JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.
JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737

JPET # 118737

Anti-obesity efficacy of a novel cannabinoid-1 receptor inverse agonist MK-0364 in rodents

Tung M. Fong, Xiao-Ming Guan, Donald J. Marsh, Chun-Pyn Shen, D.Sloan Stribling, Kim M. Rosko, Julie Lao, Hong Yu, Yue Feng, Jing C. Xiao, Lex H.T. Van der Ploeg, Mark T. Goulet, Williams K. Hagmann, Linus S. Lin, Thomas J. Lanza, Jr., James P. Jewell, Ping Liu, Shrenik K. Shah, Hongbo Qi, Xinchun Tong, Junying Wang, Suoyu S. Xu, Barbara Francis, Alison M. Strack, D.Euan MacIntyre, and Lauren P. Shearman

Departments of Metabolic Disorders (T.M.F., X.MG., D.J.M., C.P.S., J.L., H.Y., Y.F., J.C.X., L.H.T.V.P.), Pharmacology (D.S.S., K.M.R., A.M.S., D.E.M., L.P.S.), and Medicinal Chemistry (M.T.G., W.K.H., L.S.L., T.J.L., J.P.J., P.L., S.K.S., H.Q., X.T., J.W., S.S.X.), Merck Research Laboratories, Rahway, NJ 07065; and Department of Imaging Research, Merck Research Laboratories, West Point, PA 19486 (B.F.)

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

Copyright 2007 by the American Society for Pharmacology and Experimental Therapeutics.

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

Running title: MK-0364, receptor occupancy and anti-obesity efficacy

Corresponding author: Tung M. Fong, Merck Research Laboratories, R80M-213, PO Box 2000, Rahway, NJ 07065

732-594-6711

fax: 732-594-3337

tung_fong@merck.com

Number of text pages: 24

Number of tables: 2

Number of figures: 10

Number of references: 40

Number of words in Abstract: 234

Number of words in Introduction: 681

Number of words in Discussion: 825

Abbreviations:

CB1R, cannabinoid-1 receptor; CB2R, cannabinoid-2 receptor; d-Fen, dexfenfluramine; DIO, diet-induced obesity; THC, tetrahydrocannabinol.

Recommended section: Neuropharmacology

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

The cannabinoid-1 receptor (CB1R) has been implicated in the control of energy balance. To explore the pharmacological utility of CB1R inhibition for the treatment of obesity, we evaluated the efficacy of MK-0364 and determined the relationship between efficacy and brain CB1R occupancy in rodents. MK-0364 was shown to be a highly potent CB1R inverse agonist that inhibited the binding and functional activity of various agonists with a binding K_i of 0.13 nM for the human CB1R *in vitro*. MK-0364 dose-dependently inhibited food intake and weight gain, with an acute minimum effective dose of 1 mg/kg in diet-induced obese (DIO) rats. CB1R mechanism-based effect was demonstrated for MK-0364 by its lack of efficacy in CB1R-deficient mice. Chronic treatment of DIO rats with MK-0364 dose-dependently led to significant weight loss with a minimum effective dose of 0.3 mg/kg (P.O.), or a plasma C_{max} of 87 nM. Weight loss was accompanied by the loss of fat mass. Partial occupancy (30 – 40%) of brain CB1R by MK-0364 was sufficient to reduce body weight. The magnitude of weight loss was correlated with brain CB1R occupancy. The partial receptor occupancy requirement for efficacy was also consistent with the reduced food intake of the heterozygous mice carrying one disrupted allele of CB1R gene compared to the wild-type mice. These studies demonstrated that MK-0364 is a highly potent and selective CB1R inverse agonist and is orally active in rodent models of obesity.

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

Cannabinoid-1 receptor (CB1R) is a G-protein coupled receptor predominantly expressed in the nervous system, and has been identified as the brain receptor for exogenous molecules such as tetrahydrocannabinol (THC) (Berry and Mechoulam, 2002; Howlett et al., 2002; Pertwee, 2005; Thakur et al., 2005). Endogenous agonists (endocannabinoids) include anandamide and 2-arachidonyl glycerol which have been found in the brains of vertebrates. These endocannabinoids are released from membrane precursors upon stimulation and play an important role in modulating intracellular cAMP level, ion channel activity and synaptic transmission (Schlicker and Kathmann, 2001; Wilson and Nicoll, 2002).

In the species that have been studied thus far, CB1R is expressed in several brain regions including hypothalamus, amygdala, hippocampus, basal ganglia, cortex and cerebellum (Breivogel and Childers, 1998). In both amygdala and hippocampus, electrophysiological studies have revealed that CB1R agonists inhibit the release of GABA and glutamate, acting primarily at presynaptic CB1R (Schlicker and Kathmann, 2001; Wilson and Nicoll, 2002). Conversely, CB1R inverse agonists have been shown to increase synaptic transmission (Auclair et al., 2000; Wallmichrath and Szabo, 2002; Melis et al., 2004). Taken together, these data suggest that modulation of neurotransmitter release in the brain underlies the molecular and cellular basis of the *in vivo* pharmacological effect of CB1R ligands.

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

The anatomical localization of CB1R in the hypothalamus, hippocampus, amygdala and cortex suggests that CB1R and endocannabinoids may modulate food intake and energy expenditure (Berthoud, 2002; Seeley and Woods, 2003; Schwartz and Porte, 2005; Swanson, 2005). Marijuana use is associated with increased appetite in humans (Greenberg et al., 1976). Experimental animal studies indicated that the active ingredient in marijuana, Δ^9-THC, is responsible for the appetite enhancing effect through activation of CB1R (Trojniar and Wise, 1991). Cannabinoid agonists have also been shown to increase the reward value of food (Gardner and Vorel, 1998). Conversely, CB1R inverse agonist has been shown to inhibit the intake of food in lean rodents, diet-induced obese (DIO) rodents, marmosets or humans (Arnone et al., 1997; Gardner and Vorel, 1998; Simiad et al., 1998; Freedland et al., 2000; Di Marzo et al., 2001; Blundell et al., 2006). In addition to the hypophagic effect, inverse agonists were shown to increase energy expenditure (Liu et al., 2005). Upon chronic administration of CB1R inverse agonists, weight loss has been observed in rodents and humans (Colombo et al., 1998; Van Gaal et al., 2005). The involvement of CB1R in energy balance is further supported by the observation that the endocannabinoid level appears to be modulated by leptin, an adipocyte-derived hormone. Acute treatment of rats with 125 μg of leptin led to a significant reduction of endocannabinoids (anandamide and 2-arachidonyl glycerol) in the hypothalamus (Di Marzo et al., 2001).

Previous studies indicated that disruption of both copies of CB1R gene led to a lean phenotype in mice and resistance to DIO (Cota et al., 2003; Ravinet Trillou et al., 2004;

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

Osei-Hyiaman et al., 2005). However, body weight phenotype of the heterozygous mice has not been reported. In addition, it has not been reported whether complete blockade or partial inhibition of CB1R is required for chronic weight loss efficacy, while variable occupancy has been reported for different compounds to achieve acute food intake inhibition (Need et al., 2006). In the present studies, we evaluated the *in vitro* and *in vivo* pharmacological properties of a novel compound MK-0364 (Lin et al., 2006). MK-0364 is structurally distinct from previously reported inverse agonists such as rimonabant or SLV319 (Rinaldi-Carmona et al., 1994; Lange et al., 2004). MK-0364 was shown to be a highly potent and selective CB1R inverse agonist. It inhibited food intake acutely in both mice and rats, and caused reduction of body weight and fat mass following 14 days of once-a-day oral administration. It was further demonstrated that partial brain CB1R occupancy was sufficient for efficacy, and weight loss efficacy was correlated with brain CB1R occupancy. The partial receptor occupancy requirement for efficacy recapitulated the reduced food intake phenotype of the heterozygous mice carrying one copy of disrupted CB1R gene, providing consistent pharmacological and genetic validation for the development of MK-0364 as a new anti-obesity agent.

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

Methods

Materials and animals

MK-0364, AM-2233 and [^{125}I]AM-2233 (specific activity 2000 Ci/mmol) were synthesized at Merck. AM251 and CP55940 were purchased from Tocris Cookson (Ellisville, MO). AM251 is a close analog of SR141716 (Thakur et al., 2005). Dex-fenfluramine (d-Fen) was purchased from Research Biochemicals Inc (Natick, MA). All animal testing protocols were reviewed and approved by the Merck Research Laboratories Institutional Animals Care and Use Committee in Rahway, NJ.

In vitro pharmacology

The binding assay was performed by incubating various concentrations of MK-0364 with 0.5 nM [^{3}H] CP55940, 1.5 μg of recombinant human CB1R-CHO membranes (or 0.1 μg of human CB2R-CHO membranes) in 50 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 2.5mM EDTA, 0.5mg/mL fatty acid free Bovine Serum Albumin (BSA), 1 X proteinase inhibitor mix (P8340, Sigma), and 1% DMSO. After 1 hr incubation at 37 ^{o}C, the reaction was stopped by filtration and bound radioligand was separated from free radioligand by washing the filter plate. Total specifically bound radiolabel was approximately 10% of the total added radiolabel. Inhibitory IC_{50} values were calculated through non-linear curve fitting, from which K_i values were then calculated. The CB1R density (B_{max} = 5 pmol/mg based on [^{3}H] CP55940 binding) in the recombinant human CB1R-CHO membranes was close to that from rat brain membranes (3 -5 pmol/mg).

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

The intrinsic activity was measured by incubating recombinant CB1R-CHO cells with various concentrations of MK-0364 in the presence of 10 μM forskolin, 200 μM phosphodiesterase inhibitor, 500 nM 3-isobutyl-1-methylxanthine (IBMX) in the assay buffer (Earle's balanced salt solution supplemented with 5 mM $MgCl_2$, 10 mM HEPES pH7.3, 1 mg/mL BSA) at room temperature for 30 min. Cells were lysed by boiling and intracellular cAMP level was determined using the cAMP SPA kit (Amersham). Since CB1R is a Gi-coupled receptor, in the absence of forskolin, cannabinoid compounds do not change intracellular cAMP level. When adenylyl cyclase is activated by forskolin, activation of Gi by CB1R in the presence of agonist (such as CP55940) will lead to an inhibition of the forskolin-stimulated cAMP increase, and inverse agonist will lead to a further increase of the forskolin-stimulated cAMP increase. The maximal CP55940-mediated inhibition of forskolin-stimulated cAMP increase is defined as 100 % agonist efficacy, and the intrinsic activity of all other compounds is relative to the efficacy of CP55940. Negative efficacy denotes inverse agonism.

For selectivity counter-screen, 170 radioligand binding or enzymatic assays were carried at MDS Pharma Services as a contract service to Merck. A summary of each assay protocol and the reference for each assay are listed in the MDS Pharma catalog.

Body temperature measurement

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

Male Sprague-Dawley rats (139-181 g body weight; 6 weeks old) from Charles River Laboratories (Wilmington, MA) were used. Rats were fed *ad libitum* with pelleted standard rodent chow (Harlan Teklad Diet 7012, 5% fat; 3.75 kcal/gm; Madison, WI). Animals were maintained on a 12/12 hour light-dark cycle (lights off at 4:00 PM). They were randomized into groups (6 rats/group) for compound and vehicle dosing. Animals were first dosed with vehicle (10% Tween80 in water, iv) or the CB1R inverse agonist MK-0364 (3 mg/kg, iv) 30 minutes prior to challenge with vehicle (1%Tween80 in saline, ip) or the cannabinoid receptor agonist CP55940 (1 mg/kg, ip). Acute measurement of body temperature was accomplished using a microprobe thermocouple thermometer (model BAT 10 thermometer and RET-2 temperature probe; Physitemp Instruments Inc., Clifton, NJ). Measurements were performed at T = -30, 0, 60 and 75 minutes following CP55940 administration. Studies were conducted during the mid-light cycle (9:00 AM to 1:00 PM).

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

Mouse acute food intake and weight change

For the first study involving only wild-type mice, male C57BL/6N wild-type mice were used. MK-0364 was dissolved or dispersed (with sonication) as a fine homogeneous suspension in 0.225% methylcellulose/10% Tween 80 in water for subsequent oral dosing of mice. All mice were weighed, and vehicle (0.225% methylcellulose/10% Tween 80 in water) or MK-0364 (1 or 3 mg/kg) was administered by oral gavage to male mice approximately 30 min prior to the onset of the dark phase of the light cycle (n = 12 per group, age 23 weeks, mean body weight 34.14 ± 0.53 g). Mice were fed *ad libitum* in the dark phase following

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

dosing. A preweighed aliquot of a highly palatable medium high fat diet (Research Diets D12266Bi; 25% kcal from sucrose, 32% kcal from fat, 4.41 kcal/g) was provided in the food hopper of the cage 5 min prior to the onset of the dark phase of the light cycle and weighed 2 and 18 hr after the onset of the dark phase of the light cycle. Additionally, all mice were weighed 18 hr after the onset of the dark phase of the light cycle. The study was of crossover design, i.e., vehicle and 1 mk/kg groups were dosed first. After a 4-day wash out, the previous vehicle group was dosed with 3 mg/kg of MK-0364, and the previous 1 mg/kg group was dosed with vehicle.

A second study was conducted comparing drug effect in wild-type and CB1R-deficient mice which were obtained from A. Zimmer (Zimmer et al., 1999). These mice were backcrossed onto the C57BL/6J genetic background for ten generations by A. Zimmer prior to homozygous CB1R-deficient mice being rederived at Taconic Farms onto the C57BL/6N genetic background. The resulting heterozygous mice were intracrossed to yield mice of all three possible genotypes (wild-type; heterozygous; homozygous). The resulting wild-type and knockout mice were then used to set up time-matched homozygous and wild-type intracrosses. These intracrosses were used to generate age-matched wild-type and knockout mice. Mice were maintained at all times on an irradiated regular rodent chow diet (Harlan Teklads 7012) except during the time of assay. At the time of the study, average body weight was 27 g (SEM = 0.1 g) for the wild-type group, and 24 g (SEM = 0.4 g) for the CB1R-deficient group.

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

Acute studies in DIO rats

For acute experiments, male Sprague-Dawley DIO rats from Charles River Laboratories were raised from 4 weeks of age on a diet moderately high in fat (32% kcal) and high in sucrose (25% kcal) (D12266B from Research Diets, New Brunswick, NJ). Animals were used at 12 weeks of age and were maintained on a 12/12 hour light dark cycle. The rats were randomized into groups (n=6 rats/group) for compound and vehicle dosing. Rats were weighed 17 hours after dosing to determine effects on overnight body weight gain. MK-0364 was administered orally to DIO rats 1 hour before the start of the dark cycle (3 PM), at 0.3, 1, 3 mg/kg (p.o.). Vehicle was 10% Tween 80 in water, and dosing volume was 2 mL/kg. Powdered food was provided in food cups which were weighed continuously at 5 minute intervals over 18 hours and the data were recorded using a computerized system.

Chronic studies in DIO rats

For the 14-day chronic experiment, male Sprague-Dawley DIO rats were obtained as described above. Animals were used at 15 weeks of age and were maintained on a 12/12 hour light-dark cycle. Rats were conditioned to dosing for 4 days prior to baseline measurements, using an oral gavage of 10% Tween 80 in water. Thereafter, animals were dosed daily with vehicle (10% Tween 80), MK-0364 (0.3, 1, or 3 mg/kg) or dexfenfluramine (d-Fen, 3 mg/kg). Compounds or vehicle were administered at 1 hour before the dark cycle for 14 days. Body composition was measured by dual energy X-ray densitometry (DEXAscan) 5 days prior to the study and at the end of the 14-day study. Daily endpoints

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

included body weight and food intake (changes on day-14 was 18-hour change while changes on days 1-13 were 24-hour changes). Terminal endpoints (18 hours after the last dose) included DEXAscan analysis, white adipose tissue weights, and trough brain and plasma MK-0364 levels. The epididymal fat pad was associated with the testes along the vas deferens. The mesenteric fat pad was abdominal from the base of stomach and along the intestine to the colon.

In a parallel pharmacokinetic study, a subset of DIO rats with indwelling femoral arterial catheters was given MK-0364 at 0.3 mg/kg, p.o., once daily for 14 days (n = 23 cannulated rats). To determine circulating concentrations of MK-0364 on day 1 or 7, blood was drawn prior to dosing and then at 15 and 30 minutes, and 1, 2, 4, 6, and 8 hours post dosing. Some animals were euthanized at 2 hours post dose on day-14 in order to evaluate brain exposure to MK-0364.

Ex vivo receptor occupancy measurement

Ex vivo receptor occupancy was determined by dosing MK-0364 or vehicle to rats, followed by cutting brain sections for subsequent *in vitro* binding of [^{125}I]-AM2233 which is a non-selective cannabinoid agonist (Gifford et al., 2002). Occupancy of CB1R in drug-treated animals can be determined by comparison with vehicle-treated animals.

In the single dose study, non-fasted lean SD rats (male, 350-400 g) were dosed with MK-0364 (0.3, 1, 3, 10 or 30 mg/kg, p.o.) or vehicle (10% Tween 80 in water). Two hours

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

later, rats were euthanized by CO_2 asphyxiation. Plasma samples were taken by cardiac puncture, and brains were quickly dissected and frozen in isopentane at – 40°C and stored at – 80°C until use. The degree of brain receptor occupancy was determined by quantitative receptor autoradiography *in vitro*. Thin coronal brain sections (20 µm) encompassing the substantia nigra were cut on a cryostat at – 17°C, and thaw-mounted onto microslides and dried completely at room temperature. Receptor binding was initiated by incubating the brain sections in the binding buffer (Tris.HCl: 50 mM, pH. 7.3, $MgCl_2$: 2 mM, $CaCl_2$: 1 mM, KCl: 5 mM, BSA: 1.0 %) containing 0.5 nM [^{125}I]-AM2233 for 30 minutes at room temperature. Non-specific binding was defined by similar incubation conditions in the presence of 100 nM MK-0364. Brain sections were washed 3 times for 4 minutes in ice-cold washing buffer (Tris.HCl: 50 mM, pH. 7.3, $MgCl_2$: 2 mM, $CaCl_2$: 1 mM, KCl: 5 mM, Triton X-100: 0.05% (v/v)), and then rinsed briefly in ice-cold distilled water before drying at room temperature. Brain sections were exposed to X-ray film overnight. Autoradiographic images were captured and analyzed with MCID/M2 image analysis system. Relative optical density was determined for vehicle- or compound-treated groups (n=3 for vehicle or each dose of compound treatment). Specific binding was obtained by subtracting the non-specific binding (average pixel intensity of the area above substantia nigra in the presence of 100 nM MK-0364) from the corresponding total binding (average pixel intensity of the entire coronal section at the level including the substantia nigra). The maximal specific binding was defined by specific binding in the vehicle-treated rat brains. Oral dosing of MK-0364 resulted in a dose-dependent reduction of [^{125}I]-AM2233 binding to CB1R, and % receptor occupancy is derived by the reciprocal of % maximal binding.

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

To demonstrate that there was no significant dissociation of the bound MK-0364 from the tissue sections during incubation of radioligand binding, an *in vitro* control study was performed prior to the occupancy assay. Two equivalent sets (A and B) of coronal brain sections from naïve rats (7 slides each) encompassing the same regions as the occupancy assay were pre-incubated with rising concentrations of MK-0364 (0, 1, 10, 100, 1000, 5000 and 10000 nM) in the binding buffer (see above) for 30 min at room temperature. Following a brief rinse with the buffer, both sets were subjected to [^{125}I]-AM2233 binding following the same protocol as the occupancy assay except that set A contained the radioligand plus the same rising concentrations of MK-0364 as in the pre-incubation, whereas set B contained radioligand only. The absence of a significant rightward shift of competition binding curve in set B with respect to set A suggested a lack of significant dissociation of pre-bound MK-0463 from tissues under these conditions (data not shown).

In the 14-day chronic study, male DIO rats (543 g average body weight, 16% body fat, n=8 per group) were dosed orally once a day for 14 days with vehicle (10% Tween 80 in water), MK-0364 (0.1, 0.3, 1, or 3 mg/kg), or AM251 (3 mg/kg). Rats were dosed 1 hour before the beginning of the dark cycle. Food intake and body weight were recorded daily. Fourteen days later, half of each treatment group (n=4) was euthanized at either 2 or 24 hours after the last dose. Brains were collected for receptor occupancy studies. Blood was collected for compound level determination.

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

Results

In Vitro Pharmacological Characterization

The apparent binding affinity of MK-0364 for the recombinant human or rat CB1R or CB2R is summarized in Table 1. It binds to human or rat CB1R with an IC_{50} of 0.3 and 0.4 nM, respectively, corresponding to a K_i value of 0.13 nM and 0.27 nM, respectively. MK-0364 binds to the human or rat CB2R with an IC_{50} value of 290 and 470 nM, respectively, corresponding to a K_i value of 170 nM and 310 nM, respectively. The selectivity ratio of CB1R over CB2R is approximately 1000-fold. As shown in Figure 1A, MK-0364 is an inverse agonist at CB1R because it causes a further increase of forskolin-induced cAMP increase, an opposite effect compared to the effect of an agonist such as CP-55940. AM251 is also an inverse agonist, consistent with previous report (Pertwee, 2005). The competitive antagonist activity of MK-0364 was demonstrated by determining the dose response curve of the synthetic agonist CP55940 in the presence of various concentrations of MK-0364. MK-0364 shifted the agonist dose response to the right, and the K_b value from Schild's plot for MK-0364 against CP55940 is 0.5 nM (Figures 1B and 1C). MK-0364 similarly shifted the dose response curve of methanandamide (data not shown).

Off-target activity of MK-0364 was evaluated in 170 assays of receptors, enzymes, ion channels and transporters. Other than activity at CB1R and CB2R, none of the 170 assays

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

revealed more than 50% inhibition by MK-0364 at concentrations less than 1 μM. Only 11 targets were inhibited with IC_{50} values between 1 – 10 μM (Table 2). Thus, MK-0364 showed more than 1000-fold selectivity over all tested targets.

Reversal of agonist-induced hypothermia by MK-0364 in rats

Agonist-induced hypothermia is one of the typical cannabinoid agonist effects observed in rodents. MK-0364 was evaluated in rats to determine its effect in reversing agonist-induced hypothermia. The vehicle treated group had an average basal body temperature of 37 ^{o}C. The CB1/2R receptor agonist, CP-55940, elicited a robust 4 ^{o}C temperature decrease over the course of the study (60 min: - 4.2 ^{o}C; 75 min: - 4.0 ^{o}C; $p<0.0001$ vs. vehicle). Prior treatment with MK-0364 blocked this hypothermic response completely at both time points examined (60 min: 0.4 ^{o}C, $p<0.0001$ vs. CP55940; 75 min: 0.3 ^{o}C, $p<0.0001$ vs. CP55940). When monitored over the initial 30 minute period prior to CP55940 challenge, MK-0364 alone did not cause a temperature change when compared to vehicle (Figure 2).

Acute Efficacy of MK-0364 on Food Intake and Body Weight Gain in Wild-type and CB1R-Deficient Mice

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

MK-0364 dose-dependently inhibited 2 hr and overnight food intake, as well as overnight gains in body weight in C57BL/6N mice (Figure 3). At the 1 and 3 mg/kg doses (PO), MK-0364 significantly inhibited 2 hr food intake (36% and 69% reductions, respectively; $P < 0.05$ and $P < 0.00001$, respectively) and overnight food intake (13% and 40% reductions, respectively; $P < 0.05$ and $P < 0.00001$, respectively), as well as overnight gains in body weight (48% and 165% reductions, respectively; $P < 0.01$ and $P < 0.00001$, respectively). Animal behavior was not significantly affected by the treatment of MK-0364 based on visual inspection.

Based upon the dose response study described above, a second study was carried out in which *ad libitum* fed male wild-type mice ($n = 10$) were compared to male CB1R-deficient mice ($n = 9$) in their response to MK-0364 at 3 mg/kg. MK-0364 significantly inhibited 2-h food intake (65% reduction; $P < 0.00001$), overnight food intake (28% reduction; $P < 0.00001$) and overnight gains in body weight (73% reduction; $P < 0.00005$) in wild-type mice. In contrast, MK-0364 had no significant effect on any of these parameters in CB1R-deficient mice (Figure 4).

Acute Efficacy of MK-0364 on Food Intake and Body Weight Gain in Diet Induced Obese Rats

The average cumulative overnight food intake for vehicle treated DIO rats was 18.8 g and the average body weight gain was 9.5 g (Figure 5). The profile and extent of food

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

intake in animals receiving MK-0364 (0.3 mg/kg) were similar to that of vehicle treated animals. MK-0364 (1 and 3 mg/kg, p.o.) decreased cumulative food intake significantly from 2 hours post-dosing until the end of the study. Average cumulative food intake overnight in the MK-0364 (1 mg/kg, p.o.) group was 12.2 g. At this dose, food intake was reduced by 61% at 3 hours ($p=0.0003$ vs. vehicle), 60% at 6 hours ($p=0.001$ vs. vehicle), and 35% at 17 hours ($p=0.004$ vs. vehicle). Average cumulative food intake overnight for rats given MK-0364 (3 mg/kg, p.o.) was 9.6 g, and at this dose food intake was reduced by 64.4% at 3 hours ($p<0.0001$ vs. vehicle), 70% at 6 hours ($p<0.0001$ vs. vehicle), and 49% at 17 hours ($p=0.0004$ vs. vehicle). MK-0364 administration (1 and 3 mg/kg, p.o.) also led to significant decreases in body weight of 3 g ($p=0.001$ vs. vehicle) and 10 g ($p<0.0001$ vs. vehicle), respectively (Figure 5B).

Two-week Efficacy of MK-0364 on Food Intake and Body Weight Gain in Diet Induced Obese Rats

Chronic weight loss efficacy of MK-0364 was evaluated in DIO rats. At all doses tested, MK-0364 decreased food intake, body weight gain and adiposity (Figure 6). Average starting body weight was 530.0 g ± 6.8 g. Average weight gain of the vehicle treated group over 13 days was 15 ± 4 g. Final body weight changes on day-13 from starting weight in the 0.3, 1, and 3 mg/kg groups were - 3 ± 6 g, - 6 ± 4 g and - 19 ± 6 g, respectively. The relative weight loss compared to vehicle was 4 %, 5 %, 7 % for the 0.3, 1, 3 mg/kg groups,

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

respectively, at the end of the study. The final weight loss in the 1 mg/kg MK-0364 group was comparable to that of the 3 mg/kg d-Fen group, while MK-0364 at 3 mg/kg caused a bigger weight loss than d-Fen at 3 mg/kg. Mesenteric and epididymal fat pad weights decreased significantly ($p<0.05$) in a dose-dependent manner with MK-0364 treatment (Figure 6C). There was no significant effect on lean mass (data not shown).

MK-0364 treatment resulted in a dose-related decrease in food intake that achieved statistical significance for the 0.3, 1 and 3 mg/kg groups (Figure 6A). The reductions in cumulative food intake in comparison to the vehicle group were 17.5 ± 4 %, 20.7 ± 1.8 %, and 21.4 ± 2.8 % for the 0.3, 1, and 3 mg/kg groups, respectively. Significant decreases in food intake were evident on day 1 for the 1 and 3 mg/kg MK-0364 groups, while the 0.3 mg/kg group showed significant decreases as early as day 2. In all three groups, significant food intake suppression was observed in at least 7 of the 14 days of the study. During the second week of MK-0364 treatment, daily food intake inhibition was maintained between 10-25% inhibition. Administration of d-Fen markedly inhibited food intake on day 1 of administration, and the extent of daily food intake inhibition declined on subsequent days, with a 15-30 % inhibition relative to vehicle for most of the study duration.

At the end of the 14-day efficacy study, the plasma levels (18 hr after the last dose) of MK-0364 was determined to be 3, 10, and 16 nM, respectively at 0.3, 1, and 3 mg/kg. The levels of MK-0364 in brain tissue (18 hr post last dose) were determined to be 0.068, 0.051,

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

and 0.1 nmol/g of tissue, respectively. Plasma cholesterol levels measured at the end of the study in the drug-treated groups were decreased compared to the vehicle group (82±3, 77±6, 69±3, 72±6 mg/dl in the vehicle, 0.3, 1, and 3 mg/kg groups, respectively). Only the cholesterol reduction in the 1 mg/kg MK-0364 group achieved statistical significance ($p<0.05$).

In a 14-day parallel PK study at 0.3 mg/kg (p.o.) of MK-0364 in DIO rats, plasma C_{max} was 87 nM after the first dose (Tmax=2 hr), and AUC(0-8h) was 332 nM*h. The brain tissue level of MK-0364 at 2 hr after the last dose was 0.09 nmol/g of brain tissue on day 14. There was no significant difference in the plasma level of MK-0364 after 1 or 7 doses, indicating a lack of accumulation or reduction of drug level over repeated doses. It should be pointed out that even though the total drug level in brain tissue reached 0.09 nmol/g at 0.3 mg/kg, this drug level does not necessarily represent drug available for receptor binding and likely represents significant amount of drug bound to brain tissue. Hence, direct CB1R occupancy was determined utilizing an *ex vivo* occupancy procedure.

CB1R Occupancy After Single Dose in Rats

CB1R occupancy by MK-0364 was determined in lean SD rats following oral administration of MK-0364 (0.3, 1, 3, 10 or 30 mg/kg, p.o.). As shown in Figure 7, there was a dose-dependent increase of CB1R occupancy as the dose was increased, and 50% receptor

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

occupancy was reached at approximately 100 nM of MK-0364 in the plasma at 2h post dosing. More than 90% receptor occupancy was reached at a total plasma level of 1 μM.

CB1R Occupancy of after Two-week Treatment in Diet Induced Obese Rats

To further correlate the CB1R occupancy requirement with chronic weight loss efficacy, another 14-day chronic study was performed with MK-0364 (at 0.1, 0.3, 1, or 3 mg/kg, p.o.) in which both CB1R occupancy and weight loss efficacy were measured. Two hours after the last dose of the 14-day treatment period, the CB1R occupancy and plasma compound level were determined, and 50% occupancy was reached at approximately 90 nM of MK-0364 in the plasma (Figure 8). This $OCCUPANCY_{50}$ value was very similar to that after single dose treatment (Figure 7), suggesting a lack of accumulation of MK-0364 in the brain with repeated dosing. In addition, the brain CB1R occupancy by MK-0364 declined slower than its clearance from plasma, so that part of the 24 hr occupancy-plasma level curve was shifted to the left compared to the 2 hr occupancy-plasma level curve (Figure 8).

After 14 days of once-a-day treatment, reduced weight gain (0.1 mg/kg of MK-0364) or weight loss (0.3 – 3 mg/kg of MK-0364, or AM251 at 3 mg/kg) were observed (Figure 9). The dose-dependent weight loss efficacy was correlated with occupancy at 2 hr for MK-0364. Weight loss efficacy of AM251 was close to the weight loss efficacy of MK-0364 at similar occupancy.

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

Haploinsufficiency of the heterozygous mice with CB1R gene disruption

We have evaluated the phenotype of wild-type mice, heterozygous mice with one disrupted allele of the CB1R gene and homozygous CB1R-deficient mice. As shown in Figure 10, four parameters were measured, including (1) cumulative weight gain from the age of 4 weeks to 22 weeks, (2) body composition at 21 - 23 weeks of age, (3) cumulative food intake from 4 to 22 weeks of age, and (4) cumulative feed efficiency from 4 to 22 weeks of age. The heterozygous mice exhibited a trend of intermediate phenotype in all 4 parameters between the wild-type mice and CB1R-deficient mice, although only two of the parameters (food intake and body composition) were statistically different from either wild-type mice or CB1R-deficient mice. In situ binding with [^{3}H] CP55940 confirmed the absence of radioligand binding in the homozygous mouse brain and reduced radioligand binding in the heterozygous mouse brain (data not shown). CB1R density in mouse brain membranes (B_{max}) based on [^{3}H] CP55940 binding also confirmed that the receptor was present at high levels in wild-type mice, could not be detected in CB1R-deficient mice, and was significantly reduced by 40-50% in heterozygous mice (data not shown). The subtle haploinsufficiency phenotype suggested a gene dosage effect of CB1R gene disruption, and the genetic evidence was consistent with the partial receptor occupancy requirement for weight loss efficacy of MK-0364.

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

The current studies evaluated the *in vitro* and *in vivo* pharmacological properties of MK-0364. The novel CB1R inverse agonist MK-0364 is structurally distinct from previously published CB1R ligands (Lin et al., 2006). In binding assays, MK-0364 is highly potent and selective, with a binding K_i of 0.13 nM at human CB1R and a selectivity ratio of at least 1000-fold against CB2R and 168 other targets (Tables 1 and 2). In whole cell functional assays, MK-0364 exhibits inverse agonist efficacy analogous to SR141716A (Bouaboula et al., 1997; Pertwee, 2005). When MK-0364 and an agonist were present together, MK-0364 competitively inhibited the agonist effect. These data demonstrated that MK-0364 is an inverse agonist when agonist is not present, and it can also inhibit the action of agonists when both are present. Further evidence supporting the inverse agonist interpretation of the data in Figure 1A is the existence of neutral antagonist O-2050 for human CB1R (Martin et al., 2002).

Consistent with the *in vitro* pharmacological activity, MK-0364 inhibited cannabinoid agonist-induced hypothermia in rats, demonstrating that MK-0364 is brain penetrating and inhibits one of the well known *in vivo* effects mediated by CB1R agonists (Compton et al., 1992). Studies utilizing regional injection of CB1R agonist have indicated that the hypothalamus is involved in the agonist-induced hypothermia (Fitton and Pertwee, 1982). Although CB1R agonists are known to induce hypothermia, inverse agonists such as SR141716A or MK-0364 do not cause hyperthermia (Rinaldi-Carmona et al., 1994 and

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

current studies). On the other hand, agonists increase food intake (Williams and Kirkham, 2002) while inverse agonists inhibit food intake (Arnone et al, 1997 and current studies). These data suggest that either the CB1R involved in the hypothermic response is not constitutively active or there is no endogenous agonist tone. In contrast, the opposite effects of agonists and inverse agonists on food intake suggest that the CB1R involved in feeding control is either constitutively active or activated by an endogenous agonist tone.

Furthermore, MK-0364 inhibited spontaneous food intake in both mice and rats, and the anorectic effect was observed only in wild-type mice but not in CB1R-deficient mice. Thus, the *in vivo* efficacy of MK-0364 is highly selective and mediated entirely by CB1R, consistent with the more than 1000-fold selectivity profile of MK-0364 as determined by *in vitro* assays.

With chronic treatment of MK-0364 in DIO rats, significant weight loss was accompanied by loss of fat mass. Mechanistically, at least a portion of body weight loss occurs as a consequence of a decrease in food ingested. While the maximum food intake inhibition occurred after the first day of treatment, lower level of food intake inhibition was sustained for the entire 2 weeks. The minimum effective dose for body weight reduction in 14 days was 0.3 mg/kg (C_{max} = 87 nM). Such a high *in vivo* potency of MK-0364 is consistent with the high binding affinity *in vitro*.

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

To further establish the relationship between efficacy and brain CB1R occupancy, we have developed an *ex vivo* receptor occupancy procedure to determine what level of CB1R occupancy is needed for efficacy. As shown in Figure 7, 50% occupancy can be achieved at 2h post a single dose at the plasma level of 100 nM in lean rats, and more than 90% occupancy can be achieved at the plasma level of 1000 nM. Since the minimum effective dose for chronic weight inhibition was 0.3 mg/kg with a C_{max} of 87 nM in DIO rats , the occupancy data suggested that approximately 30-40% occupancy is achieved at the minimum effect dose. As shown in Figure 8, 50% occupancy can be achieved at 2h after the last dose at the plasma level of approximately 90 nM in DIO rats. The data in Figures 7 and 8 thus indicated that the 2h occupancy vs plasma level relationship in lean rats after single dose is very similar to that in DIO rats after 14 repeated doses. The data in Figure 8 further suggested that the brain elimination of MK-0384 is slower than that of plasma elimination in rats, as the 24h occupancy vs plasma level curve is shifted to the left compared to the 2h occupancy vs plasma level curve. It should be pointed out that future *in vivo* occupancy measurement and perhaps an *in vivo* Positron Emission Tomography (PET) occupancy study will be desirable to confirm the current conclusion based on *ex vivo* occupancy data.

The above conclusion that partial occupancy is sufficient for efficacy was further demonstrated in the DIO rat weight loss study where both occupancy and efficacy were measured. As shown in Figure 9, chronic weight loss efficacy can be achieved with at least 30% CB1R occupancy by MK-0364 at 2 hr post-dose. Higher occupancy is correlated with higher efficacy. These data demonstrate that complete blockade of CB1R is not required for

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

efficacy in rodents. The requirement of partial occupancy for efficacy is also consistent with the genetic evidence demonstrating a subtle haploinsufficiency phenotype of the heterozygous CB1R mice, especially food intake and body composition phenotypes (Figure 10). This would contrast the efficacy of NPY Y5 receptor antagonist where more than 90% receptor occupancy is required for efficacy (Ishihara et al., 2006).

In summary, the novel CB1R inverse agonist MK-0364 was shown to be highly potent and selective both *in vitro* and *in vivo*. Chronic weight loss was accompanied by the loss of fat mass. Significant weight loss was observed at partial receptor occupancy, and weight loss efficacy was proportional to brain CB1R occupancy. These rodent data suggest that MK-0364 has the potential to be a new agent for the treatment of obesity.

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

Acknowledgements

We would like to thank Dr. Andreas Zimmer for allowing us to use the CB1R-deficient mice in these studies.

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

References

Arnone M, Maruani J, Chaperon F, Thiebot M, Poncelet M, Soubrie P and Le Fur G (1997) Selective inhibition of sucrose and ethanol intake by SR141716, an antagonist of central cannabinoid (CB1) receptors. *Psychopharmacol.* **132**:104-106.

Auclair N, Otani S, Soubrie P and Crepel F (2000) Cannabinoids modulate synaptic strength and plasticity at glutamatergic synapses of rat prefrontal cortex pyramidal neurons. *J Neurophysiol* **83**:3287-3293.

Berry EM and Mechoulam R (2002) Tetrahydrocannabinol and endocannabinoids in feeding and appetite. *Pharmacol. Ther.* **95**:185-190.

Berthoud HR (2002) Multiple neural systems controlling food intake and body weight. *Neurosci Biobehav Rev.* **26**:393-428.

Blundell JE, Jebb SA, Stiubbs RJ, Wilding JR, Lawton CL, Browning L, Whybrow S and Halford JCG (2006) Effcet of rimonabant on energy intake, motivation to eat and body weight with or without hypocaloric diet: the REBA study. *Obesity Rev.* **7 (Suppl 2)**: 104.

Bouaboula M, Perrachon S, Milligan L, Canat X, Rinaldi-Carmona M, Portier M, Barth F, Calandra B, Pecceu F, Lupker J, Maffrand JP, Le Fur G and Casellas P (1997) A selective inverse agonist for central cannabinoid receptor inhibits mitogen-activated protein kinase activation stimulated by insulin or insulin-like growth factor 1. Evidence for a new model of receptor/ligand interactions. *J Biol Chem.* **272**:22330-22339.

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

Breivogel CS and Childers SR (1998) The functional neuroanatomy of brain cannabinoid receptors. *Neurobiol. Dis.* **5**:417-431.

Colombo G, Agabio R, Diaz G, Lobina C, Reali R and Gessa GL (1998) Appetite suppression and weight loss after the cannabinoid antagonist SR141716. *Life Sci.* **63**:113-117.

Compton DR, Johnson MR, Melvin LS and Martin BR (1992) Pharmacological profile of a series of bicyclic cannabinoid analogs: classification as cannabimimetic agents. *J Pharmacol Exp Ther.* **260**:201-209.

Cota D, Marsicano G, Tschop M, Grubler Y, Flachskamm C, Schubert M, Auer D, Yassouridis A, Thone-Reineke C, Ortmann S, Tomassoni F, Cervino C, Nisoli E, Linthorst AC, Pasquali R, Lutz B, Stalla GK and Pagotto U (2003) The endogenous cannabinoid system affects energy balance via central orexigenic drive and peripheral lipogenesis. *J Clin Invest.* **112**:423-431.

Di Marzo V, Goparaju SK, Wang L, Liu J, Batkai S, Jarai Z, Fezza F, Miura GI, Palmiter RD, Sugiura T and Kunos G (2001) Leptin-regulated endocannabinoids are involved in maintaining food intake. *Nature* **410**:822-825.

Fitton AG and Pertwee RG (1982) Changes in body temperature and oxygen consumption rate of conscious mice produced by intrahypothalamic and intracerebroventricular injections of delta 9-tetrahydrocannabinol. *Br J Pharmacol* **75**:409-414.

Freedland CS, Poston JS and Porrino LJ (2000) Effects of SR141716A, a central cannabinoid receptor antagonist, on food-maintained responding. *Pharmacol. Biochem. Behav.* **67**:265-270.

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

Gardner EL and Vorel SR (1998) Cannabinoid transmission and reward-related events. *Neurobiol. Dis.* **5**:502-533.

Gifford AN, Makriyannis A, Volkow ND and Gatley SJ (2002) In vivo imaging of the brain cannabinoid receptor. *Chem. Phys. Lipids.* **121**:65-72.

Greenberg I, Kuehnle J, Medelson J and Bernstein J (1976) Effects of Marijuana use on body weight and caloric intake in humans. *Psychopharmacol.* **49**:79-84.

Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA, Felder CC, Herkenham M, Mackie K, Martin BR, Mechoulam R and Pertwee RG (2002) International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. *Pharmacol. Rev.* **54**:161-202.

Ishihara A, Kanatani A, Mashiko S, Tanaka T, Hidaka M, Gomori A, Iwaasa H, Murai N, Egashira S, Murai T, Mitobe Y, Matsushita H, Okamoto O, Sato N, Jitsuoka M, Fukuroda T, Ohe T, Guan X, MacNeil DJ, Van der Ploeg LH, Nishikibe M, Ishii Y, Ihara M and Fukami T (2006) A neuropeptide Y Y5 antagonist selectively ameliorates body weight gain and associated parameters in diet-induced obese mice. *Proc Natl Acad Sci U S A* **103**:7154-7158.

Lange JH, Coolen HK, van Stuivenberg HH, Dijksman JA, Herremans AH, Ronken E, Keizer HG, Tipker K, McCreary AC, Veerman W, Wals HC, Stork B, Verveer PC, den Hartog AP, de Jong NM, Adolfs TJ, Hoogendoorn J and Kruse CG (2004) Synthesis, biological properties, and molecular modeling investigations of novel 3,4-diarylpyrazolines as potent and selective CB(1) cannabinoid receptor antagonists. *J Med Chem* **47**:627-643.

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

Lin LS, Lanza TJ, Jr., Jewell JP, Liu P, Shah SK, Qi H, Tong X, Wang J, Xu SS, Fong TM, Shen CP, Lao J, Xiao JC, Shearman LP, Stribling DS, Rosko K, Strack A, Marsh DJ, Feng Y, Kumar S, Samuel K, Yin W, Ploeg LH, Goulet MT and Hagmann WK (2006) Discovery of N-[(1S,2S)-3-(4-chlorophenyl)-2- (3-cyanophenyl)-1-methylpropyl]-2-methyl-2- {[5-(trifluoromethyl)pyridin-2-yl]oxy}propanamide (MK-0364), a novel, acyclic cannabinoid-1 receptor inverse agonist for the treatment of obesity. *J Med Chem* **49**:7584-7587.

Liu YL, Connoley IP, Wilson CA and Stock MJ (2005) Effects of the cannabinoid CB1 receptor antagonist SR141716 on oxygen consumption and soleus muscle glucose uptake in Lep(ob)/Lep(ob) mice. *Int J Obesity* **29**:183-187.

Martin BR, Stevenson LA, Pertwee RG, Breivogel CS, Williams W, Mahadevan A and Razdan RK (2002) Agonists and silent antagonists in a series of cannabinoid sulfonamides., in *Symposium on the Cannabinoids,* p 2, International Cannabinoid Research Society, Burlington, Vermont.

Melis M, Pistis M, Perra S, Muntoni AL, Pillolla G and Gessa GL (2004) Endocannabinoids mediate presynaptic inhibition of glutamatergic transmission in rat ventral tegmental area dopamine neurons through activation of CB1 receptors. *J Neurosci* **24**:53-62.

Need AB, Davis RJ, Alexander-Chacko JT, Eastwood B, Chernet E, Phebus LA, Sindelar DK and Nomikos GG (2006) The relationship of in vivo central CB1 receptor occupancy to changes in cortical monoamine release and feeding elicited by CB1 receptor antagonists in rats. *Psychopharmacology (Berl)* **184**:26-35.

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

Osei-Hyiaman D, DePetrillo M, Pacher P, Liu J, Radaeva S, Batkai S, Harvey-White J, Mackie K, Offertaler L, Wang L and Kunos G (2005) Endocannabinoid activation at hepatic CB1 receptors stimulates fatty acid synthesis and contributes to diet-induced obesity. *J Clin Invest* **115**:1298-1305.

Pertwee RG (2005) Inverse agonism and neutral antagonism at cannabinoid CB1 receptors. *Life Sci.* **76**:1307-1324.

Ravinet Trillou C, Delgorge C, Menet C, Arnone M and Soubrie P (2004) CB1 cannabinoid receptor knockout in mice leads to leanness, resistance to diet-induced obesity and enhanced leptin sensitivity. *Int J Obes Relat Metab Disord.* **28**:640-648.

Rinaldi-Carmona M, Barth F, Heaulme M, Shire D, Calandra B, Congy C, Martinez S, Maruani J, Neliat G, Caput D, Ferrara P, Soubrie P, Breliere JC and Le Fur G (1994) SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. *FEBS Lett.* **350**:240-244.

Schlicker E and Kathmann M (2001) Modulation of transmitter release via presynaptic cannabinoid receptors. *Trends Pharmacol. Sci.* **22**:565-572.

Schwartz MW and Porte DJ (2005) Diabetes, obesity, and the brain. *Science* **307**:375-379.

Seeley RJ and Woods SC (2003) Monitoring of stored and available fuel by the CNS: implications for obesity. *Nat. Rev. Neurosci.* **4**:901-909.

Simiad J, Keane M, Keane PE and Soubrie P (1998) SR141716, a CB1 cannabinoid receptor antagonist, selectively reduces sweet food intake in marmoset. *Behav. Pharmacol.* **9**:179-181.

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

Swanson LW (2005) Anatomy of the soul as reflected in the cerebral hemispheres: neural circuits underlying voluntary control of basic motivated behaviors. *J Comp Neurol.* **493**:122-131.

Thakur GA, Nikas SP and Makriyannis A (2005) CB1 cannabinoid receptor ligands. *Mini. Rev Med. Chem.* **5**:631-640.

Trojniar W and Wise RA (1991) Facilitory effect of delta9-tetrahydrocannabinol on hypothalamically induced feeding. *Psychopharmacol.* **103**:172-176.

Van Gaal LF, Rissanen AM, Scheen AJ, Ziegler O, Rossner S and RIO Europe Study Group (2005) Effects of the cannabinoid-1 receptor blocker rimonabant on weight reduction and cardiovascular risk factors in overweight patients: 1-year experience from the RIO-Europe study. *Lancet* **365**:1389-1397.

Wallmichrath I and Szabo B (2002) Analysis of the effect of cannabinoids on GABAergic neurotransmission in the substantia nigra pars reticulata. *Naunyn Schmiedebergs Arch Pharmacol* **365**:326-334.

Williams CM and Kirkham TC (2002) Observational analysis of feeding induced by Delta9-THC and anandamide. *Physiol Behav* **76**:241-250.

Wilson RI and Nicoll RA (2002) Endocannabinoid signaling in the brain. *Science* **296**:678-682.

Zimmer A, Zimmer AM, Hohmann AG, Herkenham M and Bonner TI (1999) Increased mortality, hypoactivity, and hypoalgesia in cannabinoid CB1 receptor knockout mice. *Proc. Natl. Acad. Sci.* **96**:5780-5785.

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes

Reprint request:

Tung M. Fong

Merck Research Laboratories, R80M-213

PO Box 2000

Rahway, NJ 07065

tung_fong@merck.com

The current address of LHTVdP, MTG and JPJ are: Merck Research Laboratories, Boston, MA 02115

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

Legends for Figures

Figure 1: *In vitro* functional assay of MK-0364. (A) Inverse agonist activity of MK-0364 (diamond) or AM251 (open square) at the human CB1R. (B) Effect of MK-0364 on the dose response curves of the synthetic agonist CP55940 at the human CB1R. The dose response curve of CP55940 was determined in the absence (◊) or presence of various concentrations of MK-0364 (■, 1 nM; ▲, 10 nM; ♦, 100 nM; ●, 1000 nM). Data represent mean and SEM. (C) Schild's plot for MK-0364 antagonizing the response of CP55940. Diamond (♦) represents data derived from (B). Square (■) represents data from an independent experiment analogous to that of (B). (D) Structure of MK-0364.

Figure 2: MK-0364 (3 mg/kg, i.v.) inhibited cannabinoid receptor agonist CP55940 (1 mg/kg, ip)-induced hypothermia in Sprague-Dawley rats. Data represent mean values ± SEM (n = 6 per group). Circles (●) represent vehicle/vehicle treatment. Squares (■) represent vehicle/CP55940 treatment. Triangles (▲) represent MK-0364/CP55940 treatment. *, $p<0.0001$ vs. veh/veh. Statistical test was unpaired 2-tailed t-test.

Figure 3: Acute efficacy of MK-0364 in mice. (A) Inhibition of 2 hour food intake by MK-0364 (1 and 3 mg/kg, p.o.). (B) Inhibition of overnight food intake. (C) Inhibition of overnight weight gain. Data represent mean ± SEM (n=12 mice per group). * $P < 0.05$ vs. vehicle (Student t-test, 2-tailed 2-sample equal variance test).

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 4: Acute efficacy of MK-0364 in wild-type or CB1R-deficient mice. (A) Effect of MK-0364 (3 mg/kg, p.o.) on 2 hour food intake. (B) Effect of MK-0364 (3 mg/kg, p.o.) on overnight food intake. (C) Effect of MK-0364 (3 mg/kg, p.o.) on overnight body weight change. Data represent mean ± SEM (n=10 for wild-type mice, n=9 for CB1R-deficient mice). * $P < 0.0001$ vs. corresponding vehicle. # $P < 0.05$ vs. WT vehicle (Student t-test, 2-tailed 2-sample equal variance test).

Figure 5: Acute efficacy of MK-0364 in DIO rats. (A) Cumulative food intake. (B) Overnight body weight change. Data represent mean ± SEM (n = 6 rats per group). Statistical test was unpaired 2-tailed t-test. * $P < 0.01$, ** $P < 0.0001$ vs. vehicle.

Figure 6: Chronic MK-0364 effects in DIO rats. (A) Daily food intake. $P < 0.05$ vs vehicle was observed on days 2, 4, 7, 11-13 at 0.3 mg/kg of MK-0364, on days 1-4, 7-8 at 1 mg/kg of MK-0364, on days 1-4, 6-8, 11 at 3 mg/kg of MK-0364, on days 1-4, 6-8, 10-13 for d-Fen. (B) Body weight change. $P < 0.05$ vs vehicle was observed on days 7, 13 at 0.3 mg/kg of MK-0364, on all days at 1 and 3 mg/kg of MK-0364 and at 3 mg/kg of d-Fen. (C) Fat pad weight at end of study. * denotes $P < 0.05$ vs vehicle, ** denotes $P < 0.01$ vs vehicle. In all graphs, data represent mean ± SEM (n=7 to 8 rats per group).

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 7: *Ex vivo* CB1R occuapncy at 2 hr post dosing in rats as a function of plasma level of MK-0364 at 2 hr post dosing. Data represent mean ± SEM (n=3 rats).

Figure 8: *Ex vivo* CB1R occupancy at 2 hr or 24 hr after the last dose of 14-day treatment period. Data represent mean ± SEM (n=4 rats).

Figure 9: Correlation between 14-day weight loss efficacy and CB1R occupancy. Data represent mean ± SEM (n=4 rats for vehicle and MK-0364, n=3 rats for AM251). From regression analysis, $R^2 = 0.87$, $p = 0.02$.

Figure 10: Haploinsufficiency of male heterozygous mice with one disrupted allele of CB1R gene compared to male wild-type mice and CB1R-deficient mice. (A) Cumulative body weight gain over 18 weeks from 4 to 22 weeks of age. (B) Body composition analysis performed between 21 and 23 weeks of age. (C) Cumulative food intake over the same period as in A. (D) Cumulative feed efficiency over the same period as in A. * denotes $p<0.05$ vs the wild-type. # denotes $p<0.05$ vs the CB1R-deficient mice. N=11-18 per genotype.

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

Tables

Table 1

Binding K_i , inverse agonism EC_{50} and % activation values of MK-0364 [a].

	K_i , nM	EC_{50} , nM (%activation)
	mean ± SEM (n)	
Human CB1R	0.13 ± 0.01 (16)	2 ± 0.4 (- 120 %) (n=14)
Human CB2R	170 ± 9 (14)	230 ± 30 (- 230 %) (n=4)
Rat CB1R	0.27 ± 0.04 (6)	2 ± 0.4 (- 130 %) (n=2)
Rat CB2R	310 ± 36 (2)	nd (- 3 % @ 10 μM) (n=3)

[a] Binding K_i values were calculated from binding IC_{50} values based on the Cheng-Prussoff equation in which the K_d of CP55940 was determined by saturation binding of [^{3}H]CP55940. The 100% activation reference is defined by the maximal inhibition of forskolin-stimulated cAMP in response to the agonist CP55940. A negative value of % activation represents inverse agonism. Data represent mean ± SEM values with the number of independent measurements indicated in parentheses. nd, not determined.

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

Table 2

Selectivity profile of MK-0364 against 170 targets.

Target	K_i (μM) [a]
CB1R	0.00013
CB2R	0.17
angiotensin AT1R	4.7
adenosine A3R	3.6
melatonin MT1R	5.4
dopamines D1R	3.4
dopamine D3R	1.9
neurokinin NK2R	0.5
acetylcholinesterase	6.7 [b]
norepinephrine transporter	9.5
Ca channel (diltiazem site)	2.7
Na channel (batrachotoxine site)	1.3
K channel (MK-499 site)	2.3
157 other tested targets	>10.0 [b]

[a] K_i values are calculated from IC_{50} values based on the Cheng-Prussoff equation, and IC_{50} value represents the concentration of MK-0364 that inhibited radiolabeled ligand binding or

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

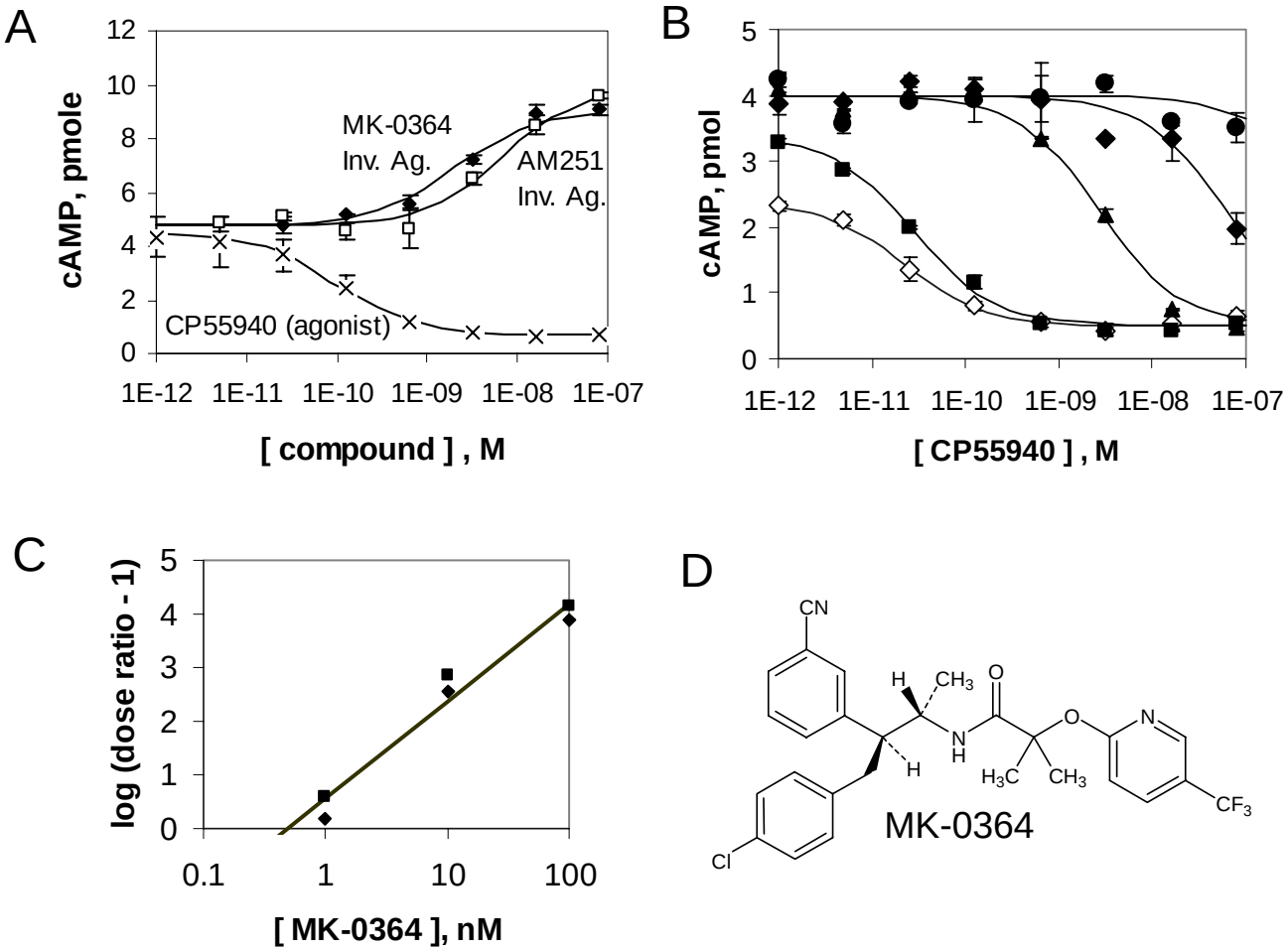
JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

enzyme activity by 50%. The list of tested targets can be found in the supplemental description. [b] These values are IC_{50} values.

Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

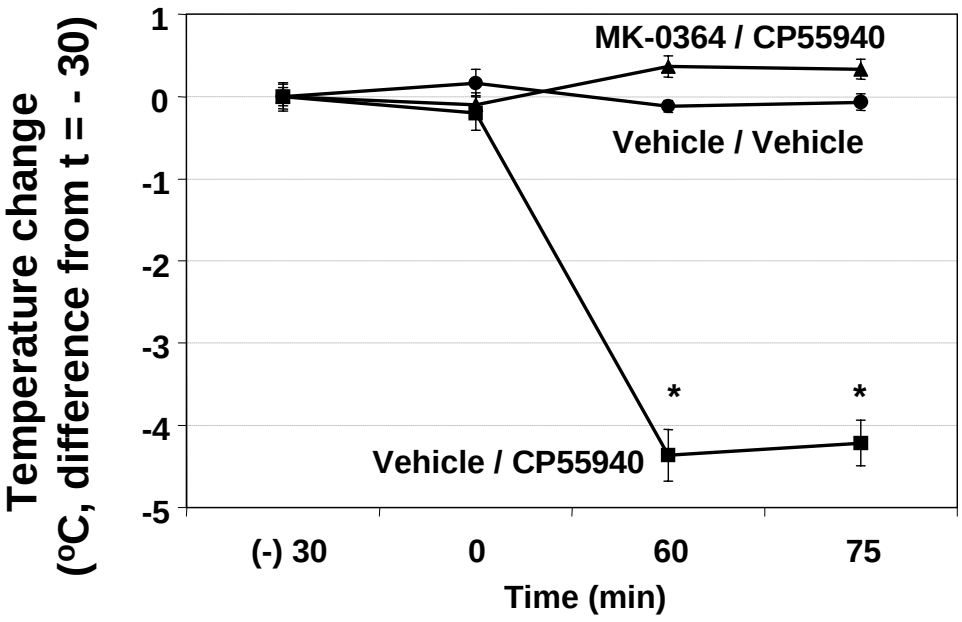
Figure 1



Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

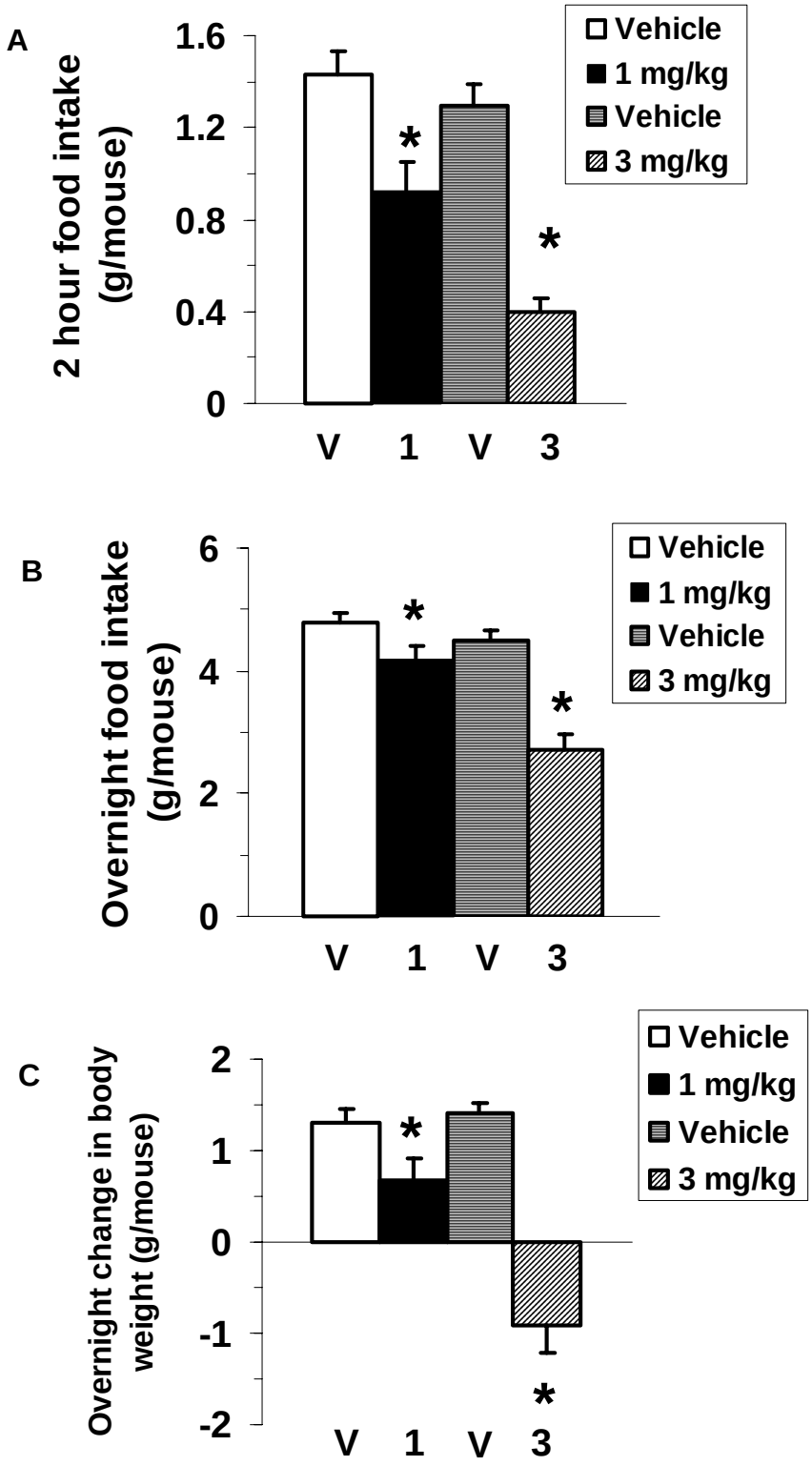
Figure 2



Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

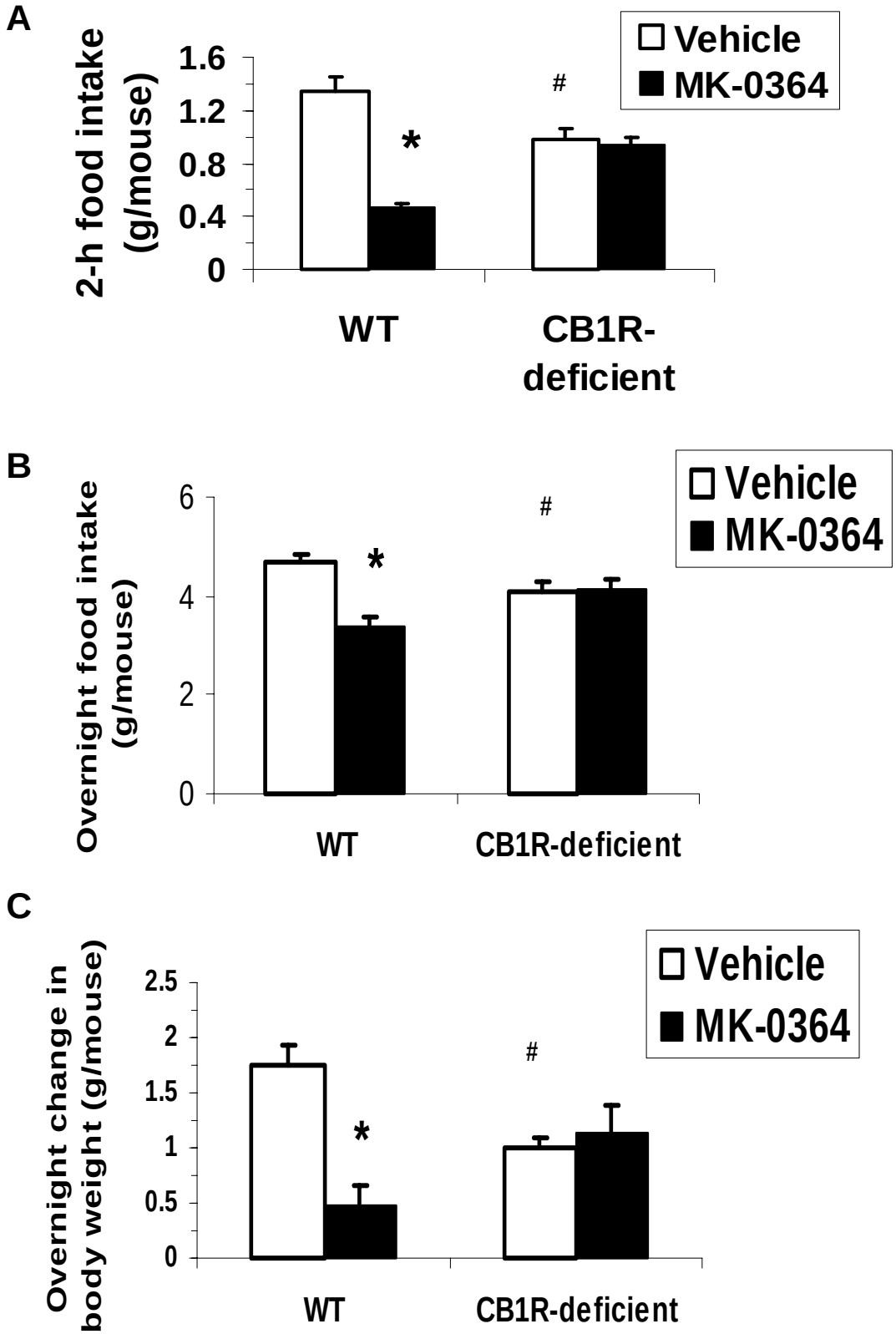
Figure 3



Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

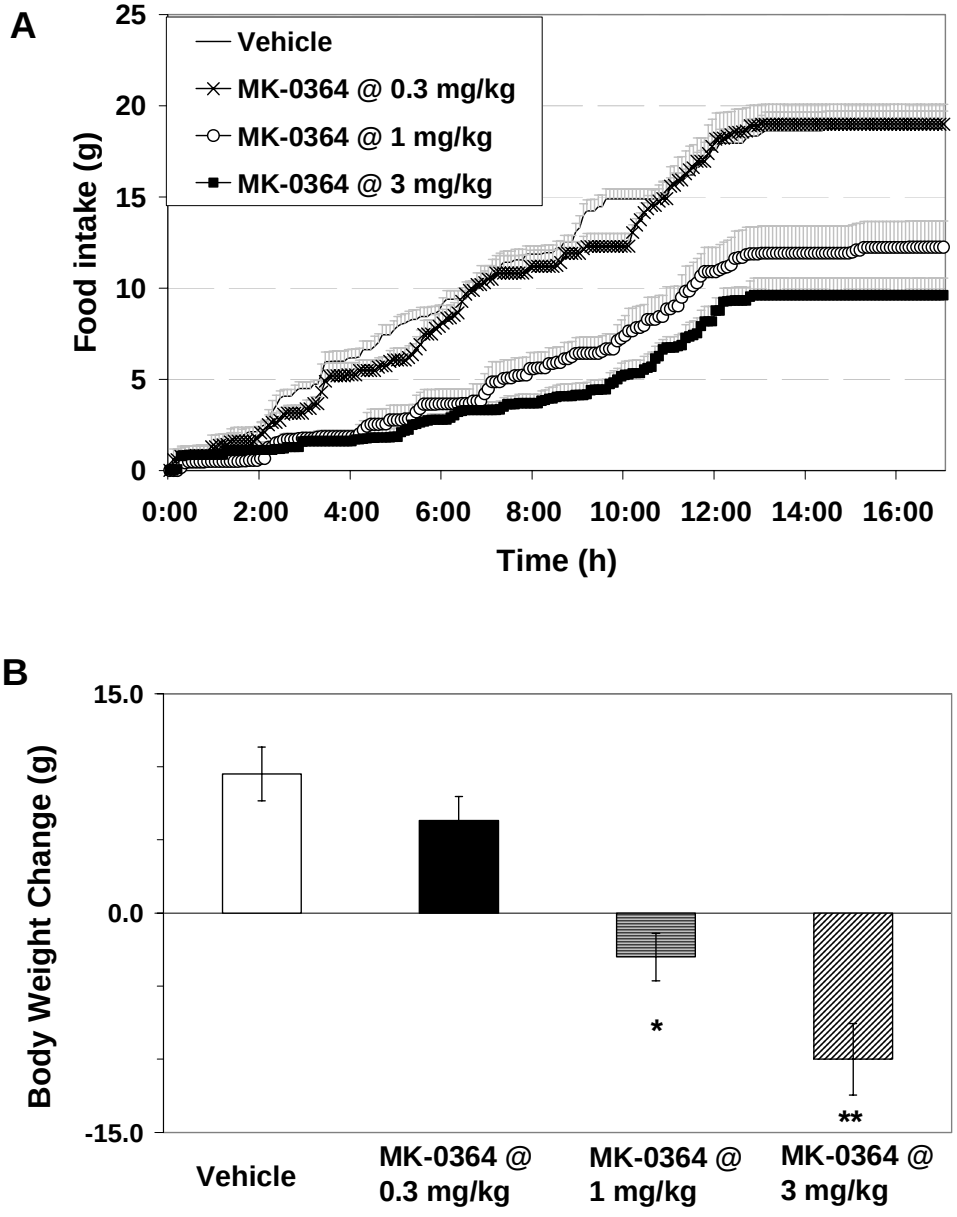
Figure 4



Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

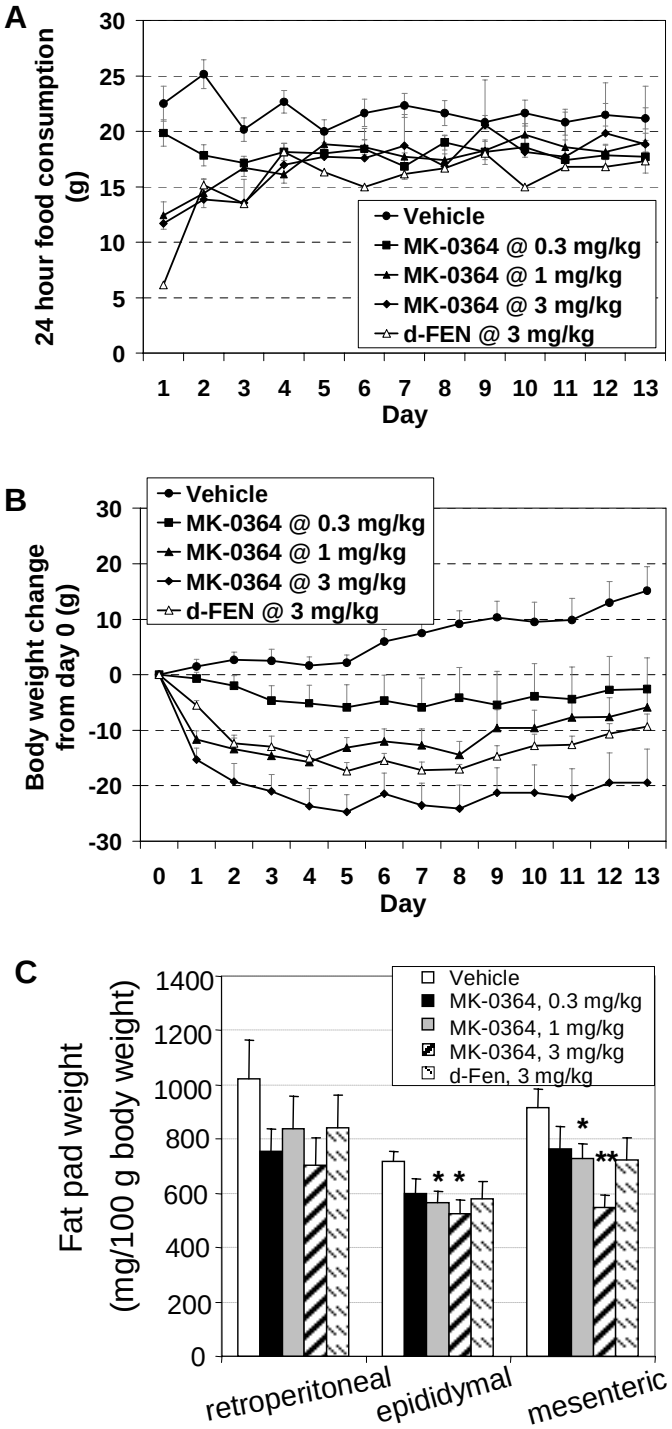
Figure 5



Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

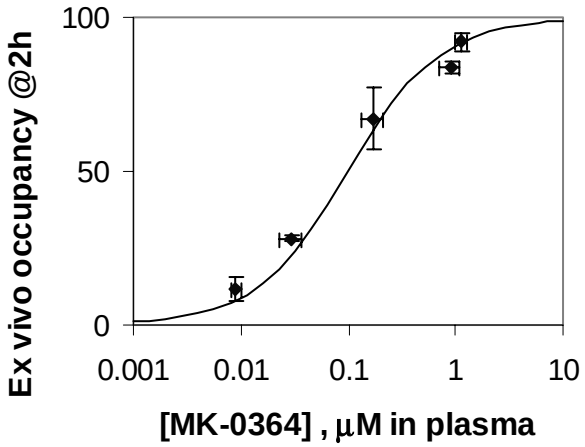
Figure 6



Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

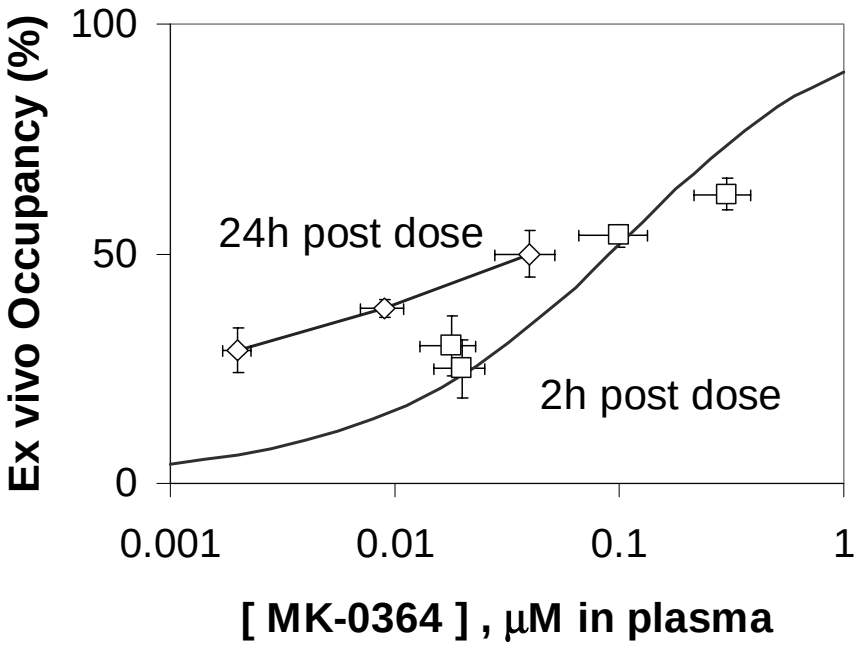
Figure 7



Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

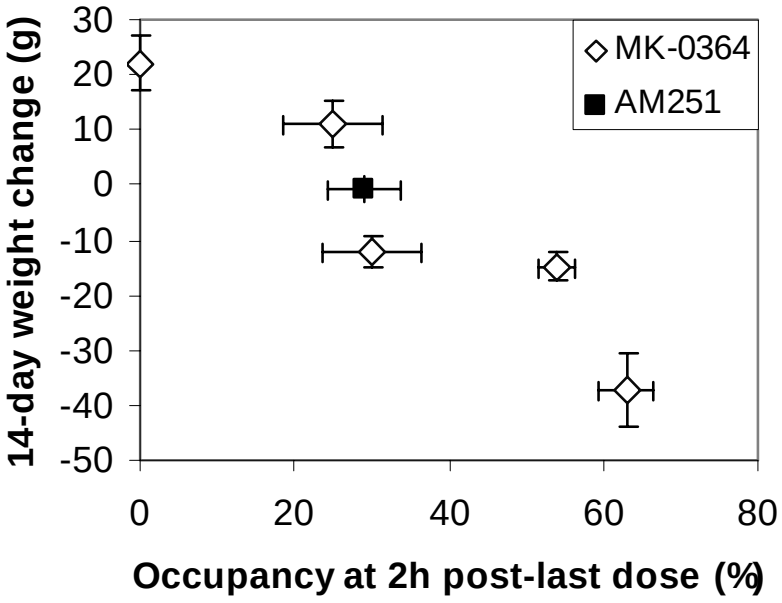
Figure 8



Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

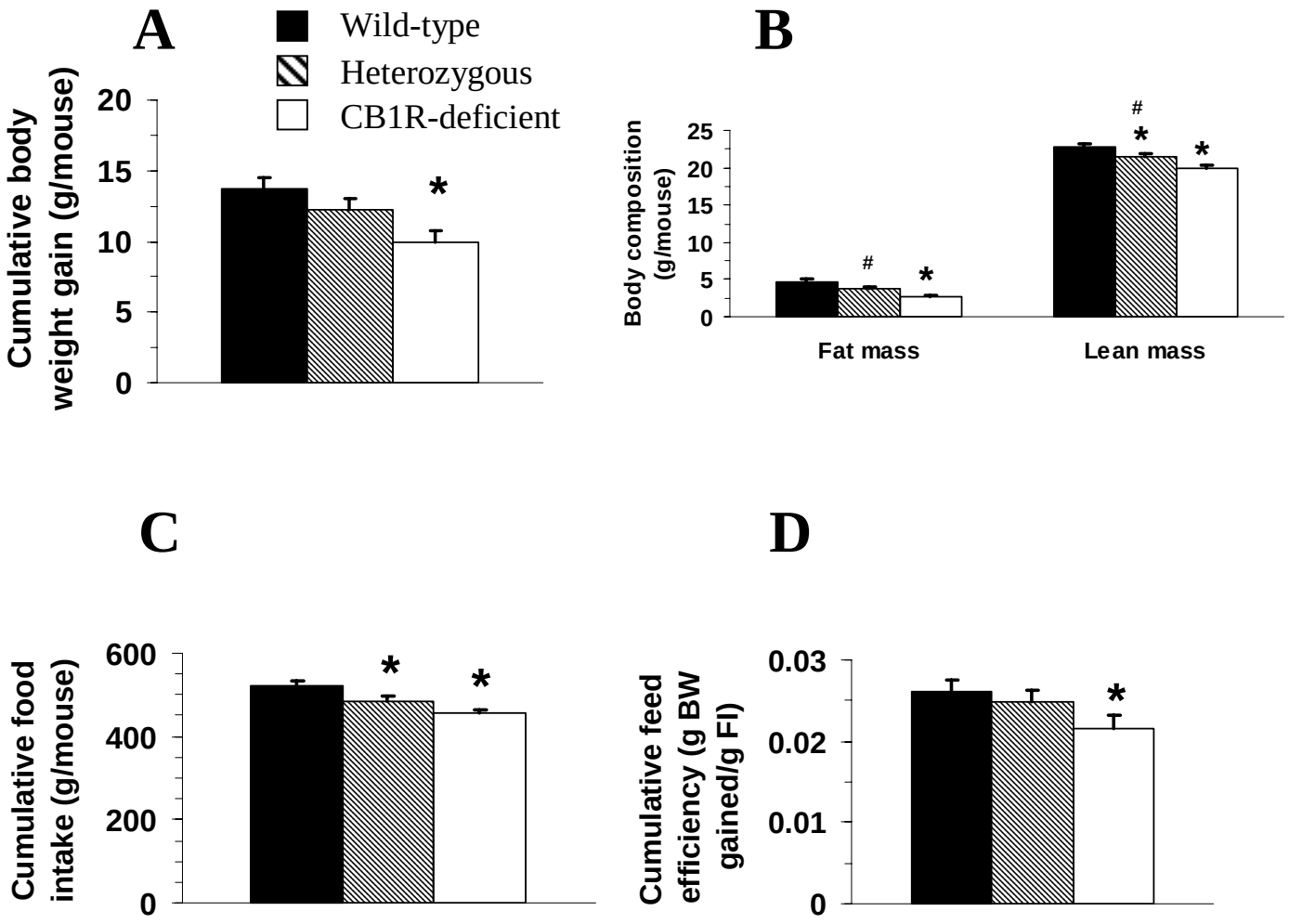
Figure 9



Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016

JPET Fast Forward. Published on February 27, 2007 as DOI: 10.1124/jpet.106.118737
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 10



Downloaded from jpet.aspetjournals.org at ASPET Journals on May 12, 2016