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RESEARCH PAPER

A novel peripherally restricted cannabinoid receptor antagonist, AM6545, reduces food intake and body weight, but does not cause malaise, in rodents

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BACKGROUND AND PURPOSE
Cannabinoid CB_1 receptor antagonists reduce food intake and body weight, but clinical use in humans is limited by effects on the CNS. We have evaluated a novel cannabinoid antagonist (AM6545) designed to have limited CNS penetration, to see if it would inhibit food intake in rodents, without aversive effects.

EXPERIMENTAL APPROACH
Cannabinoid receptor binding studies, cAMP assays, brain penetration studies and gastrointestinal motility studies were carried out to assess the activity profile of AM6545. The potential for AM6545 to induce malaise in rats and the actions of AM6545 on food intake and body weight were also investigated.

KEY RESULTS
AM6545 binds to CB_1 receptors with a K_i of 1.7 nM and CB_2 receptors with a K_i of 523 nM. AM6545 is a neutral antagonist, having no effect on cAMP levels in transfected cells and was less centrally penetrant than AM4113, a comparable CB_1 receptor antagonist. AM6545 reversed the effects of WIN55212-2 in an assay of colonic motility. In contrast to AM251, AM6545 did not produce conditioned gaping or conditioned taste avoidance in rats. In rats and mice, AM6545 dose-dependently reduced food intake and induced a sustained reduction in body weight. The effect on food intake was maintained in rats with a complete subdiaphragmatic vagotomy. AM6545 inhibited food intake in CB_1 receptor gene-deficient mice, but not in CB_1/CB_2 receptor double knockout mice.

CONCLUSIONS AND IMPLICATIONS
Peripherally active, cannabinoid receptor antagonists with limited brain penetration may be useful agents for the treatment of obesity and its complications.

Abbreviations
CB_1, cannabinoid 1 receptor; CB_2, cannabinoid 2 receptor; GI, gastrointestinal; i.c.v., intracerebroventricular

Introduction

Agonist activation of cannabinoid 1 (CB_1) receptors (nomenclature follows Alexander *et al.*, 2009) by cannabinoids stimulates food intake in humans and rodents (Jarbe and DiPatrizio, 2005), and conversely antagonists/inverse agonists and neutral antagonists inhibit food intake (Salamone *et al.*, 2007). With obesity-related medical problems becoming an increasing burden on health-care systems in the Western world, there has been a great deal of research into the cannabinoid system as a therapeutic target in obesity. The CB_1 receptor antagonist/inverse agonist SR141716 (rimonabant) (Colombo *et al.*, 1998; Vickers *et al.*, 2003) and the pharmacologically and structurally similar CB_1 receptor antagonist/inverse agonist, AM251 (Hildebrandt *et al.*, 2003; McLaughlin *et al.*, 2003; Chambers *et al.*, 2004; 2006), have well characterized anorexigenic actions in rodents. Unfortunately, CB_1 receptor antagonist/inverse agonists also have the potential to induce centrally mediated effects and, owing to these side effects, rimonabant as an anti-obesity agent has been removed from the European market and did not receive approval from the Food and Drug Administration in the United States (Bifulco and Pisanti, 2009). Despite this, studies with rimonabant support the overall finding that antagonist/inverse agonist actions at CB_1 receptors in humans can be used as a successful weight-loss therapy.

It has been shown that the peripheral endocannabinoid system is influenced by both feeding status and obesity. The levels of CB_1 receptor mRNA and protein expression in vagal afferent neurons that innervate the gut of food-deprived lean rats are greatly increased compared with the levels during the fed state (Burdyga *et al.*, 2004). Similarly, during periods of fasting, levels of the endocannabinoid, anandamide, are elevated in the rat duodenum (Gómez *et al.*, 2002). Furthermore, in obese rodents, an increase in mRNA for CB_1 receptors is observed in the stomach (Di Marzo *et al.*, 2008) and in the nodose ganglia (Paulino *et al.*, 2009). Endocannabinoid levels in the duodenum, pancreas and liver are similarly elevated in obese rodents (Izzo *et al.*, 2009). These reports suggest that the peripheral endocannabinoid system is implicated in the regulation of energy balance.

It has been suggested that peripheral, as opposed to central, CB_1 receptors mediate the inhibition of food intake observed with CB_1 receptor antagonism/inverse agonism (Gómez *et al.*, 2002). Rimonabant, when injected intracerebroventricularly (i.c.v.) did not inhibit food intake in rats while similar and lower doses injected intraperitoneally (i.p.) induced hypophagia (Gómez *et al.*, 2002). Furthermore, this study demonstrated that rats treated with the neurotoxin capsaicin, which destroys primary afferent fibres innervating the gastrointestinal (GI) tract, did not display hypophagia to rimonabant (Gómez *et al.*, 2002). However, the delivery of rimonabant directly into the CNS by another group was shown to inhibit food intake and body weight (Nogueiras *et al.*, 2008), while a study by Madsen *et al.* (2009) found that neither subdiaphragmatic vagotomy nor subdiaphragmatic vagal de-afferentation prevented the effects of rimonabant on food intake and body weight. Therefore, while negative psychological effects of CB_1 receptor antagonists are no doubt mediated through actions in the brain, the role of peripheral CB_1 receptors on the control of food intake and body weight remains controversial. LoVerme *et al.*, (2009) have recently synthesized a mixed CB_1 receptor neutral antagonist/CB_2 receptor agonist with limited access to the CNS; URB447. This compound was shown to inhibit food intake and body weight gain in mice (LoVerme *et al.*, 2009), but whether this was due to CB_1 receptor antagonism or CB_2 receptor stimulation was not determined.

In the present study, we describe a novel cannabinoid CB_1 receptor specific neutral antagonist with limited CNS penetration: AM6545 (Makriyannis *et al.*, 2010; Tam *et al.*, 2010). We tested the hypothesis that AM6545 would inhibit food intake in rodents in the absence of adverse effects. We show that AM6545 is a neutral CB_1 antagonist with low CNS penetration. It inhibited food intake and body weight gain in rodents and at these doses it did not appear to have aversive side effects.

Methods

Animals

All animal care and experimental procedures were in accordance with the guidelines of the Canadian Council on Animal Care and approved by the University of Calgary Animal Care Committee or University of Guelph Institutional Animal Care Committee. Male Sprague Dawley (SD) rats with or without a complete subdiaphragmatic truncal vagotomy and male and female C57BL/6 mice were obtained from Charles River (Montreal, Quebec, Canada). Two breeding pairs of heterozygous $CB_1^{+/-C57BL/6N}$ mice were obtained from B. Lutz (University Medical Center Mainz) and bred in our facility to obtain $CB_1^{-/-C57BL/6N}$ mice (Marsicano *et al.*, 2002). Animals used in these studies were backcrossed to C57BL/6N for six generations and were used at the same age (female, 9–14 weeks) and maintained

under the same conditions as the wild-type mice. All $CB_1^{-/-}$ mice were genotyped using established protocols and were confirmed as homozygous gene deficient animals ($CB_1^{-/-C57BL/6N}$) prior to inclusion in the study. $CB_1^{-/-}/CB_2^{-/-}$ double knockout mice, bred on a C57BL/6J background, were obtained from A. Zimmer (University of Bonn) and bred in our facility. All animals were housed, under a 12 h light-dark cycle (lights off 19:00, unless otherwise stated), in plastic sawdust floor cages and allowed free access to tap water and standard laboratory chow, unless otherwise stated.

Cannabinoid receptor binding assay

AM6545 was tested for its affinity for the cannabinoid receptors using membrane preparations made from rat brain (CB_1) or HEK293 cells expressing either mouse CB_2 (mCB_2) or human CB_2 (hCB_2) and [^{3}H]CP-55,940, as previously described (Morse *et al.*, 1995; Lan *et al.*, 1999; McLaughlin *et al.*, 2006).

cAMP assay

The binding of an inverse agonist to cannabinoid receptors raises levels of intracellular cyclic AMP (cAMP) whereas a neutral antagonist has no effect on cAMP levels (Janero and Makriyannis, 2009). We looked at the effect of AM6545 on forskolin-induced cAMP levels to determine whether it is a neutral antagonist. The effects of an inverse agonist, AM251, were also investigated as a positive control. Intracellular cAMP levels were measured with a competitive binding assay using intact HEK293 cells expressing hCB_1 or hCB_2 as previously described (McLaughlin *et al.*, 2006). After lysing the cells and centrifugation, a cAMP assay kit (Sigma-Aldrich, St. Louis, MO, USA) was used to determine cAMP released in the resulting supernatant.

Brain penetration assay

Rats (280–310 g) were killed (sodium pentobarbital, 80–100 mg·kg^{-1}, i.p.) 1, 3 and 5 h after receiving an i.p. injection of vehicle (4% DMSO, 1% Tween 80 in physiological saline; $n = 2$), AM6545 (10 mg·kg^{-1}; $n = 4$) or AM4113 (10 mg·kg^{-1}; $n = 4$). In further experiments, female C57BL/6 mice (18.5–21.0 g) were administered an i.p. injection of vehicle ($n = 2$) or AM6545 (5 mg·kg^{-1}; $n = 4$) and were killed 1, 3 and 17 h post-injection. From both rats and mice, blood was collected and centrifuged for plasma, and brains were removed. All samples were flash-frozen in liquid nitrogen and stored at –80°C. Tissues (plasma or brain) were extracted following published procedures (Folch *et al.*, 1957) and analysed in SRM mode after APCI+ ionization using a Thermo-Finnigan Quantum Ultra triple quadrupole mass spectrometer (Thermo Electron, San Jose, CA, USA) with an Agilent 1100 HPLC (Agilent Technologies, Wilmington, DE, USA) front-end. Compounds were eluted from the Phenomenex Gemini C18 column (Phenomenex, Torrance, CA, USA) (2 × 50 mm, 5 µ) with a C18 guard column using a gradient elution consisting of 0.1% formic acid in both methanol (A) and water (B). The detection limits in this assay for AM6545 were: rat plasma, 9 ng·mL^{-1}; mouse plasma, 2.6 ng·mL^{-1} and for AM4113, in rat plasma, 1.5 ng·mL^{-1}. For brain samples, the corresponding lower limits were AM6545 (rat) 7.5 ng·g^{-1} (mouse) 5.4 ng·g^{-1} and AM4113 (rat) 5.3 ng·g^{-1}

Colonic propulsion assay

Cannabinoid agonists slow GI transit and thus the actions of AM6545 on WIN55212-2-induced slowing of colonic propulsion was used to confirm the functional blockade of peripheral CB_1 receptors by AM6545 (Pinto *et al.*, 2002). Male C57BL/6 mice (19–26 g) were lightly anesthetized with isoflurane (4% in air) before a 2.5 mm spherical glass bead was inserted 2 cm intrarectally. The time to the expulsion of the bead was recorded. AM6545 (10 mg·kg^{-1}, n = 5–10 or 20 mg·kg^{-1}, n = 6–7) or vehicle (4% DMSO, 1% Tween 80 in physiological saline, n = 9–18) was injected i.p. 60 min prior to the administration of WIN55212-2 (1 mg·kg^{-1}, n = 7–16), loperamide (1 mg·kg^{-1}, n = 5–9) or vehicle (n = 7–18). Twenty minutes later, colonic propulsion was measured. Doses of WIN55212-2 (Pinto *et al.*, 2002) and loperamide (Yamada and Onoda, 1993) were based on previous work.

Conditioned gaping

All rats were implanted with intra-oral cannulae, according to the procedure previously described (Rock *et al.*, 2009). For 3 days following surgery, the rats were weighed and their cannulae flushed daily with chlorhexidine. Three days after the intraoral cannulation surgery, the rats received an adaptation trial; they were individually placed in the Plexiglas taste reactivity (TR) chamber (22.5 × 26 × 20 cm) with the cannula attached to an infusion pump (Harvard Apparatus, South Natick, MA, USA) for fluid delivery. Water was infused into the intra-oral cannulae over a period of 2 min at the rate of 1 mL·min^{-1}.

Rats received the conditioning trial 24 h after the adaptation trial. They were individually placed in the TR chamber and were intra-orally infused with 0.1% saccharin for 2 min at a rate of 1 mL·min^{-1}. During the infusion, their orofacial and somatic responses were videotaped from a mirror at a 45° angle below the TR chamber. Immediately following the saccharin infusion, the rats were injected (8 mL·kg^{-1}) with vehicle (2-HPBCD, n = 7), AM251 (n = 7) or AM6545 (n = 7). We have previously

demonstrated that a dose of 8 mg·kg^{-1}, of the inverse agonist, AM251, produces conditioned gaping reactions (McLaughlin *et al.*, 2005). Classically, conditioned responses, such as conditioned gaping, are most readily formed when the conditioned stimulus (saccharin) precedes the unconditioned stimulus (potential nausea induced by the antagonist). Therefore, the temporal arrangement ensured the optimal opportunity to detect the nauseating property of the antagonist. Had the drug been administered prior to the saccharin, then the arrangement would have been one of backward conditioning which is much less likely to result in an association between taste and nausea.

The animals were given a second adaptation trial with a 2 min intra-oral infusion of water 48 h following the conditioning trial, prior to TR testing. The drug free TR testing occurred 72 h following the conditioning trial. The rats were placed in the TR chamber and infused with 0.1% saccharin over a period of 2 min (1 mL·min^{-1}) while their orofacial reactions were videorecorded [Sony DCR-HC48 (Noldus, Inc., Leesburg, VA, USA), with firewire feed to a PC] from the mirror beneath the chamber. The videos were later scored by an observer unaware of the experimental conditions using 'The Observer' (Noldus, Inc.) software for the aversive behaviour of gaping (number of large openings of the mouth and jaw, with lower incisors exposed). Additionally, the number of 2 s bouts of tongue protrusions (forward and lateral protrusions of the tongue from the mouth) and mouth movements (movement of the mandible with a closed mouth) were summated to provide a positive hedonic reaction score.

Conditioned taste avoidance

Different flavors of Ensure Plus™ Liquid diet (53.3% carbohydrate, 29% fat, 16.7% protein, 1.41 kcal·g^{-1}) (Abbot Laboratories, Abbott Park, IL, USA) were used to assess whether rats would avoid a flavour associated with the effect of AM6545. Naive male SD rats (250–275 g) were restricted to 15 g of rodent chow per day in order to promote food intake during training and testing trials. On the first day of the experiment, rats were given access to vanilla-flavoured Ensure Plus in order to become familiar with the diet. The vanilla flavour was deliberately chosen to be different to those that would be used in the conditioning study. On the second day, rats were given access to either chocolate- or strawberry-flavoured Ensure Plus followed 45 min later by an i.p. vehicle injection. On the third day, rats were given access to the opposite flavour from the day before followed 45 min later by AM6545 (10 mg·kg^{-1}, n = 7). The dose of AM6545 was based on that used in the conditioned gaping studies. An additional group of rats were treated the same way with saline vehicle and LiCl (10 mL·kg^{-1} of 0.15 M, n = 6) to provide a positive control. On the fourth day, each rat was given access to each flavour for 45 min. Evidence of conditioned taste avoidance was observed by the preference for one flavour over another, and expressed as food intake of a flavour (paired to a treatment) as a percentage of total food consumed.

Short-term food intake in rats

Rats were individually housed and fed chocolate-flavoured Ensure Plus Liquid diet. Rats were habituated to testing and handling procedures for 3 days prior to the start of the study. Food and water were presented in drip-free inverted glass bottles that attached to the outside of the cage. Food was available for 18 h each day starting at 16:00 h (12 h light-dark cycle; lights off 16:00 h). Prior to the first day of treatment, rats were assigned to vehicle (1 mL·kg^{-1}) (mean body weight ± s.e.mean; 252 ± 5 g, n = 6), 5 mg·kg^{-1} AM6545 (253 ± 6 g; n = 6) or 10 mg·kg^{-1} AM6545 (252 ± 3 g, n = 6) treatment groups. Drugs were administered i.p. starting at 15:45. Food intake was measured at 1, 3 and 5 h. Doses were used based on the conditioned gaping and conditioned taste avoidance studies.

Chronic feeding study in rats

Daily food intake and body weight were monitored in rats treated with vehicle (256 ± 6 g, n = 4) or 10 mg·kg^{-1} AM6545 (255 ± 6 g, n = 4) to determine if the peripherally restricted antagonist would affect body weight over 7 days. Rats were maintained on vanilla-flavoured Ensure Plus Liquid diet as described previously. Injections were given i.p. each day starting at 15:45. Food intake and body weight were measured each day. The dose of AM6545 was chosen based on the results from the short-term food intake study and conditioned taste avoidance assay.

Short-term food intake in vagotomized rats

Upon arrival from Charles River, vagotomized rats were immediately placed on vanilla-flavoured Ensure Plus Liquid diet and allowed to recover for 10 days. Food was available each day from 16:00 to 09:00. Food intake and body weight were monitored daily. The day before the experiment, rats were assigned to either vehicle (281.2 ± 13 g; n = 5) or 10 mg·kg^{-1} AM6545 (281.8 ± 15 g; n = 5) treatment groups. The dose was chosen based on results from the early feeding study and conditioned taste avoidance assay. On the day of the first experiment, rats were injected i.p. at 15:30. Food intake was measured at 1, 3, and 5 h post-injection.

Short-term food intake in CB_1 and CB_1/CB_2 receptor knockout mice

Female $CB_1^{-/-}$ (Marsicano *et al.*, 2002) and littermate control mice, $CB_1^{-/-}/CB_2^{-/-}$ (Jarai *et al.*, 1999) and C57BL/6J control mice were individually housed 4 days prior to the experiment and placed on a medium fat diet (51.4% carbohydrate, 31.8% fat, 16.8% protein; 4.41 kcal·g^{-1}; Diet # D12266B, Research Diets, New Brunswick, NJ, USA). Access to food was restricted to the hours of 16:30 to 09:30 with free access to tap water at all times. The day before testing the effect of 5 mg·kg^{-1} AM6545 on food intake, mice were assigned to either vehicle (wild-type, 23.4 ± 2.0 g; n = 9; $CB_1^{-/-}$, 21.9 ± 0.9 g; n = 8; C57BL/6, 27.5 ± 0.8 g; n = 5; $CB_1^{-/-}/CB_2^{-/-}$, 21.6 ± 1.1 g; n = 5) or AM6545 (wild-type, 23.9 ± 3.9 g; n = 9; $CB_1^{-/-}$, 22.28 ± 1.6 g; n = 9; C57BL/6, 27.7 ± 0.8 g; n = 6; $CB_1^{-/-}/CB_2^{-/-}$, 21.9 ± 2.0 g; n = 6) treatment groups. Similarly, the day before testing the effect of 20 mg·kg^{-1} AM6545 on food intake, mice were assigned to either vehicle (wild-type, 23.0 ± 1.9 g; n = 8; $CB_1^{-/-}$, 22.2 ± 0.9 g; n = 10) or AM6545 (wild-type, 23.3 ± 1.5 g; n = 11; $CB_1^{-/-}$, 21.6 ± 1.7 g; n = 12) treatment groups. Drugs were administered i.p. starting at 16:00. Food intake was measured at 1, 2, 3 and 17 h. Doses were chosen based on the colonic propulsion study in mice and feeding studies in rats.

Data analysis

Results from the competition binding assays were analysed using non-linear regression to determine the IC_{50} values for the ligand; K_i values were calculated from the IC_{50} (Cheng and Prusoff, 1973) (Prism by GraphPad Software, Inc., La Jolla, CA, USA). Each experiment was performed in triplicate and K_i values determined from at least two independent experiments. The results from the cAMP assay were expressed as percent inhibition of forskolin-stimulated cAMP accumulation and EC_{50} curves were generated with the use of GraphPad Prism software. All other data were expressed as the mean ± s.e.mean. Data were analysed (GraphPad Prism software) using *t*-tests, one-way ANOVA or Kruskal–Wallis test followed by Bonferroni's or Dunn's multiple comparisons *post hoc* test or by two-way mixed design ANOVA with time as the repeated measure followed by Bonferroni's *post hoc* test, as appropriate.

Materials

1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-(piperidin-1-yl)-1*H*-pyrazole-3-carboxamide (AM251), *N*-piperidin-1-yl-2,4-dichlorophenyl-1*H*-pyrazole-3-carboxamide analogue (AM4113) and 5-(4-(4-cyanobut-1-ynyl)phenyl)-1-(2,4-dichlorophenyl)-4-methyl-*N*-(1,1-ioxothiomorpholino)-1*H*-pyrazole-3-carboxamide (AM6545) were synthesized at the Center for Drug Discovery, Northeastern University. The cannabinoid receptor agonist (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate (WIN55212-2 mesylate) was purchased from Tocris (Ellisville, MI, USA) and the μ-opioid receptor agonist, loperamide hydrochloride, was purchased from Sigma-Aldrich. Sodium pentobarbital was supplied by Ceva Sante Animale, Libourne, France and isoflurane by Halocarbon Products Corporation, NJ, USA. Lithium chloride (LiCl) was purchased from Sigma-Aldrich, made up to a concentration of 0.15 M in sterile water and injected i.p., at 10 mL kg^{-1} rat. All other drugs were dissolved in a vehicle of 4% DMSO, 1% Tween 80 in physiological saline unless otherwise stated. Drugs used in the conditioned gaping studies were dissolved in 45% 2-hydroxypropyl-β-cyclodextrin (2-HPBCD) with sterile water to 1 mg·mL^{-1}. Injections were administered i.p. to mice at 100 μL·10 g^{-1} body weight and to rats at 100 μL·100 g^{-1} body weight.

Results

The novel compound AM6545 was synthesized (Makriyannis *et al.*, 2010) as a neutral antagonist of the cannabinoid receptors, with selectivity for CB_1 over CB_2 receptors, and with limited brain penetration. Pharmacological and functional assays were used to verify these properties.

Cannabinoid receptor binding assay

Binding assays were performed to determine the affinity of AM6545 for the cannabinoid receptors. AM6545 bound with high affinity to CB_1 receptors exhibiting 302-fold selectivity for CB_1 versus mCB_2 receptors and 38-fold selectivity for CB_1 versus hCB_2 receptors (Table 1).

cAMP assay

A cAMP assay was used to demonstrate that AM6545 acts as a neutral antagonist (as opposed to an inverse agonist) of the CB_1 receptors. When carried out in HEK293 cell cultures expressing hCB_1, AM6545, at concentrations up to 3 μM, produced no change in forskolin-stimulated cAMP levels, while the inverse agonist AM251 further stimulated cAMP production (Figure 1).

Brain penetration assay

A unique property of the novel CB_1 receptor neutral antagonist, AM6545, is its limited penetration of

Table 1
Binding data for AM6545

Assay	AM6545
CB_1 binding K_i	1.73 ± 0.92
95% confidence	(0.94, 3.20)
r^2-value	0.942
mCB_2 binding K_i	523 ± 143
95% confidence	(288, 1,050)
r^2-value	0.929
hCB_2 binding K_i	65.2 ± 123
95% confidence	(43.0, 99.0)
r^2-value	0.963

Values for K_i are in nM ± standard deviation of four (CB_1 receptors), two (mCB_2 receptors) or three (hCB_2 receptors) assays done in triplicate (shown with 95% confidence intervals and r^2-value).

the CNS. To verify this, the levels in the brain and plasma of AM6545 and AM4113 (a centrally active compound) were measured after the systemic administration of each compound. Neither AM6545 nor AM4113 were detected in the samples taken from rats receiving a vehicle injection. AM6545 was not detected in brain or plasma taken from mice following vehicle administration. The brain : plasma ratios demonstrate that AM4113 has a much greater brain penetration than AM6545 (Table 2). Similarly, low brain : plasma ratios of AM6545 were measured in mouse tissues (Table 2).

Colonic propulsion assay

Experiments were carried out to confirm the functional blockade of peripheral CB_1 receptors by AM6545. The cannabinoid receptor agonist WIN55212-2, when systemically administered, has been shown to slow GI transit through the activation of peripheral CB_1 receptors (Izzo *et al.*, 2000). The ability of AM6545 to reverse WIN55212-2-induced actions on the GI tract was investigated. WIN55212-2 (1 mg·kg^{-1}) slowed colonic propulsion ($P < 0.001$; Figure 2) and this effect was reversed in a dose-dependent manner by AM6545 (Figure 2). To further confirm the specificity of AM6545, it was tested against the action of loperamide, a μ–opioid receptor agonist. Loperamide (1 mg·kg^{-1}) slowed colonic propulsion ($P < 0.001$) to a similar magnitude as WIN55212-2 and this effect was not modified by AM6545 (Figure 2). AM6545 (10 or 20 mg·kg^{-1}) alone did not modify colonic propulsion ($P > 0.05$; Figure 2).

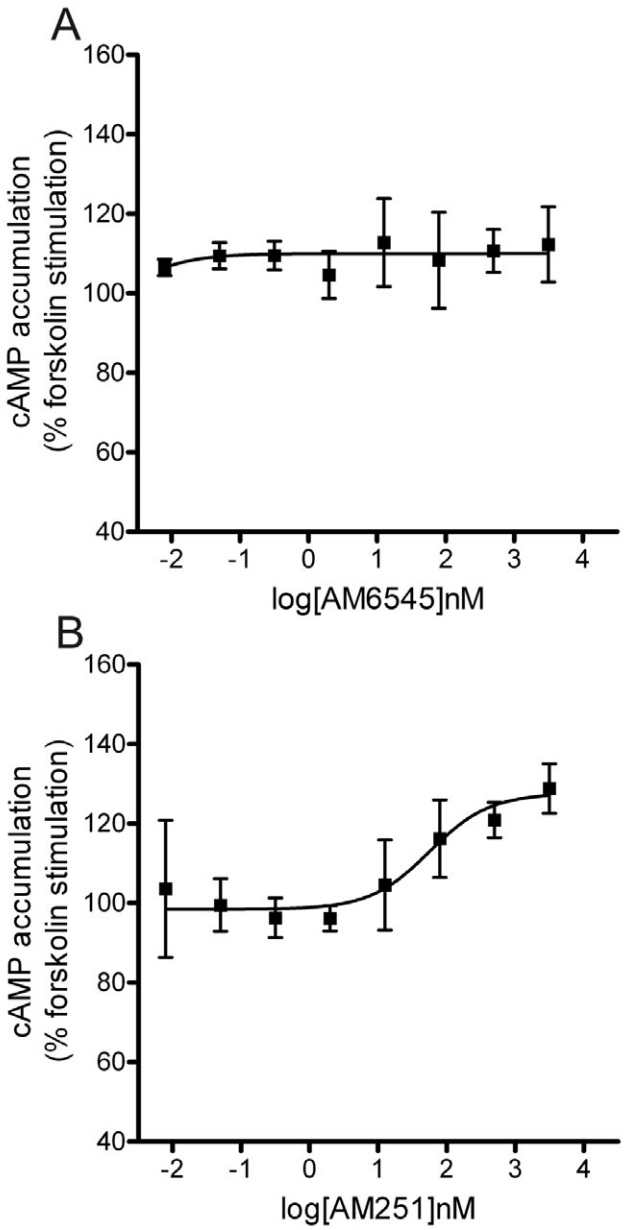

Figure 1
Representative cAMP assay using hCB1-HEK293 cells for AM6545 (A) and AM251 (B). $n = 3$.

Conditioned gaping and conditioned taste avoidance

In order to confirm whether AM6545 induces signs of nausea, we examined the potential for AM6545 to generate conditioned gaping and conditioned taste avoidance in rats. There was a significant difference between treatments in the number of conditioned gaping reactions, $F(2,16) = 14.43$, $P = 0.003$, and hedonic reactions, $F(2,16) = 5.41$, $P = 0.016$ (Figure 3A,B). *Post hoc* analysis revealed that AM251 (8 mg·kg^{-1}; $P < 0.05$), but not AM6545 (8 mg·kg^{-1};

Table 2

AM6545 or AM4113 levels in the brain and plasma of rats or mice at specified time points after the i.p. administration of the antagonist

Species	Time point (h)	Treatment ($mg \cdot kg^{-1}$)	Brain [antagonist] ($ng \cdot g^{-1}$)	Plasma [antagonist] ($ng \cdot mL^{-1}$)	Brain : plasma ratio
Rat	1	10 AM6545	29.7 ± 9.8	178.1 ± 99.4	0.18 ± 0.11
		10 AM4113	63.6 ± 6.2	49.3 ± 5.9	1.30 ± 0.18
	3	10 AM6545	58.8 ± 4.8	267.9 ± 91.9	0.23 ± 0.06
		10 AM4113	123.4 ± 15.6	36.2 ± 8.5	4.73 ± 2.62
	5	10 AM6545	41.9 ± 26.3	123.5 ± 114.0	0.41 ± 0.12
		10 AM4113	79.5 ± 27.3	22.7 ± 14.1	3.56 ± 0.61
Mouse	1	5 AM6545	20.7 ± 5.5	197.0 ± 0.1	0.13 ± 0.08
	3	5 AM6545	39.4 ± 11.4	195.0 ± 0.2	0.10 ± 0.01
	17	5 AM6545	11.3 ± 1.4	153.4 ± 0.2	0.20 ± 0.06

Data represent the mean ± s.e.mean, $n = 3–4$.

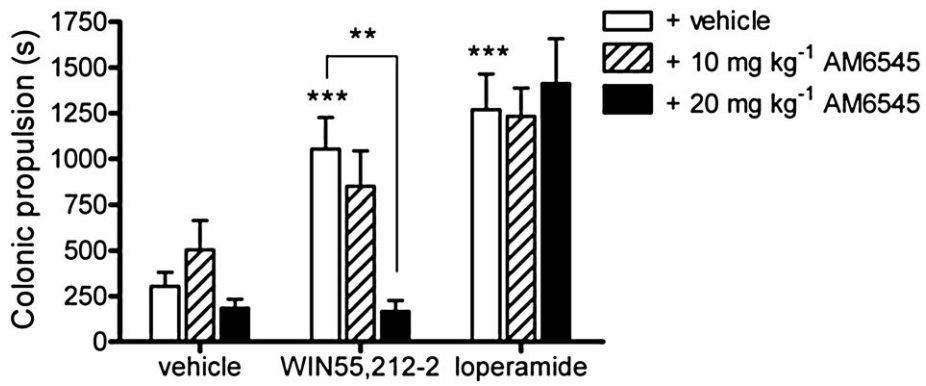

Figure 2

The effect of vehicle (4% DMSO, 1% Tween 80 in physiological saline), 10 $mg \cdot kg^{-1}$ or 20 $mg \cdot kg^{-1}$ AM6545 administered 1 h prior to vehicle, WIN55212-2 (1 $mg \cdot kg^{-1}$) or loperamide (1 $mg \cdot kg^{-1}$) on colonic propulsion in mice. Bars represent the mean ± s.e.mean, $n = 6–7$. $^{**}P < 0.01$ and $^{***}P < 0.001$ denote a significant difference between groups analysed using one-way ANOVA followed by Bonferroni's multiple comparisons *post hoc* test.

$P > 0.05$) produced aversive conditioned gaping reactions (Figure 3A) and suppressed positive conditioned ingestive reactions (Figure 3B) relative to vehicle. Furthermore, while the positive control LiCl produced conditioned taste avoidance ($P < 0.001$; Figure 3C), AM6545 (10 $mg \cdot kg^{-1}$; $P > 0.05$) did not (Figure 3D).

Short-term food intake in rats

Investigations were carried out to examine whether AM6545 could inhibit food intake in rats. Two-way repeated measures ANOVA showed there was a significant treatment effect [$F(2,32) = 5.83$, $P = 0.0126$]. *Post hoc* analysis revealed that AM6545 (5 $mg \cdot kg^{-1}$) had no effect on food intake in rats at 1, 3 and 5 h post-injection (Figure 4A) and that food intake in rats was significantly ($P < 0.001$) inhibited 3 h after the administration of a higher dose of AM6545 (10 $mg \cdot kg^{-1}$) and, while the trend for inhibition was still evident, the action was not significant ($P > 0.05$) at 5 h (Figure 4A).

Chronic feeding study with AM6545

Following the observation that AM6545 could inhibit short-term food intake after an acute injection, investigations were carried out into the long-term effect on food intake and body weight that chronic administration produced. There was a significant (treatment-time) interaction, $F(7,42) = 3.25$, $P = 0.0075$, of daily AM6545 treatment on food intake (Figure 4B). Daily administration of AM6545 (10 $mg \cdot kg^{-1}$) inhibited food intake in rats, $F(1,42) = 21.98$, $P = 0.003$, with significant ($P < 0.05$) differences between vehicle- and AM6545-treated animals

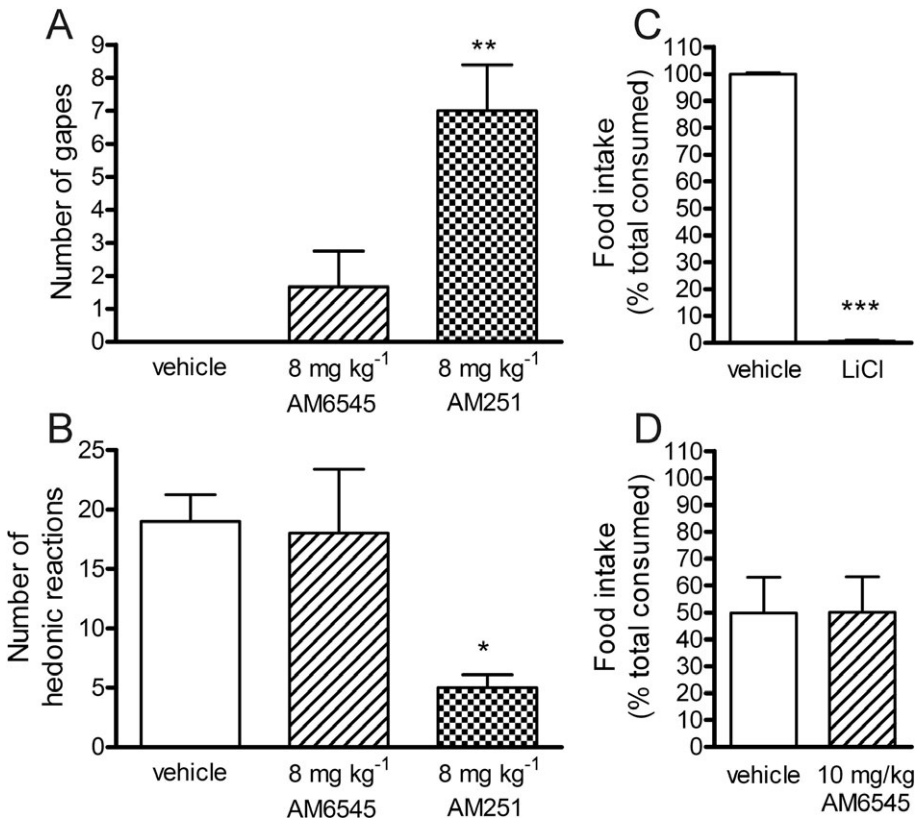

Figure 3

The effect of vehicle (2-HPBCD), AM6545 (8 mg·kg^{-1}) or AM251 (8 mg·kg^{-1}) on subsequent conditioned gaping (A) and positive hedonic reactions (B) elicited by 0.1% saccharin solution. The effect of AM6545 (10 mg·kg^{-1}) on the consumption of flavoured food previously paired to vehicle, lithium chloride (LiCl, C) or 10 mg·kg^{-1} AM6545 (D) in naive rats. Bars represent the mean ± s.e.mean, n = 6–7. *P < 0.05, **P < 0.01 and ***P < 0.001 denote a significant difference from the vehicle control analysed using either one-way ANOVA followed by Dunnett's *post hoc* test (A and B) or using paired *t*-test (C and D).

observed at days 1 through 5 (Figure 4B). There was also a significant (treatment-time) interaction, $F(7,42) = 6.88$, $P < 0.0001$, on body weight change induced by daily AM6545 (Figure 4C). The rats treated daily with AM6545 (10 mg·kg^{-1}) exhibited a reduction in weight change, $F(1,42) = 12.04$, $P = 0.013$, with significant ($P < 0.05$) differences between vehicle- and AM6545-treated animals seen at days 4 to 7 (Figure 4C).

Short-term food intake in vagotomized rats

Experiments were carried out in vagotomized rats to investigate the role of the vagus nerve in the action of AM6545 on food intake. A two-way repeated measures ANOVA revealed a significant (treatment-time) interaction, $F(2,16) = 7.19$, $P = 0.006$. AM6545 (10 mg·kg^{-1}) significantly inhibited short-term food intake in vagotomized rats, $F(1,16) = 53.01$, $P < 0.0001$, with *post hoc* tests showing there was a significant difference from vehicle-treated rats at all time points (Figure 5).

Short-term food intake in CB_1 and CB_1/CB_2 receptor knockout mice

CB_1 receptor knockout and CB_1/CB_2 receptor knockout mice were used in an attempt to specify the mechanism of action of AM6545 on food intake. AM6545 (20 mg·kg^{-1}) inhibited food intake in wild-type mice. Two-way repeated measures ANOVA showed a significant (treatment-time) interaction, $F(3,48) = 11.86$, $P < 0.0001$. Also, there was a significant treatment effect, $F(1,48) = 29.14$, $P < 0.0001$. Bonferroni's *post hoc* analysis showed there was a significant inhibition ($P < 0.001$) in food intake induced by AM6545 (1.79 ± 0.39 g) at 17 h as compared with vehicle-treated mice (3.90 ± 0.29 g). At the earlier time points of 1, 2 and 3 h, there was no difference in food intake ($P > 0.05$) between vehicle-treated mice (1 h, 0.25 ± 0.08 g; 2 h, 0.48 ± 0.10 g; 3 h, 0.70 ± 0.08 g) and 20 mg·kg^{-1} AM6545-treated mice (1 h, 0.07 ± 0.03 g; 2 h, 0.11 ± 0.04 g; 3 h, 0.12 ± 0.04 g). AM6545 (20 mg·kg^{-1}) also inhibited food intake in

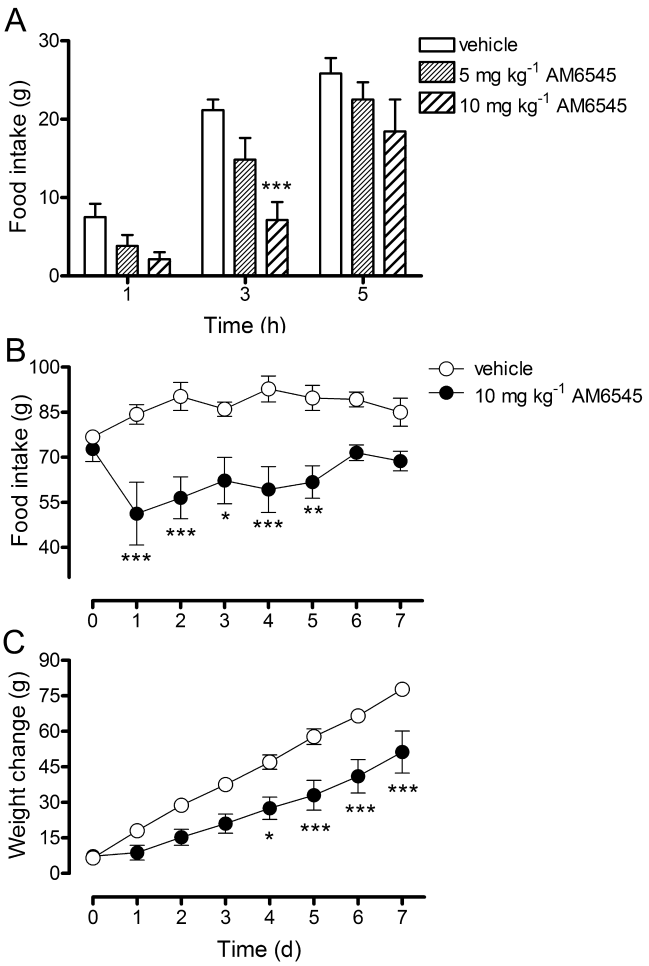

Figure 4

The effect of vehicle (4% DMSO, 1% Tween 80 in physiological saline) or AM6545 (5 or 10 mg·kg^{-1}) on short-term food intake in rats (A). Bars represent the mean ± s.e.mean, n = 6–7. ***P < 0.001 denotes a significant difference to vehicle control analysed using one -way ANOVA followed by Bonferroni's *post hoc* test. The effect of vehicle (4% DMSO, 1% Tween 80 in physiological saline) or AM6545 (10 mg·kg^{-1}) on food intake (B) and weight change (C) in rats. Data points represent the mean ± s.e.mean, n = 4. *P < 0.05, **P < 0.01 and ***P < 0.001 denote a significant difference from vehicle control analysed using two-way mixed design ANOVA with time as the repeated measure followed by Bonferroni's *post hoc* test.

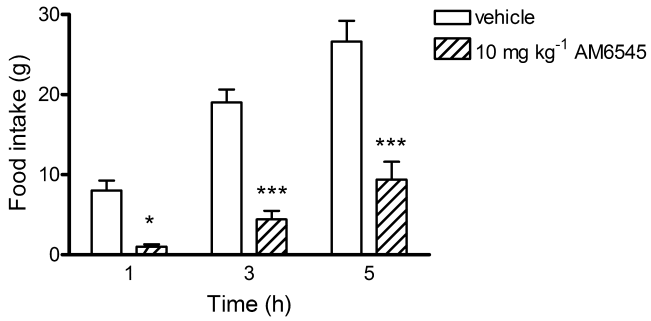

Figure 5

The effect of vehicle (4% DMSO, 1% Tween 80 in physiological saline) or AM6545 (10 mg·kg^{-1}) on food intake in vagotomized rats. Bars represent the mean ± s.e.mean, n = 5. *P < 0.05 and ***P < 0.001 denote a significant difference from vehicle control analysed using two-way mixed design ANOVA with time as the repeated measure followed by Bonferroni's *post hoc* test.

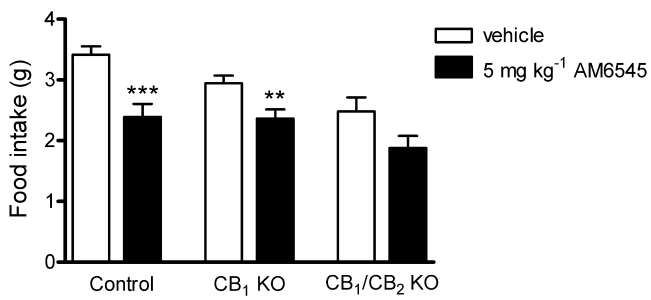

Figure 6

The effect of vehicle (4% DMSO, 1% Tween 80 in physiological saline) or AM6545 (5 mg·kg^{-1}) on food intake in control (wild-type or C57BL/6J), CB_1 receptor knockout (CB_1 KO) or CB_1/CB_2 receptor double knockout (CB_1/CB_2 KO) mice. Bars represent the mean ± s.e.mean, n = 5–14. **P < 0.01 and ***P < 0.001 denote a significant difference from vehicle control analysed using *t*-test.

CB_1 knockout mice with a significant (treatment-time) interaction, $F(3,63) = 17.15$, $P < 0.0001$, and significant treatment effect, $F(1,63) = 30.84$, $P < 0.0001$. Food intake was reduced at 17 h ($P < 0.001$) in mice receiving 20 mg·kg^{-1} AM6545 (1.65 ± 0.21 g) compared with vehicle-treated mice (3.12 ± 0.24 g). At earlier time points, AM6545 (20 mg·kg^{-1}) did not modify food intake ($P > 0.05$) in CB_1 knockout mice (1 h, 0.08 ± 0.02 g; 2 h, 0.09 ± 0.02 g; 3 h, 0.11 ± 0.03 g) compared with vehicle-treated mice (1 h, 0.16 ± 0.03 g; 2 h, 0.29 ± 0.03 g; 3 h, 0.50 ± 0.06 g). AM6545 at the lower dose of 5 mg·kg^{-1} inhibited 17 h food intake in control mice (P = 0.0004) and in CB_1 knockout mice (P = 0.009), but had no effect in CB_1/CB_2 double knockout mice (P = 0.08) compared with vehicle (Figure 6).

Discussion

We report the actions of a novel cannabinoid CB_1 receptor antagonist, AM6545, on food intake and body weight in rats and mice. This compound is a neutral antagonist of CB_1 receptors and shows limited penetration into the CNS. Furthermore, we demonstrated the ability of AM6545 to inhibit short-term food intake, and to reduce body weight gain, in rats without the potential for inducing aversive reactions. As AM6545 has limited brain penetrance, these findings suggest that the hypophagic

actions of CB_1 receptor antagonists are not dependent on actions in the CNS, making AM6545 of potential therapeutic value for the treatment of obesity and its complications.

Brain penetration studies showed that at doses, route of administration and time points which were relevant to the other investigations in the present study, the levels of AM6545 in the brain were much lower than the centrally active compound, AM4113, suggesting limited penetration of the brain by this compound. AM6545 showed selectivity for CB_1 receptors over CB_2. To determine the CB_1 selective, functional activity of AM6545, we examined its ability to block a well-established cannabinoid-mediated action in the GI tract. The action of the cannabinoid CB_1/CB_2 receptor agonist WIN55212-2 on the motility of the GI tract is well documented, with this compound slowing gastric emptying (Fichna *et al.*, 2009), upper GI transit (Carai *et al.*, 2006), colonic propulsion (Pinto *et al.*, 2002) and whole GI transit (Carai *et al.*, 2006) in mice. Furthermore, the action of the cannabinoid-induced inhibition of physiological GI transit has been shown to be mediated by peripherally located CB_1 receptors (Izzo *et al.*, 2000). AM6545 blocked the inhibitory action of WIN55212-2 on colonic propulsion in a dose-dependent manner while having no effect on the inhibitory action of a μ-opioid receptor agonist, confirming its functionality as a peripherally active, specific CB_1 receptor antagonist. Cannabinoid receptor antagonists such as AM251 (present study) and rimonabant (Landsman *et al.*, 1997) are in fact inverse agonists, blocking constitutive activity of the receptor, while others, such as AM4113 (Chambers *et al.*, 2007; Sink *et al.*, 2008), are neutral antagonists which block the receptor binding site preventing agonist stimulation. A forskolin-stimulated cAMP assay confirmed that while AM251 acts as an inverse agonist, further stimulating the production of cAMP, AM6545 behaved as a neutral antagonist. Together, these data confirm the CB_1 receptor specific, neutral antagonist characteristics of AM6545.

Antagonist/inverse agonist actions at CB_1 receptors are widely acknowledged to reduce food intake in both lean and obese laboratory animals and in obese humans. However, CB_1 receptor antagonist/inverse agonists can induce such side effects as nausea. Rimonabant was shown to induce nausea in humans during clinical trials, with 8.7% of patients reporting the side effect compared with 3.6% of patients who received placebo (Rosenstock *et al.*, 2008). Investigating the potential of a compound to induce nausea in laboratory rodents is made more difficult by the subjective nature of the sensation; however, models do exist. One such model is the conditioned taste avoidance test. Here, a flavoured food or liquid is paired with a compound and subsequent avoidance of this flavour can reflect the potential of the compound to be noxious and induce illness. Rimonabant (10 $mg \cdot kg^{-1}$) (De Vry *et al.*, 2004) and AM251 (8 $mg \cdot kg^{-1}$) (McLaughlin *et al.*, 2005) have been shown to induce conditioned taste avoidance in rats, suggesting they possess the potential to induce nausea. In the present study, AM6545 did not produce conditioned taste avoidance when paired with a flavour of Ensure Liquid diet, whereas in separate experiments, rats avoided consumption of the flavour of diet that was paired to the reference compound, LiCl. However, one drawback to this model is that conditioned taste avoidance is also observed with compounds which possess rewarding properties such as amphetamine (Berger, 1972). To further confirm that AM6545 does not have the potential to induce nausea, it was also tested in a more selective model of nausea, the taste reactivity test in rats. When noxious compounds are paired with an intra-oral infusion of saccharin solution, rats display gaping of the mouth and a reduction in the number of hedonic behaviours; these effects are only observed in response to compounds which produce emesis in animals capable of vomiting and not to rewarding drugs (Parker, 2003; Parker *et al.*, 2008). The CB_1 receptor antagonist/inverse agonist AM251 induced gaping in the taste reactivity test in rats (McLaughlin *et al.*, 2005); however, the neutral antagonist AM4113 did not demonstrate a potential to induce nausea in this paradigm (Sink *et al.*, 2008). We also did not observe gaping in response to AM6545 in the present study, suggesting that this peripherally restricted, neutral CB_1 antagonist does not induce nausea in two rat models.

In rats fed a chow diet, acute systemic administration of rimonabant inhibits short-term food intake (Arnone *et al.*, 1997; Ward *et al.*, 2009) and operant food intake (McLaughlin *et al.*, 2003; De Vry *et al.*, 2004). Acute systemic administration of AM251 also inhibits short-term food intake in rats (McLaughlin *et al.*, 2003; 2005) and mice (Riedel *et al.*, 2009), and in rats, this effect is still observed 5 days post-injection (Chambers *et al.*, 2004). We report that acute systemic administration of AM6545 dose-dependently inhibited food intake in the short term, with significant reductions observed 3 h after an injection of a 10 $mg \cdot kg^{-1}$ dose in rats. A reduction in food intake by AM6545 in rats has also been demonstrated by another group (Randall *et al.*, 2010), further confirming the effect. In an attempt to further characterize the mechanism of action of AM6545 on short-term food intake, we investigated its action in CB_1 knockout and CB_1/CB_2 receptors double knockout mice and

found that while food intake was reduced in the absence of CB_1 receptors, there was no effect of AM6545 on feeding in the absence of both CB_1 and CB_2 receptors. It has been shown that CB_2 receptor ligands can inhibit food intake in C57BL/6 mice (Onaivi *et al.*, 2008), suggesting that the hypophagic action of AM6545 may involve the CB_2 receptor. Furthermore, AM6545 showed no affinity for an additional 61 targets investigated in the Nova Screen 'side effect' profile assay (Caliper Life-Sciences, Hanover, MD, USA; data not shown), further suggesting the specificity of the compound.

To further characterize the effect of AM6545 on feeding, we investigated the effect of a 7 day chronic treatment schedule on body weight change and food intake. After 4 days of daily injections of AM6545, body weight gain was inhibited and this effect persisted until the end of the study despite the fact that there was not a significant inhibition in food intake after day 5. This trend was also observed in lean rats administered rimonabant once daily for 14 days (Colombo *et al.*, 1998). Tolerance to the effect on food intake was observed at treatment day 5 while the inhibitory action on body weight gain continued for 7 days after rimonabant treatment ended (Colombo *et al.*, 1998). However, tolerance did not develop to the inhibitory action of AM251 (Chambers *et al.*, 2006) and AM4113 (Chambers *et al.*, 2007) on food intake in chronic studies where the compound was administered daily for 15 and 5 days, respectively. The current findings suggest that the inhibitory actions of AM6545 on body weight gain may not be solely due to an inhibition of food intake. Further investigations are required to determine the effect of AM6545 on energy expenditure.

As we have shown that AM6545 inhibits food intake and has limited brain penetration, it was of interest to investigate the role of the vagus nerve in the mediation of the hypophagic action of AM6545. Short-term food intake was inhibited by AM6545 in rats with a complete subdiaphragmatic truncal vagotomy implying that an intact vagus nerve is not essential in the mediation of this effect. The site of action of AM6545 in these studies remains to be determined. The circumventricular organs (CVOs) that lack a blood-brain-barrier are one potential site mediating the effects of AM6545. The area postrema is believed to be an important CVO site in the regulation of feeding (Cottrell and Ferguson, 2004). While it is not known whether cannabinoid CB_1 receptors are expressed in other CVOs, it has been shown that they are present in the area postrema (Partosoedarso *et al.*, 2003; Van Sickle *et al.*, 2003). The present study also revealed the interesting finding that AM6545-induced hypophagia in vagotomized rats was enhanced compared with rats with an intact vagus nerve. Indeed, the effect of AM6545 on food intake in naïve rats was only seen 3 h post-injection whereas in vagotomized rats AM6545 inhibited food intake sooner (at 1 h post-injection) and the effect was observed for as long as 5 h post-injection. Gómez *et al.* (2002) reported that the inhibitory action of rimonabant on food intake in rodents was abolished by capsaicin-induced deafferentation, suggesting that the effect was mediated in the periphery. Receptor targets for peripherally released orexigenic and anorexigenic compounds are found in the nodose ganglia and these receptors are translocated to either central or peripheral terminals (Dockray, 2009). It is known that gene and protein expression levels of these receptors in the nodose ganglia are altered in response to differing feeding states (Dockray, 2009). This suggests that the hypophagic action of AM6545 may be suppressed by the action of endogenous compounds acting via the vagus nerve.

Pavon *et al.*, (2006) reported that a neutral CB_1 receptor antagonist LH-21 with limited penetration to the CNS inhibited food intake and body weight gain in obese rats. However, it has since been reported that LH-21 is an inverse agonist at CB_1 receptors and that the brain to plasma ratio following a single intravenous injection of LH-21 is 1:1, demonstrating that it is highly brain penetrant (Chen *et al.*, 2008). It was also reported that CB_1 receptor antagonists with little potential to cross the blood-brain-barrier reduced body weight of obese mice (McElroy *et al.*, 2008). Recently, LoVerme *et al.* (2009) have described the effects of a peripherally restricted mixed CB_1 receptor antagonist/CB_2 receptor agonist, URB447, on food intake. They showed that URB447 inhibited short-term food intake in lean mice and inhibited food intake and reduced body weight in genetically obese *ob/ob* mice during a chronic treatment period (LoVerme *et al.*, 2009). The authors attribute these findings to the antagonism of peripheral CB_1 receptors rather than the agonist stimulation of CB_2 receptors. However, we have shown in the present study that while AM6545 has a much greater affinity for CB_1 over CB_2 receptors, and that AM6545 blocks CB_1 receptor-mediated inhibition of colonic propulsion, its effects on food intake may involve actions at CB_2 receptors.

In conclusion, the present study has revealed that hypophagia mediated by cannabinoid receptors is not solely dependent on central activation. AM6545 is a neutral cannabinoid receptor antagonist with limited penetration into the CNS that is also devoid of aversive effects. These valuable

characteristics indicate that peripherally restricted neutral cannabinoid receptor antagonists could be developed for the treatment of obesity and its complications, potentially without the psychiatric side effects observed with centrally active CB_1 receptor antagonists.

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Conflicts of interest

Two authors (AM and VKV) are co-applicants for a patent application which covers the synthesis and characterization of AM6545 (Makriyannis A, Vemuri VK, Thotapally R, Olszewska T. International Patent Application No: PCT/US09/01054) to be published in 2010. There are no other conflicts to declare.

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