Title Page

Title: Pharmacologic inhibition of ghrelin receptor signaling is insulin sparing and promotes insulin sensitivity

Authors: Kenneth A. Longo, Ph.D.
Elizabeth K. Govek, M.S.
Anna Nolan, Ph.D.
Thomas McDonagh, B.S.
Soratree Charoenthongtrakul, B.S.
Derek J. Giuliana, M.S.
Kristen Morgan
Jeffrey Hixon
Chaoseng Zhou
Bruce Kelder
John J Kopchick
Jeffrey O Saunders, Ph.D.
Manuel Navia, Ph.D.
Rory Curtis, Ph.D.
Peter S. DiStefano, Ph.D.
Brad J. Geddes, Ph.D.

Laboratories of Origin:
Elixir Pharmaceuticals, Inc., 300 Putnam Ave., Cambridge, MA 02139, USA: (KAL, EKG, AN, TM, SC, DJG, KM, JH, CZ, JOS, MN, RC, PSD, BJG)
Edison Biotechnology Institute, Ohio University, Athens, OH, 45701, USA: (BK, JJK)
Running Title Page

Running title: Ghrelin antagonism promotes insulin sensitivity

Corresponding author:

Dr. Brad J. Geddes
Current Address:
Proteostasis Therapeutics, Inc.
200 Technology Sq., Suite 402
Cambridge, MA 02139
Telephone: 617.225.0096
Email: bgeddes@proteostasis.com

Text pages: 30

Number of Tables: 1

Number of Figures: 6

Number of References: 32

Number of words in:

Abstract: 250

Introduction: 483

Discussion: 1608

Non-standard abbreviations: AUC (Area Under the Curve), BW (Body Weight), FR (Food Restricted), FI (Food Intake), GhrR (Ghrelin Receptor), GTT (Glucose Tolerance Test), HFD (High Fat Diet), HG clamp (Hyperglycemic clamp), ip (Intraperitoneal), iv (Intravenous), KO (Knockout), LFD (Low Fat Diet), PF (Pair Fed), po (per oral), WT Wildtype), TC (Total Cholesterol)

Recommended Section: Drug Discovery and Translational Medicine
Abstract
Ghrelin influences a variety of metabolic functions through a direct action at its receptor, the GhrR (aka, GhrR-1a). Ghrelin knockout (KO) and GhrR KO mice are resistant to the negative effects of high fat diet (HFD) feeding. We have generated several classes of small molecule GhrR antagonists and evaluated whether pharmacologic blockade of ghrelin signaling can recapitulate the phenotype of ghrelin/GhrR KO mice. Antagonist treatment blocked ghrelin-induced and spontaneous food intake; however, the effects on spontaneous feeding were absent in GhrR KO mice, suggesting target-specific effects of the antagonists. Oral administration of antagonists to HFD-fed mice improved insulin sensitivity in both glucose tolerance and glycemic clamp tests. The insulin sensitivity observed was characterized by improved glucose disposal with dramatically decreased insulin secretion. Importantly, these results mimic those obtained in similar tests of HFD-fed GhrR KO mice. HFD-fed mice treated for 56 days with antagonist experienced a transient decrease in food intake but a sustained body weight decrease resulting from decreased white adipose, but not lean tissue. They also had improved glucose disposal and a striking reduction in the amount of insulin needed to achieve this. These mice had reduced hepatic steatosis, improved liver function, and no evidence of systemic toxicity relative to controls. Furthermore, GhrR KO mice placed on low- or high-fat diets had lifespans similar to the wild type, emphasizing the long term safety of ghrelin receptor blockade. We have therefore demonstrated that chronic pharmacologic blockade of the GhrR is an effective and safe strategy for treating metabolic syndrome.
Introduction

Ghrelin is a 28 amino acid peptide synthesized in the stomach and pancreas of mammals. Although the ghrelin receptor (GhrR; aka GHSR-1a) was originally cloned as the growth hormone secretagogue receptor (Howard et al, 1996), ghrelin has been shown to affect a variety of metabolic functions including increased food intake (FI), fat storage, gastrointestinal motility and growth hormone release. Uniquely, ghrelin is post-translationally modified with an octanoyl side chain on serine position 3 which is required for its activity at the GhrR (Smith, 2005). Removal of the acyl group renders the molecule completely inactive at the cognate GhrR. The GhrR is a member of the GPCR super family and its localization is consistent with its known biological functions. The highest concentrations of the receptor mRNA are in the arcuate nucleus of the hypothalamus and in the pituitary gland; lower but significant amounts are found in the peripheral neuraxis such as the nodose ganglia, nucleus tractus solitarius and pancreas (Zigman et al, 2006).

A growing body of evidence suggests that a loss of GhrR signaling improves the health of animals that have been fed a ‘westernized’ diet that is rich in fats and carbohydrates. GhrR knockout (KO) mice fed high-fat diets (HFDs) accumulated less adipose tissue but retained their lean tissue mass (Zigman et al., 2005; Longo et al., 2008). We recently reported that GhrR KO mice resisted diet-induced hepatic steatosis, and had higher insulin sensitivity and lower hyperinsulinemia (Longo et al., 2008). Lower FI, and not changes in energy expenditure, appeared to explain partially the mechanism by which these mice resisted diet-induced obesity (Longo et al., 2008). Interestingly, GhrR KO mice exhibited greater metabolic flexibility and a lower rate of intestinal dietary lipid absorption/secretion, which suggested that alterations in fuel usage and partitioning, as well as gastrointestinal effects, contributed to their relatively healthy phenotype (Longo et al., 2008). A subsequent analysis of multiple cohorts of HFD-fed GhrR mice confirmed the improved insulin sensitivity of GhrR KO mice and showed how this may be independent of decreases in BW (BW; Qi et al, 2011). Furthermore, ghrelin KO mice also had a better metabolic profile when fed a western diet (Wortley et al., 2004; Wortley et al., 2005). Thus, antagonism of the GhrR may constitute a unique and robust approach to managing the metabolic syndrome.
Indeed, reports have published recently describing classes of ghrelin antagonist that impart improvements in metabolic function. These include peptide-based inhibitors (Asakawa, 2003) and small molecules (Esler et al, 2007; Moulin et al, 2007; Rudolph et al, 2007) that affect BW gain FI and energy expenditure. In this work, we present evidence that antagonism of the GhrR with orally bio-available small molecules provides a new therapeutic modality for the simultaneous treatment of obesity and insulin resistance in mice. The results corroborate those observed in KO mice and provide further support that ghrelin signaling is important in the control of metabolic stress.

Methods

Animals

All animal studies were approved by Elixir Pharmaceuticals’ animal care and use committee. Starting at five weeks of age, male C57BL/6 mice (Taconic, Germantown, NY) were fed a high fat diet (HFD; 5.24 kcal/g, or 60% kcal, from fat; D12492; Research Diets, New Brunswick, NJ) for 14-16 weeks, with food and water available ad libitum. Mice were group-housed in ventilated cages (Thoren Caging Systems, Hazelton, PA) with enrichment (Igloos®, Bioserv, Frenchtown, NJ; Enviro-dri, PharmaServ, Framingham, MA) in a controlled environment (72°F, ~40% humidity, 12 hr light-dark cycles). Wild type (WT) and GhrR KO mice on a C57BL/6 background were bred from a single male founder at Charles River Laboratories (Wilmington, MA) (Sun et al., 2004). Mice were genotyped by a polymerase chain reaction assay (TransnetYX; Cordova, TN). Additionally, loss of functional GhrR signaling in these mice was verified by demonstrating lack of ghrelin-stimulated food intake (FI), growth hormone release and insulin resistance (Supplemental Figure 1). Mice were weaned at three weeks of age and then maintained on PicoLab rodent diet 20 (LFD; Purina; St. Louis, MO) until eight weeks of age. Starting at eight weeks of age, some male GhrR KO and WT mice were placed on HFD as described above. All experimental mice were housed individually for at least 5 days prior to study initiation. Except where noted in the text mice were tested in groups of n=8 (glucose tolerance test, GTT) or n=10 (FI and BW studies).
Pharmacology

Clonal Chinese hamster ovary cells expressing the human GhrR-aequorin system were obtained from Euroscreen (Belgium). Cells were cultured in complete Ham’s F12 media containing 10% fetal bovine serum, 100 IU/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL fungizone antimycotic in 0.85% saline, 400 µg/mL G418 (Geneticin), and 250 µg/mL Zeocin. Cells were cultured as monolayers in 75cm² cell culture flasks at 37°C with 5% CO₂, and were split and fed every 2 to 3 days. Cells grown to mid-log phase were removed from flasks by a gentle wash with PBS containing 5 mM EDTA (PBS-EDTA), followed by a 10 min incubation at 37°C in PBS-EDTA. Cells were recovered by centrifugation, then counted and resuspended at a density of 5 x 10⁶ cells/mL in BSA medium (DMEM/Ham’s F12 with HEPES, without phenol red, containing 0.1% bovine serum albumin). Coelenterazine h was added to the cells at 5 µM final concentration. The cells were protected from light and incubated at room temperature for 4-16 hr. The cells were diluted in BSA medium (1/10, v/v) and incubated with stirring for 1 hr at room temperature. Human ghrelin (Phoenix Pharmaceuticals, Inc., Burlingame, CA) was diluted in BSA medium and dispensed into black 96-well plates (50 µl/well). Test compounds were diluted to 100x final concentration in 100% DMSO, and 1 µl was added to each well. We injected 50 µl of stirring cell suspension into each well using a Luminoskan luminescent plate reader equipped with injectors (Thermo Fisher Scientific, Inc., Waltham, MA). Light emissions were recorded for 30 sec, integrated and analyzed using Luminoskan Ascent® software, resulting in one value representing the intensity of emitted light per test well. We generated IC₅₀ curves and Ki values for each compound with XLfit software (IDBS, Alameda, CA).

Effect of GhrR antagonist on ghrelin-induced FI

Individually housed, 10-week old, ad libitum LFD-fed, male C57BL/6 mice were used in these studies. On the morning of the experiment, food was temporarily removed from the cages and weighed. Mice then received an intraperitoneal (ip) injection (5 mL/Kg) of either vehicle-1 (4% DMSO, 10% β-OH-cyclodextran) or CpdD at 3, 10, or 30 mg/Kg. One minute later, vehicle-treated mice received a second ip injection of either vehicle-2 (ddH₂O) or ghrelin (5 mg/Kg). Immediately after the second ip injection, mice
were returned to their home cages and their food was returned. FI was measured 6 hr after the injections. In a separate experiment, continuous, overnight endogenous FI was measured in LFD-fed GhrR KO and WT mice dosed ip with vehicle or CpdD (30 mg/Kg, dosed 3 and 1 hr prior to lights off) using an Oxymax™ system (Columbus Instruments, Inc., Columbus, OH).

Repeated treatment of HFD-fed mice with GhrR antagonists

One week prior to study initiation, mice that exceeded 42 g BW were individually housed and acclimated to oral dosing with vehicle (bid, 5 mL/Kg) for one week at 09:00 and 17:00 hr. Body and food weights were recorded during afternoon dosing. (Mice that lost ≥ 10% of their original BW during the acclimation period were eliminated from the study.) Next, mice were sorted into four groups of equivalent mean BW (n=10/group). We then commenced vehicle and compound dosing for periods of 7, 14, 28, or 56 days, followed by a glucose tolerance test (GTT; see below). In order to determine whether constant dosing was required for observed effects of GhrR antagonist treatment an additional 7 day compound dosing study was performed in which one group of mice received all doses compound except for the final, pre-GTT dose. Similarly, in the 56 day study an additional group was included in which mice received only a single pre-GTT dose of compound or vehicle.

In some experiments, additional analyses were performed. The percent of glycated hemoglobin (%HbA1c) was determined using a DCA2000+ analyzer (Bayer, Indianapolis, IN). Plasma triglycerides, lipoproteins and cholesterol were determined using a Cholestech LDX analyzer (Cholestech, Hayward, CA). Mouse IGF-1 was measured by ELISA (R&D Systems, Minneapolis, MN). Plasma free fatty acids were measured using a kit from Sigma-Aldrich (St. Louis, MO). Additional biochemical markers in plasma were assessed by AniLytics (Gaithersburg, MD). Insulin resistance was calculated using the homeostasis model assessment of insulin resistance (HOMA-IR) using fasting plasma insulin (FPI, mU/L) and fasting blood glucose (FBG, mmol/L) in the following equation: HOMA-IR = (FPIxFBG)/22.5 (Matthews et al., 1985). Liver lipid content was determined by chloroform:methanol (2:1) extraction (Folch et al., 1957).
Glucose tolerance test (GTT)

On the evening prior to the experiment, BW was measured in all mice followed by an overnight (16 hr) fast. On the morning of the GTT, a 40 μL blood sample was collected by tail nick for determination of fasted blood glucose and plasma insulin. One hour later, mice were dosed with glucose (dose and route as indicated in text). Mice in the pharmacology studies received their normal dose of compound following the initial tail bleed, one hour prior to being given glucose. Tail blood was sampled at 15, 30, 60 and 120 min after the glucose dose for the determination of blood glucose and plasma insulin. Blood glucose was measured using Ascencia Elite glucometers (Bayer, Indianapolis, IN), and plasma insulin was measured by ELISA (CrystalChem, Downer’s Grove, IL). In one experiment, the capacity of mice to secrete insulin was tested using the insulin secretagogue repaglinide (R9028; Sigma-Aldrich, St. Louis, MO).

Hyperglycemic clamp (HG clamp)

HG clamps were performed on mice that had been dosed orally for 7 days with either vehicle or CpdB (45 mg/Kg, bid). The HG clamp methodology has been described previously (Qi et al., 2011).

Pair-feeding

Groups of mice were administered vehicle or CpdB and BWs and FI were recorded daily for 8 days. A third group was administered vehicle, and each animal within this group was fed daily with an amount of food equivalent to the mean daily FI of the CpdB-treated group (PF to CpdB). A fourth group of food restriction (FR to 60% Veh) was administered vehicle and fed daily with an amount of food equivalent to 60% of the mean daily FI of vehicle-dosed mice.

Immunohistochemistry

Liver and pancreas were fixed in 10% buffered formalin, embedded in paraffin and cut into 5 μm sections (Mass Histology, Worcester, MA). Sections were de-waxed in xylene, hydrated and stained with hematoxylin and eosin (H&E) for examination of liver morphology. Images of liver were acquired using a DP70, 12.5MP digital camera from
Olympus. Pancreas sections were de-waxed in xylene, hydrated and boiled for 10 minutes in a microwave oven in 10 mM sodium citrate (pH 6) for antigen retrieval. Primary antibodies used for pancreas staining were guinea-pig anti-insulin (Millipore, Billerica, MA) diluted 1:1000 and rabbit anti-glucagon diluted 1:200 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Biotinylated anti-guinea-pig or anti-rabbit secondary antibodies were used and detected with the Vectastain ABC kit and diaminobenzidine tetrahydrochloride (Vector Laboratories, Burlingame, CA). Sections were then counter-stained with H & E. Stained sections were permanently mounted with clarion mounting media (Sigma-Aldrich, St. Louis, MO). Images were acquired with a Spot digital camera (Micro Video Instruments, Avon, MA).

Statistical analyses of data

Data collected over time were analyzed by repeated measures two-way analysis of variance with Bonferroni post hoc tests. End-of-study plasma values were compared using Student’s t-test. Unless otherwise noted, data were considered statistically significant at P<0.05. All analyses were performed using GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego CA).

Results

Pharmacology

We generated multiple chemical series of GhrR antagonists in order to test whether the favorable metabolic phenotype of the GhrR KO mouse fed a high fat diet could be recapitulated by pharmacologic blockade of the receptor. Several representatives of these series were chosen for studies in vivo. These compounds were screened for their ability to inhibit ghrelin-stimulated activation of the human GhrR using a luminescence-based reporter assay system. Ki values for each compound were calculated from IC₅₀ values using the Cheng-Prussoff equation and confirmed through ¹²⁵I-ghrelin binding studies using membranes from cells that over-expressed the human or rodent GhrRs. Compounds showed a strong correlation of aequorin assay Ki at human and rodent receptors (r=0.7, p<0.05; data not shown). Properties of select competitive antagonist molecules are reported in Supplemental Table 1; oral bioavailability (%F) is also shown.
for several compounds. A representative structure of a GhrR antagonist (Compound D; CpdD) is shown in Supplemental Figure 2. In addition, compounds were routinely counter-screned against 20-100 other drug like targets (GPCR’s, transporters, ion channels, enzymes) and found to be 100->2000-fold selective for the human GhrR (data not shown).

Blockade of ghrelin-induced responses

We have shown that ghrelin administration stimulates FI and growth hormone release, responses that are absent in GhrR KO mice (Supplemental Figure 1A and 1B). Additionally, the ability of a single ip injection of ghrelin to induce acute, transient glucose intolerance was also absent in HFD-fed GhrR KO mice (Supplemental Figure 1C). Thus, a series of experiments was carried out to determine if antagonist compounds could block ghrelin-induced responses in a dose-dependent and selective manner. We initially used FI assays to determine the acute efficacy of ghrelin antagonist compounds. Consistent with previous reports in a variety of species, ghrelin administration (5 mg/Kg, ip) stimulated a significant increase in FI in mice over a 6 hr period and concomitant ip dosing with CpdD was able to reduce significantly the ghrelin-stimulated FI in a dose-dependent manner (Figure 1A). In this in vivo assay format we have been able to show that numerous compounds with in vitro antagonist activity are able to block ghrelin-induced FI (not shown).

To examine the selectivity of antagonists for effects on the GhrR we monitored the effect of antagonist treatment on spontaneous feeding using a metabolic chamber apparatus that records continual food consumption. Eight week-old WT and GhrR KO mice with access to a normal chow diet were dosed orally with either vehicle or CpdD at 3 hr and 1 hr before the beginning of the dark cycle. We found that mice treated with CpdD had decreased nocturnal FI during the 12 hr dark phase period (Figure 1B). Interestingly, the spontaneous FI in GhrR KO mice administered vehicle was lower compared to the WT and equal to WT mice treated with CpdD. However, administration of CpdD to KO mice showed no further effect on FI, showing that the effects of the compound on FI were selective for the GhrR (Figure 1B).
Repeated treatment of HFD-fed mice with GhrR antagonists

We and others have previously shown that GhrR KO mice resist many of the negative effects of HFD, including the development of insulin resistance. To determine if GhrR antagonist treatment of HFD-fed mice could recapitulate this phenotype in WT mice, a potent antagonist CpdB (Supplemental Table 1) was administered to HFD-fed mice and a variety of parameters was measured. HFD-fed mice were dosed for 7 days with either vehicle or CpdB (30 or 60mg/Kg, bid, po) and the effects were evaluated. CpdB administration resulted in a dose-dependent decrease in BW and FI (Figure 2A and 2B, respectively). In this study, CpdB had only modest, non-significant effects on glucose disposal (Figure 2C); however, the insulin required to dispose the glucose was significantly reduced (Figure 2D). We have observed the insulin sparing effects of both genetic and pharmacologic blockade of GhrR signaling in GTTs using either oral or ip glucose loads (Supplemental Figure 3) indicating that the incretin effect of the oral glucose load (Pratley et al, 2008) is intact in the context of GhrR blockade.

To further analyze the results of CpdB in terms of an insulin sensitivity index and in terms of specificity for the GhrR we transformed the data from the GTT using the HOMA-IR. HOMA-IR transformation shows that mice treated with CpdB have a lower insulin resistance score compared to vehicle, as expected (Figure 2E). In a separate study, we compared the effect of 7 day oral CpdB (60 mg/Kg, bid) vs vehicle treatment in GhrR KO mice to demonstrate the selectivity of the response. As shown in Figure 2F, GhrR KO mice showed a lower HOMA-IR score compared to the WT when placed on the HFD, corroborating previous reports of increased insulin sensitivity in the KO. Additionally, we found that CpdB had equivalent HOMA-IR scores relative to both vehicle and CpdB-treated HFD-fed GhrR KO mice, establishing its GhrR selectivity. However, in this experiment the differences between GhrR KO mice, as well as the CpdB-treated WT mice failed to reach statistical significance relative to the vehicle-treated WT mice. Nevertheless, this study, coupled with the observations in Figure 1B, support that CpdB is likely acting specifically through the GhrR to improve insulin sensitivity.

Repeated dosing of GhrR antagonists is often associated with decreases in BW and FI. However, the effects during a 7 day treatment period can be subtle and BW
changes are not always observed (Supplemental Figure 4A and 4B). Despite this, improvements in glucose homeostasis are consistently observed (Supplemental Figure 4C and 4D). Nevertheless, to more fully investigate the contributions of decreased BW and FI to the increased insulin sensitivity response to GhrR antagonist treatment we performed a pair feeding study, comparing vehicle and CpdB in the various feeding paradigms in a 2x2 design. The results show that, while pair-fed animals mirrored the BW changes induced by CpdB as expected, they did not show any improvement in insulin or glucose homeostasis (Figure 43), indicating that the effect of CpdB on apparent insulin sensitivity was not due to a loss in body mass, rather they suggest an alteration of metabolic set point, much like what is observed in GhrR KO mice on HFD.

To evaluate whether the insulin sensitizing effects of GhrR antagonist treatment is sustained after dosing is discontinued, HFD-fed mice were treated bid for 7 days with CpdB (60mg/Kg, po) as above except the last pre-GTT dose was eliminated. In these animals the effect on insulin sensitivity was still manifest but the magnitude of the response was diminished (Supplemental Figure 5A). In a related experiment we dosed animals with only a single, pre-GTT dose of CpdB and found that there was no efficacy with respect to improved glucose homeostasis (Supplemental Figure 5B). Taken together these result suggest that relatively constant blockade of the receptor is required for maintained efficacy.

To confirm the improved glucose homeostasis evident in the GTT assays, CpdB-treated, HFD-fed mice were evaluated in an HG clamp. Figures 34A and 34B show that there were no differences in first phase insulin release following the initial glucose load in CpdB and vehicle-treated mice. However, during the second, glycemic clamp (300mg/dL) phase of the experiment (see Figure 34C), CpdB-treated mice displayed significant reduction (AUC, p<0.05) in the insulin required to maintain the clamp (Figure 34D). Furthermore, the glucose infusion rate was increased in CpdB-treated animals consistent with improved insulin sensitivity (Figure 34F). Thus, pharmacologic blockade of GhrR signaling is associated with increased insulin sensitivity and this is consistent with HG clamp studies obtained in GhrR KO mice.

We next addressed the effects of longer term treatment with a ghrelin antagonist on metabolic homeostasis. Mice were treated with vehicle or CpdB (60 mg/Kg, po) for 28
and 56 days. As observed with shorter term (7 day) treatment, CpdB caused a transient decrease in FI that was completely resolved by 7 days (Figure 5A). Interestingly, animals lost 9-10% of their BW by 7 days and maintained this level of weight loss throughout the 56 day study (Figure 5B). After 28 or 56 days of treatment an oral GTT was performed. With 28 and 56 day treatment periods we noticed greater efficiencies in glucose disposal (Figure 5C and 5E, respectively) and even more remarkable reductions in the 2nd phase plasma insulin response (Figure 5D and 5F).

That the flat insulin response observed after long term treatment was the result of a CpdB-induced toxicity is unlikely since glucose disposal is greatly improved after 28 and 56 days. Also, immunohistochemical staining for insulin (Figure 6C and 6D) or glucagon (Figure 6E, F) revealed no discernable differences in vehicle- or CpdB-treated mice in this study. Additionally, HG clamp experiments have demonstrated that first phase insulin release does indeed occur in animals dosed with GhrR antagonists (Figure 34A). Nevertheless, to further investigate this issue we performed a pancreas challenge study using the short-acting insulin secretagogue, repaglinide, to determine if the ability of the pancreas to release insulin was affected by antagonist treatment. Mice treated for 14 days with CpdB (30 mg/Kg, bid, po) were given repaglinide (2mg/Kg) at the time of the last dose of CpdB and the insulin and glucose responses recorded during a GTT. As expected, mice dosed chronically with CpdB were capable of secreting insulin in response to repaglinide, comparable to the vehicle treated mice (Supplemental Figure 6). Taken together, these experiments further demonstrate the safety of the compound on pancreas function.

Upon further examination of organs from long-term GhrR antagonist-treated mice we observed two very striking features. First, the amount of white fat in these animals was significantly reduced (Table 1). We found that subcutaneous fat was not significantly changed but omental fat was reduced by 25-45% relative to vehicle-treated controls. These changes likely contributed substantially to the sustained, reduced total BW of the animals since the mass of different muscle beds was not changed (Table 1). Pancreas wet weight was not significantly altered; however, brown fat pads were slightly reduced with CpdB treatment (Table 1). We and others have shown in GhrR KO mice that white adipose tissue, and total BW, are significantly reduced relative to WT controls during
chronic HFD feeding. Thus the effects of chronic CpdB treatment replicate the effects of long-term genetic blockade of GhrR signaling.

A second remarkable observation was the extent to which CpdB treatment diminished fat content of the liver of HFD-fed mice. Livers from CpdB-treated mice had a more normal, redish appearance compared to the pink color characteristic of steatotic livers of vehicle-treated mice on HFD. Upon gross examination, livers from vehicle-treated animals were highly enlarged compared to those of CpdB-treated mice, as determined by wet weight measurement (Table 1). H&E staining of liver sections revealed widespread steatosis in the vehicle-treated mice fed the HFD whereas mice treated with CpdB were remarkably devoid of the characteristic lipid inclusions (Figure 6A and 6B, respectively). Finally, biochemical measurement of total fat showed that the livers of CpdB-treated mice had 50% reduced fat content compared to controls (Table 1).

Plasma analysis revealed other important observations in CpdB-treated mice (Table 1). Accompanying the improved insulin sensitivity was a slight but significant decrease in HbA1c of 0.46%. We also observed significant decreases in triglycerides and total cholesterol:HDL cholesterol ratios; no significant effects were observed for HDL or FFA levels, however. Interestingly, steady state plasma IGF-1 levels were not significantly reduced by GhrR antagonist treatment (Table 1). Therefore improvement in several metabolic parameters and functions were evident with long-term pharmacologic blockade of GhrR signaling.

We also assessed the safety profile of the mice after long term GhrR antagonist treatment by making note of the outward appearance of the mice and by measuring a series of plasma analytes. Other than the obvious BW effect, there was no detectable difference in overall appearance of CpdB-treated mice versus those treated with vehicle. Supplemental Table 2 shows that electrolytes and several metabolites were not changed. There was however a significant decrease in serum ALT and AST, in keeping with a protective effect on liver function and morphology (ie, prevention of steatosis). LDH, a marker of cardiac and skeletal muscle damage, was also significantly reduced. Overall, the health of the animals treated with CpdB was, if anything, improved and reflects the point that GhrR antagonism does not appear to be deleterious. In support of this we have performed lifespan studies with male and female GhrR KO and WT mice, fed either a
Discussion

We describe the development and assessment of potent, orally bioavailable small molecule antagonists of the GhrR. These molecules have low nanomolar affinity for the GhrR and show a high degree of selectivity against a broad panel of other GPCRs and molecular targets. GhrR antagonists were able to block both ghrelin-stimulated and spontaneous feeding in mice. Repeat oral dosing resulted in variable and transient decreases in FI and BW during the first week of dosing. However, a consistent observation was that repeated exposure of HFD-fed mice to these GhrR antagonists for 7 or more days resulted in dramatic improvements in insulin sensitivity that was accompanied by significantly decreased insulin secretion, independent of effects on BW. Collectively, these results show that pharmacological GhrR inhibition recapitulates the phenotype of GhrR KO mice fed a HFD (Wortley et al, 2004 and 2005; Zigman et al, 2005; Longo et al, 2008; Qi et al, 2011).

Experimental evidence gathered over the last several years has shown that ghrelin, an acylated peptide secreted by the stomach, is a unique regulator of energy balance and metabolic function. Ghrelin acts centrally as a potent orexigen in rodents and humans, and promotes positive energy balance and weight gain when administered chronically (Tschop et al., 2000; Nakazato et al., 2001; Wren et al., 2001; Cowley et al., 2003). Conversely, both ghrelin and GhrR KO mice had lower BWs when compared to controls, following several weeks of high-fat diet feeding (Wortley et al., 2005; Zigman et al., 2005; Longo et al., 2008). Lower total FI and equal total energy expenditure in GhrR KO mice has confirmed that these animals were in negative energy balance (Zigman et al., 2005; Longo et al., 2008). Interestingly, ghrelin KO mice had similar total FI and total energy expenditure, suggesting that there was a net loss of energy through decreased nutrient absorption in these animals (Wortley et al., 2005). In fact, GhrR KO mice have also demonstrated higher fecal excretion of lipid and a lower intestinal absorption/secrection rate of dietary lipid, indicating that a loss of ghrelin signaling limits
the rate of systemic energy absorption (Longo et al., 2008). Ghrelin also promotes gastric acid secretion in the stomach, gastric motility, and gastric emptying (Masuda et al., 2000; Levin et al., 2005; Levin et al., 2006; Charoenthongtrakul et al., 2008). Specific antagonism of the GhrR with [D-Lys-3]-GHRP-6 was shown previously to lower the rate of gastric emptying in mice (Asakawa et al., 2003). A delayed systemic exposure to ingested nutrients might confer some protection against postprandial hyperglycemia in these mice, in a manner similar to that which has been described for GLP-1, or its analogue, Exenatide (Wettergren et al., 1993; Willms et al., 1996; Kolterman et al., 2003). Transgenic mouse studies have also revealed the potential therapeutic benefit of blocking ghrelin signaling in improving insulin sensitivity (Wortly et al, 2004 and 2005; Zigman et al, 2005; Longo et al, 2008; Qi et al, 2011). It has therefore been proposed that loss of ghrelin signaling or antagonism of the GhrR may prevent the development of diabetes in mice and humans. Results of pharmacological studies with ghrelin antagonists in HFD-fed mice presented here substantiate this hypothesis.

The GhrR antagonist treatment-induced improvements in glucose homeostasis were also confirmed by hyperglycemic clamp experiments (Figure 34). These studies revealed that while there were no initial differences in glucose-stimulated insulin release in HFD-fed mice dosed for 7 days with CpdB or vehicle, CpdB-treated mice required significantly less insulin in order to sustain the clamped glucose levels. The glucose infusion rates were also significantly higher in GhrR antagonist-treated mice. Thus, the clamp data recapitulated the observations made during the GTTs of compound-treated mice as well as similar clamp analyses of HFD-fed GhrR KO mice (Qi et al, 2011).

To determine the long-term effects of chemical antagonism of the GhrR, we performed extended dosing with CpdB for 56 days. We noted again that the effects of this antagonist on FI were transient, and restricted to the first six days of dosing (Figure 5A). Despite a return to control FI levels after seven days, CpdB- treated mice had a lower BW that was sustained throughout the dosing period (Figure 5B). In response to a GTT, mice dosed with CpdB for 28 and 56 days (Figure 5C and 5E, respectively) showed a significant improvement in glucose disposal that was associated with remarkable reductions in glucose-induced insulin secretion (Figure 5D and 5F). These results provide further demonstration that extended CpdB treatment promoted a much greater insulin
sensitivity in these animals. If antagonist treatment resulted in lower insulin secretion without also dramatically improving insulin sensitivity the glucose excursions in these mice would be expected to dramatically increase, not decrease. The pancreatic islets from the CpdB-treated mice were similar in terms of their morphology and insulin content when compared to control mice (Figure 6). GhrR antagonist treatment also had no effect on the incretin response or a secretagogue challenge with repaglinide, further substantiating that the pancreas of these animals was not compromised by antagonist-treatment.

Long-term dosing with CpdB had several other metabolic benefits. Antagonist-treated mice had lower %HbA1c relative to controls, consistent with their improved insulin sensitivity (Table 1). Plasma triglycerides and total cholesterol (TC) were lowered significantly, with a trend of decreased hepatic VLDL production. Furthermore, the TC/HDL-C ratio was reduced significantly in the compound-treated mice. The collective reversal of diet-induced dyslipidemia in these mice suggests that GhrR antagonism may have cardio-protective benefits. Perhaps most striking was the dramatic decrease in hepatic lipid, steatosis, and markers of liver dysfunction such as ALT and AST (Table 1, Supplemental Table 2 and Figures 6A and 6B). While we did not explore directly insulin signaling in the liver, the inverse relationship between steatosis and hepatic insulin sensitivity has been well established (den Boer et al., 2004). In turn, the BW loss observed in the antagonist-treated animals is likely attributed to reductions in both liver and adipose tissue mass, but not skeletal muscle mass (Table 1). Thus, chemical antagonism of the GhrR limited the accumulation of adipose tissue and fatty liver which would normally contribute to peripheral insulin resistance, emphasizing the point that blockade of ghrelin signaling has more widespread effects beyond energy, insulin and glucose homeostasis.

The duration and timing of GhrR antagonist dosing was critical to the insulin-sparing effect of these compounds. GhrR antagonist dosing for 7 or more days was shown repeatedly to improve insulin sensitivity. However, mice that did not receive their final morning dose of CpdB before a GTT had glucose-induced insulin levels that were intermediate between those of the vehicle control mice and the mice that did receive their final dose (Supplemental Figure 5A). Therefore, sustained tonicity in terms of chemical
antagonism of the GhrR may be required in order to maintain the insulin sparing effect. On the other hand, an acute dose of the antagonist CpdB an hour before a GTT led to higher glucose-induced insulin secretion (Supplemental Figure 5B). This result is consistent with the observations that the GhrR antagonist [D-Lys3]-GHRP-6 stimulated glucose-induced insulin release during a GTT (Dezaki et al., 2004) or in perfused pancreas (Dezaki et al., 2006), while the acute administration of ghrelin opposed glucose-induced insulin release (Dezaki et al., 2004; Dezaki et al., 2006; Sun et al., 2006). In contrast, chronic administration of [D-Lys3]-GHRP-6 was shown to decrease both blood glucose and plasma insulin levels, while chronic administration with ghrelin had the opposite effect (Asakawa et al., 2003).

While it remains unclear why the effects of chronic versus acute GhrR antagonism on glucose-induced insulin secretion differ, these results highlight the complex role of ghrelin signaling in islet function (reviewed in Dezaki et al., 2008). It is important to note that while a previous study indicates that ghrelin KO mice had lower glucose and higher insulin during a GTT, the mice used in that experiment were eight weeks old and were fed a low-fat chow diet (Sun et al., 2006). However, ghrelin KO mice that were fed HFD for several weeks had lower fasting glucose and insulin (Wortley et al., 2005). Additionally, HFD-fed GhrR KO mice were euglycemic and had lower insulin during a GTT (Longo et al., 2008). Based on these results, it is clear that diet macronutrient composition impacts significantly the role of ghrelin signaling in the regulation of insulin secretion. Given that some of the highest levels of ghrelin receptor are found in hypothalamic regions involved in energy regulation (Zigman et al, 2006), we postulate that genetic deletion or chronic chemical antagonism of the GhrR has centrally-mediated effects on both peripheral insulin sensitivity and islet function that are separable from, and opposite to, their acute or localized effects in the pancreas.

In summary, these experiments inform us of several things: First, that continuous exposure of a GhrR antagonist is an effective means of improving a broad range of pathologic features of metabolic syndrome, such as insulin resistance, obesity and hepatic steatosis; Second, that the effects of these pharmacologic antagonists are not acutely therapeutic, and require continuous dosing to maintain benefits; Third, the reduced insulin secretion upon extended exposure to these GhrR antagonists is not the result of
pancreas dysfunction; Fourth, that decreased BW is not required for the observed improvements in insulin sensitivity; Finally, blockade of ghrelin receptor signaling is safe and well tolerated in metabolically stressed animals. Furthermore, deletion of the GhrR does not compromise lifespan regardless of whether animals are given a HFD or LFD, supporting the safety of blocking this mechanism for extended periods. Therefore, the pharmacologic GhrR antagonists described here are able to improve glucose disposal while decreasing glucose-stimulated insulin secretion and may therefore represent an insulin sparing therapeutic strategy for treating type 2 diabetes.

Acknowledgements
We would like thank to Tim Morrison for his superb oversight of Elixir’s animal facility.

Authorship Contributions
Participated in research design: BK, JJK, RC, EKG, JH, MN, AN, KAL, JS, PSD, BJG
Conducted experiments: BK, EKG, SC, TM, JH, DG, MN, AN, KAL, CZ, KM, BJG
Contributed new reagents or analytic tools: JS,
Performed data analysis: BK, JJK, RC, EKG, TM, JH, DG, MN, AN, KAL, JS, PSD, BJG
Wrote or contributed to the writing of the manuscript: KAL, JS, PSD, BJG
References


Figure legends

**Figure 1.** Effect of GhrR antagonism on acute ghrelin-stimulated and spontaneous FI. **A),** Effect of a single ip dose of the GhrR antagonist CpdD (1 or 3 mg/Kg) or vehicle on ghrelin-stimulated FI. Ghrelin was administered ip at 5 mg/Kg and FI was measured 6 hr later. Data are presented as mean FI ± SEM, *p<0.05 vs. ghrelin + vehicle group. **B),** Effect of CpdD dosed orally at 30 mg/Kg at 3 hr and 1 hr (arrows) before the dark cycle (shaded) on un-stimulated, nocturnal FI in WT and GhrR KO mice (n=8/group). Nocturnal feeding was greater in WT versus GhrR KO mice. Treatment with CpdD reduced nocturnal feeding in WT mice. CpdD did not cause any further reduction in nocturnal feeding in GhrR KO mice. Data are presented as mean cumulative FI ±SEM, (*p<0.05 vs. CpdD-treated WT mice group).

**Figure 2.** Effects of 7-day dosing with GhrR antagonist CpdB (10, 30 or 60 mg/Kg, bid, po) on BW, FI, and glucose homeostasis in HFD-fed C57BL/6 mice. One week of oral dosing with CpdB at 60 mg/Kg resulted in significant decreases in both BW (**A**) and cumulative FI (**B**) compared to vehicle-treated controls. A GTT performed at the end of the day 7 dosing period revealed no significant improvements in glucose disposal in the CpdB-treated mice (**C**). However, mice receiving either 30 or 60 mg/Kg of CpdB required significantly less insulin for glucose disposal than vehicle-treated controls. Expression of the same glucose and insulin data as a HOMA-IR score (**E**) revealed a significant decrease in insulin resistance in the 60 mg/Kg CpdB-treated group vs controls. GhrR KO mice showed less insulin resistance vs. WT and treatment of GhrR KO mice with CpdB gave no further effect on insulin resistance (**F**). (GTT, Glucose Tolerance Test;
HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; n=8/group; *P<0.05 vs. vehicle group; n=8/group).

**Figure 3.** GhrR antagonist treatment improves insulin sensitivity in a hyperglycemic clamp. HFD-fed C57BL/6 mice were treated orally with vehicle or CpdB (45 mg/Kg, bid) for 7 days. At the end of the dosing period, mice were anesthetized and a hyperglycemic (HG) was performed. The HG clamps were performed over a 90 min period as previously described (Qi et al, 2011), with the exception of modifications described in the methods section. The hyperglycemic target was 300 mg/dl. Changes in plasma glucose and insulin concentration (A and B, respectively) during the HG clamp (C and D, respectively) are shown. Data are the means ± SEM, n = 8/group. (*p < 0.05 vs. vehicle-treated control mice).

**Figure 4:** Reduced FI and BW does not account for improved insulin sensitivity in HFD-fed mice treated with a GhrR antagonist. HFD-fed C57BL/6 mice were dosed orally with the GhrR antagonist CpdB (60 mg/Kg, bid) for 8 days. A third group was pair-fed with the CpdB group (PF to CpdB), and a fourth group was given vehicle and food-restricted with 60% of the ad libitum fed vehicle group’s daily intake (FR to 60% Veh). 

A) Daily FI. Mice dosed CpdB and pair fed to the CpdB group showed a significant decrease in FI from days 1-5. As expected, food intake of FR 60% was significantly reduced relative to the vehicle group. 

B) CpdB-treated, pair fed and food restricted mice all had decreased BW relative to ad libitum fed vehicle-treated mice, though the decreases only reached statistical significance in the food restricted mice (days 5 to 8, inclusive). 

C) HOMA-IR
expression of glucose and insulin data during a oral GTT. Mice treated with CpdB and food restricted mice showed a similar, significant reduction in insulin resistance during the GTT. Data are the means ± SEM, n = 8/group. (*p < 0.05 vs. vehicle-treated control mice).

**Figure 5.** Effects of chronic dosing with GhrR antagonist CpdB. HFD-fed C57BL/6 mice were dosed orally with vehicle or CpdB (60 mg/Kg, bid) for 28 or 56 days. Daily FI (A) and BW (B) were recorded. Blood glucose and plasma insulin levels during an oral GTT were recorded for mice dosed for 28 days (C and D) or 56 days (E and F). Data are the means ± SEM, n = 8/group. (*p < 0.05 vs. vehicle-treated control mice).

**Figure 6.** Chronic oral dosing with the GhrR antagonist CpdB reduces hepatic steatosis. Representative H&E histologic sections from mice (n=6/group) dosed for 56 days with vehicle or CpdB (60 mg/Kg, bid). A) Liver from vehicle-treated mouse. B) Liver from CpdB-treated mouse showing dramatically reduced fat globules. Immunohistochemical staining for insulin (C, D) or glucagon (E, F) show no differences between vehicle and CpdB-treated mice. Pancreas from vehicle-treated (C) and CpdB-treated (D) mice show no differences in islet insulin levels. Similarly islet glucagon levels were not different in vehicle-treated (E) versus CpdB-treated (F) mice.
Tables:

**Table 1:** Chronic antagonism of GhrR signaling for 56 days improves numerous parameters of metabolic function. (GTT, glucose tolerance test; TG, triglyceride; TC/HDL-C, total cholesterol to HDL cholesterol ratio; IGF-1, insulin-like growth factor-1; FFA, free fatty acids). Data are the means ± SEM, n=8/group.

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Compound A</th>
<th>%Δ</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>46.8 ± 2.8</td>
<td>42.0 ± 4.1</td>
<td>↓ 10.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>GTT Insulin (AUC)</td>
<td>536 ± 78</td>
<td>308 ± 75</td>
<td>↓ 42.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>%HbA1c</td>
<td>4.01 ± 0.15</td>
<td>3.55 ± 0.27</td>
<td>↓ 11.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TG-plasma (mg/dL)</td>
<td>82 ± 12</td>
<td>71 ± 4</td>
<td>↓ 13.6</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>TC/HDL-C</td>
<td>1.36 ± 0.07</td>
<td>1.16 ± 0.09</td>
<td>↓ 14.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td>179 ± 16</td>
<td>167 ± 11</td>
<td>↓ 6.8</td>
<td>n.s.</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>2.13 ± 0.20</td>
<td>1.17 ± 0.15</td>
<td>↓ 45.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Liver % fat</td>
<td>28 ± 2.5</td>
<td>15.9 ± 4.2</td>
<td>↓ 43.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IGF-1</td>
<td>616 ± 39</td>
<td>564 ± 54</td>
<td>↓ 8.5</td>
<td>n.s.</td>
</tr>
<tr>
<td>Plasma FFA (g)</td>
<td>0.67 ± 0.15</td>
<td>0.72 ± 0.24</td>
<td>7.5</td>
<td>n.s.</td>
</tr>
<tr>
<td>Pancreas (g)</td>
<td>0.27 ± 0.02</td>
<td>0.23 ± 0.02</td>
<td>0</td>
<td>n.s.</td>
</tr>
<tr>
<td>Skeletal muscle (g)</td>
<td>0.34 ± 0.02</td>
<td>0.32 ± 0.02</td>
<td>↓ 5.5</td>
<td>n.s.</td>
</tr>
<tr>
<td>White fat-omental g</td>
<td>0.78 ± 0.14</td>
<td>0.46 ± 0.17</td>
<td>↓ 41.7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Brown fat (g)</td>
<td>0.3 ± 0.02</td>
<td>0.2 ± 0.01</td>
<td>↓ 33.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Spleen (g)</td>
<td>0.1 ± 0.006</td>
<td>0.09 ± 0.005</td>
<td>↓ 0.9</td>
<td>n.s.</td>
</tr>
</tbody>
</table>
Figure 1

A

Food Intake (g)

ghrelin (mg/Kg)  
-  5  5  5

CpdD (mg/Kg)  
-  -  1  3

B

Dark Phase

- WT, Veh
- WT, CpdD
- KO, Veh
- KO, CpdD

Food intake (g)

0.0  0.5  1.0  1.5  2.0  2.5  3.0

Time (h)  
0  3  6  9  12

* indicates statistical significance.
Figure 2

A

BWG (g)

<table>
<thead>
<tr>
<th></th>
<th>Veh</th>
<th>10</th>
<th>30</th>
<th>60 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veh</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

Food Intake (g)

<table>
<thead>
<tr>
<th></th>
<th>Veh</th>
<th>10</th>
<th>30</th>
<th>60 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veh</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C

Glucose (mg/dL)

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>10 mpk CpdB</th>
<th>30 mpk CpdB</th>
<th>60 mpk CpdB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minutes After Glucose Challenge</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

D

Insulin (ng/mL)

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>10 mpk CpdB</th>
<th>30 mpk CpdB</th>
<th>60 mpk CpdB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minutes After Glucose Challenge</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

E

AUC, HOMA-IR

<table>
<thead>
<tr>
<th></th>
<th>Veh</th>
<th>10</th>
<th>30</th>
<th>60 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veh</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

F

AUC HOMA-IR

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>KO</th>
<th>WT</th>
<th>KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CpdB</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3
Figure 5