectively. Panel D shows the cumulative fat content across the abdominal images as described under Methods. *$P < 0.05$ compared to high-fat fed mice, based on ANOVA followed by Dunnett's test.

kg p.o., b.i.d.) on day 26 of the study (Fig. 6). High-dose treatment with A-331440 reduced leptin levels below those observed in the low-fat diet mice (day 0 values of 5.4 ± 1.3 ng/ml, Fig. 6).

At the conclusion of the 28-day study, whole body, liver, kidney and epididymal fat pad weights were determined. Table 6 shows that there was no difference between groups on liver weight and no change in liver weight caused by drug treatment. Similarly, kidney weights were smaller only in the low-fat diet-fed vehicle group (Table 6). In contrast, epididymal fat pad weight was reduced significantly in both the high- and mid-dose A-331440 group (15 and 5 mg/kg p.o., b.i.d., respectively) and in the dexfenfluramine-treated group. The high dose of A-331440 reduced fat pad weights to levels actually lower than those found in the low-fat diet-fed vehicle group (Table 6). Thus, A-331440 appeared to cause a weight loss that was not generalized across tissues, but was apparent in fatty tissue exemplified by the epididymal fat pads.

3.5. MRI evaluation of body fat

Fig. 7 displays MR images from high-fat diet-fed mice treated with either vehicle or A-331440 (5 and 15 mg/kg) and a low-fat diet-fed mouse. Images are presented from slices 6, 9, 12 and 15, with the latter images at the level of the kidney. In each slice from high-fat vehicle-treated animals (panels B, F, J and N), fat is clearly visible as the white areas, with less white observed in images from the low-fat diet-fed mouse (Fig. 7, panels A, E, I and M). A-331440 (5 mg/kg) reduced the amount of fat seen in the MR images (Fig. 7, panels C, G, K and O), while the amount of visible fat in the images from the mouse treated with A-331440 at 15 mg/kg (Fig. 7, panels D, H, L and P) are less than the fat observed in the low-fat diet-fed mouse (Fig. 7, panels A, E, I and M).

Fig. 8 shows effects of dexfenfluramine and A-331440 on subcutaneous (panel A), abdominal (panel B) and total (panel C) fat across the slices obtained with MR imaging and quantified as described under Methods. Summary data for all slices are indicated in panel D. Dexfenfluramine did not reduce body fat significantly, based on MRI analysis, even though epididymal fat pad weight was reduced by approximately 0.5 g (Table 6). In contrast, both the 5 and 15 mg/kg p.o., b.i.d. doses of A-331440 significantly reduced total, abdominal and subcutaneous fat. Fat content was reduced by the high dose of A-331440 to levels less than or equal to the fat content in low-fat diet-fed mice (Fig. 8).

4. Discussion

Although antagonists of both histamine H_1 and histamine H_2 receptors have been in clinical use for several decades for their anti-allergy and gastroprotective efficacy, respectively, to date, no agents that modulate activity at histamine H_3 receptors have been approved for clinical use. This is despite the fact that the receptor was pharmacologically defined two decades ago (Arrang et al., 1983). Anatomical data localizing the histamine H_3 receptor to nerve endings in the CNS and peripheral nervous system, and pharmacological data demonstrating modulation of release of key neurotransmitters (Leurs et al., 1998), suggest several potential therapeutic applications for antagonists of the histamine H_3 receptor. These include cognitive and attentional disorders, epilepsy, sleep disorders or obesity (Leurs et al., 1998; Karlstedt et al., 2001). In contrast, the more recent cloning of histamine H_4 receptors and their newly emerging pharmacology (Hough, 2001) have yet to define a potential therapeutic role.

For the histamine H_3 receptor, various compounds, including thioperamide, ciproxifan and, more recently, GT-2331 (Tedford et al., 1999), have been advanced toward clinical development for one or more of the potential therapeutic indications. These compounds share the imidazole moiety with the natural agonist, histamine. In addition, these compounds have high affinity for several additional receptors, e.g., $5HT_3$ receptors (Leurs et al., 1998), α_{2C}-adrenoceptors (Esbenshade et al., 2001) and histamine H_2 receptors (Hough, 2001). In contrast, A-331440 is a novel, non-imidazole-based antagonist of the histamine H_3 receptor with low affinity for many other receptors from diverse pharmacological classes (Krueger et al., 2002).

Recent studies have revealed that the rodent and human histamine H_3 receptors, although highly homologous proteins, have distinctive pharmacological characteristics that appear related to two amino acid differences in transmembrane domain 3 (Ligneau et al., 2000; Lovenberg et al., 2000; Yao et al., 2003). For many compounds, including thioperamide, ciproxifan, GT-2016 and GT-2331, this sequence change results in markedly lower affinity for the human compared to the rat receptor, often as much as 100-fold. For A-331440, there appears to be no appreciable difference in the radioligand binding affinity of the compound for the human and rat receptor, either when using cortical homogenate binding assays or the cloned receptor. This also was shown in several measures of in vitro antagonist potency (FLIPR-based calcium assays or blockade of agonist-stimulated GTPγS binding) or in measures of inverse agonist activity (reduction of basal GTPγS binding). For A-331440, comparable potency values were obtained at both human and rat histamine H_3 receptors in the GTPγS assays, and these potencies were similar to those found in radioligand binding. In contrast, both ciproxifan and thioperamide were more potent at rat, compared to human, histamine H_3 receptors in both radioligand binding and GTPγS functional assays. Both compounds were also less potent at the human receptor in FLIPR-based assays, while A-331440 was more nearly equipotent at human and rat receptors in the FLIPR assay.

From a potential therapeutic perspective, conflicting data have been published on the role of histamine H_3 receptor

antagonists and obesity. Thioperamide has shown mixed effects in several feeding or obesity tests (Oishi et al., 1990; Sakata et al., 1997; Itoh et al., 1999). In our hands, ciproxifan administration (3 and 10 mg/kg s.c., q.d. for 15 days) reduced weight gain and food consumption 6% and 10%, respectively, in adult Sprague–Dawley rats on a normal diet (Fox et al., 2001). Additional data on appetite suppressive effects of other imidazole-based histamine H_3 receptor antagonists have been reported (Itoh et al., 1999; Bjenning et al., 2000a,b; Yates et al., 2000; Leonhardt et al., 1999). A-331440 is the first non-imidazole histamine H_3 receptor antagonist shown to reduce weight, particularly in a model of diet-induced obesity, the high-fat diet-fed mouse. At both the intermediate and high doses, A-331440 reduced weight by a statistically significant extent to levels either comparable to or in excess of the weight loss observed with dexfenfluramine. These are important findings since many imidazole compounds inhibit either their own metabolism or that of other compounds via blockade of hepatic cytochrome P450 isoenzymes (Lin and Lu, 1998). This is particularly the case with highly lipophilic imidazoles (Lin and Lu, 1998), and high lipophilicity is an important attribute for compounds targeting CNS receptors. Thus, inhibition of cytochrome P450 isoenzymes may be less likely with non-imidazole-based compounds, and in fact, A-331440 is not an inhibitor, but rather a substrate for P450 isoenzymes (Hancock and Pan, unpublished observations).

One question regarding the weight-loss effects of A-331440 is the mechanism of action, which we presume is via CNS histamine H_3 receptor antagonism. Clearly, A-331440 can block histamine H_3 receptors based on the FLIPR and GTPγS assays. In addition, we have observed that A-331440 concentration-dependently blocks the effects of histamine H_3-agonist-induced inhibition of forskolin-stimulated adenylate cyclase activity (data not shown). Also, the compound shows efficacy as an antagonist and an inverse agonist at rat and human histamine H_3 receptors in vitro. However, models of in vivo functional antagonism of histamine H_3 receptors are often indirect (e.g., micro-dialysis). One functional in vivo model is the reversal of (*R*)-α-methylhistamine-induced drinking behavior (Fox et al., 2002). Ciproxifan and thioperamide have previously been shown to dose-dependently reverse agonist-induced water consumption (Fox et al., 2002). Likewise, doses of A-331440 that are efficacious in lowering body weight in obese mice (5–15 mg/kg p.o., b.i.d.) are comparable to doses that are efficacious in this mouse dipsogenia model (data not shown), although full inhibition of the dipsogenic response to (*R*)-α-methylhistamine was not achieved by any dose of A-331440, which alone had no effect on drinking behavior (data not shown). While additional receptor interactions could be responsible for the weight loss induced by A-331440, this effect is probably not the result of any effects of the compound on histamine H_1 receptors since at concentrations up to and including 10 μM, no agonistic or antagonistic effect of A-331440 could be demonstrated in a FLIPR assay of human histamine H_1 receptor function (data not shown). Moreover, while we have not tested the affinity of the compound at rat histamine H_1 receptors, A-331440 has low affinity for not only the human (Table 1), but also the guinea pig histamine H_1 receptor (data not shown).

The efficacy of A-331440 to block CNS histamine H_3 receptors may result from very high concentrations of A-331440 achieved within the CNS, especially compared to either ciproxifan or thioperamide. Independent studies show that 1 h after A-331440, 5 mg/kg, i.v., concentrations of 17 μg/g were achieved in the rat brain, more than 160-fold higher than plasma concentrations at the same time point (0.105 μg/ml, Hancock et al., unpublished observations). In contrast, thioperamide brain and plasma concentrations (obtained 60 min after a 10 mg/kg i.p. dose) were 0.48 μg/g and 2.04 μg/ml, respectively (Silva et al., 1997), resulting in a low brain/plasma ratio (0.24 ×) for thioperamide compared to A-331440 (160 ×). Similar experiments with ciproxifan showed that a 10 mg/kg p.o. dose was associated with brain levels of approximately 5 μg/g and plasma levels of approximately 1.4 μg/ml, (ratio of 3.6 ×, Hancock et al., unpublished observations). Thus, A-331440 exhibits a 45–660-fold higher brain/plasma ratio than either ciproxifan (160 ×:3.6 × = 45 ×) or thioperamide (160 ×:0.24 × = 666 ×), respectively, in rats, consistent with more facile access to the CNS receptor target. In analogous studies in diet-induced obese mice, brain levels were between 14.4 and 9.85 μg/g after either 17 or 21 days, respectively, of dosing with 15 mg/kg b.i.d. A-331440 and were 9.02 μg/g in brains of obese mice dosed q.d. with 30 mg/kg A-331440 (Wetter et al., unpublished observations). Thus, high levels of compound are also achieved in mouse CNS. The compound does not appear to accumulate in the CNS, based not only on the data in obese mice from 17 to 28 days, but also on additional studies that showed that approximately 97% of the compound was cleared from the brain in 24 h (Wetter et al., unpublished observations). Enhanced CNS access for A-331440 to block of histamine H_3 receptors in hypothalamic appetite controlling pathways may be important for the efficacy of the compound to reduce weight in diet-induced obesity in mice since ciproxifan is ineffective in such tests (Bush et al., unpublished observations).

The efficacy of the high dose of A-331440 to reduce body weight to a level equivalent to that of the low-fat diet-fed mice is particularly impressive. Moreover, the effects of the high dose of A-331440 were salutary in terms of normalization of insulin tolerance and the reduction of leptin levels to those observed in lean mice. To the best of our knowledge, this is the first time a histamine H_3 receptor antagonist has been demonstrated to improve these parameters that could suggest potential improvement in clinical outcomes in both diabetes and obesity.

It has been shown (Ross et al., 1991) that MRI can be used to accurately measure both abdominal and subcutane-

ous fat in rats, and the technique has also been validated in several animal tissues compared to chemical extraction and analysis techniques (Laurent et al., 2000). Ross et al. (1991) measured abdominal and subcutaneous fat by collecting transverse images of the entire length of the rat and segmenting the adipose fat from the subcutaneous fat. The summation of the adipose and subcutaneous fat correlated very well with the adipose and subcutaneous fat as determined by X-ray computerized tomography. Both of these measurements showed a strong correlation with the percent fat determined by chemical analysis. Similarly, the utility of MRI to quantify fat tissue in rats was demonstrated (Osculati et al., 1989; DeSouza et al., 2001). In the latter study, weight change in obese female Zucker rats was associated with regionally specific changes in fat content with a rank order of ovarian>retroperitoneal>subcutaneous fat (DeSouza et al., 2001). Studies in mice also validated the utility of MRI technology to quantify fat content (Changani et al., 2003; Beckman et al., 2001). Together, these data indicate the possibility to accurately measure subcutaneous and abdominal fat in rodents using MRI.

A-331440 appeared to exert its effects by specific diminution of the contribution of fat content to total body weight. This was determined both by the absolute tissue weight of epididymal fat pads and by analysis of MR images of fat in the abdominal visceral area. We believe this is the first use of MR imaging analysis in obese mice to determine their distribution of body fat following treatment with potential antiobesity agents. By comparing the reduction in total fat in the treated groups compared to the high-fat vehicle group, the data from the MRI analysis indicate that approximately 50% of the weight loss in the treated mice in each group is accounted for by a reduction in fat in the abdominal region. It should be noted that each slice is 1 mm thick and only every other slice from the MRI data was analyzed. In addition, the density of fat is assumed to be 0.90 mg/ml (Ross et al., 1991). The remainder of the weight loss may be associated with a reduction in fat in regions not imaged as well as other sources. The data from both the MRI analysis and epididymal fat pad weights indicate that the effects of A-331440 were primarily limited to fat and not to generalized weight loss since the liver and kidney weights were unaffected. Notably, there were no symptoms of fatty stools or diarrhea in any of the mice, thus differentiating the histamine H_3 receptor antagonist mechanism from the effects observed with lipase inhibitors like orlistat (Clapham et al., 2001). While additional tests of potential confounding effects of appetite suppression (e.g., conditioned taste aversion or kaolin ingestion) need to be performed to help validate that histamine H_3 antagonists have a beneficial mechanism of action to reduce body weight, the findings of only minimal effects on food consumption would suggest that compounds like A-331440 would not demonstrate the same kinds of effects as, for example, lithium chloride on these tests or on food consumption. In addition, behavioral studies with A-331440 showed no effects on normal behaviors of grooming, eating (in the absence of diet-induced obesity), locomotor activity, core body temperature or basal metabolic rate (Bush and Dickinson, unpublished observations).

When the final body weights of the mice were obtained at the end of the study, there was no statistically significant difference in body weights between any of the treatment groups, excepting those mice receiving the highest dose of A-331440 (although net changes in body weight revealed statistically significant changes for the dexfenfluramine-treated mice and those treated with both the mid- and high-dose of A-331440). However, both the MRI and epididymal fat pad data analysis demonstrated a statistically significant effect on fat content at both the mid and high doses of A-331440. In addition, MRI analysis of the fat content showed that approximately 50% of the loss of body weight could be accounted for by a loss in the total fat content in the abdominal region of the mouse. This suggests that measurement of fat loss, rather than simply measuring body weight, may be a more sensitive measure of the antiobesity effects of compounds in the preclinical setting and may be relevant to the clinical development of such agents.

In summary, these data demonstrate that the novel, non-imidazole histamine H_3 receptor antagonist, A-331440, exhibits a profile of weight reduction in a model of diet-induced obesity that is predictive of a favorable profile in clinical use. To our knowledge, this is the first demonstration of the effect of a histamine H_3 receptor antagonist in a relevant model of human obesity, diet-induced obesity in mice. It is known that histamine H_3 receptor antagonists do not produce stimulant-like activity of amphetamine-like diet aids, and A-331440 specifically causes normalization of leptin levels, insulin tolerance and a specific reduction in fat content in obese mice. These studies validate that histamine H_3 receptor antagonists such as A-331440, acting presumably via blockade of CNS histamine H_3 receptors, have potential therapeutic utility in the treatment of obesity in man.

Acknowledgements

This research was conducted by scientists employed by Abbott Laboratories. Statistical analysis of obesity-related data was performed upon consultations with C. Thomas Lin, Yanhui Hu and Richard Hanrahan of the Department of Non-Clinical Statistics. Vincent Hradil (Department of Information Technology) developed software used for MRI analysis. Measurements of compound levels were determined by Jill McVey Wetter and Dr. Kennan Marsh in the Department of Experimental Kinetics, Abbott Laboratories. Dr. Sheila Collins, Duke University Medical Center, originally proposed the idea of using diet-induced obese animals as a model of human obesity.

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