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A novel Dual Amylin and Calcitonin Receptor Agonist (DACRA), KBP-089, induces weight loss through a reduction in fat, but not lean mass, while improving food preference

Running title: Novel DACRA, KBP-089, potently reduces body weight and fat deposition

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Abstract

Background and Purpose

Obesity and associated co-morbidities, such as type 2 diabetes and non-alcoholic fatty liver disease, are major health challenges – hence, development of weight loss therapies with the ability to reduce the co-morbidities is key.

Experimental Approach

The effect of the dual amylin and calcitonin receptor agonist (DACRA), KBP-089, on bodyweight, glucose homeostasis, and fatty acid accumulation in liver and muscle tissue, food preference was investigated. Further, we elucidate weight-independent effects of KBP-089 using a weight-matched group.

Key Results

High fat diet fed rats were treated with KBP-089 s.c., at 0.625, 1.25, 2.5 $\mu\text{g}\cdot\text{kg}^{-1}$ and vehicle resulting in a dose-dependent and sustained ~17% weight loss by the 2.5 $\mu\text{g}\cdot\text{kg}^{-1}$ ($p<0.001$). Moreover, KBP-089 reduced fat depot size and reduced lipid accumulation in muscle and liver.

In Zucker Diabetic Fatty rats, KBP-089 improved glucose homeostasis through improved insulin action.

To obtain a weight-matched group, significantly less food was offered (9% less than in the KBP-089 group). Weight-matching led to improved glucose homeostasis through lowered plasma insulin; however, these were inferior to the effect of KBP-089.

In the food preference test, normal diet rats obtained 74% of their calories from chocolate. KBP-089 administration reduced total caloric intake, and induced a relative increase in chow consumption while drastically lowering the chocolate compared to vehicle.

Conclusion

The novel DACRA, KBP-089 induces a sustained weight loss, leading to improved metabolic parameters including food preference, and these are beyond those observed simply by diet-induced weight loss.

Keywords: Obesity, Amylin, DACRA, adiposity, insulin sensitivity, body composition

Abbreviations

AUC, area under the curve; DACRA, dual amylin and calcitonin receptor agonist; EDTA, ethylenediaminetetraacetic acid; FPG, fasting plasma glucose; HbA1c, glycated haemoglobin; HFD, high fat diet; HOMA-IR, homeostatic model assessment for insulin resistance; IPITT, intraperitoneal insulin tolerance test; i.v., intravenous; IVGTT, intravenous glucose tolerance test; n.s., non-significant; NASH, non-alcoholic steatohepatitis; ND, normal diet; OCT, optimal cutting temperature compound; OGTT, oral glucose tolerance test; p.o., per oral; PW, pair weighed; s.c., subcutaneous; ZDF, Zucker Diabetic Fatty ZDF-Lepr^{fa}/Crl.

LIGANDS	
<u>Amylin</u>	
<u>Calcitonin</u>	
<u>Pramlintide</u>	
<u>Insulin</u>	

Introduction

Obesity and associated morbidities, such as diabetes, non-alcoholic fatty liver disease, cardiovascular disease and cancer are among this century's greatest health challenges (Pi-Sunyer, 1999; Cohen et al., 2008; Aballay et al., 2013). The incidence is increasing and treatment of obesity is in most cases limited to lifestyle interventions. However, when these fail, bariatric surgery and a few pharmacotherapies are available, although these are only used in cases of severe obesity (Fried et al., 2007). Furthermore, due to the potential complications of surgery, novel therapies with an improved efficacy in terms of weight loss and reduction of co-morbidities are of great interest (Batterham and Cummings, 2016).

The most recently developed therapy for obesity is high dose liraglutide, which leads to sustained weight loss at least partially due to a reduction in appetite. Furthermore, liraglutide reduces hyperglycaemia, albeit it is still somewhat limited in terms of efficacy and has challenges on tolerability (Kanoski et al., 2012; Lean et al., 2014). Another molecule that induces weight loss, or at least prevents weight gain is the amylin receptor agonist pramlintide (Aronne et al., 2007). pramlintide, due to its appetite regulating capability, has been shown to reduce insulin induced weight gain, while regulating post-prandial glucose excursions, and therefore has been approved as adjunct therapy to insulin for the treatment of type 2 diabetes (Weyer et al., 2001; Ryan et al., 2009). However, pramlintide use is limited significantly by lack of potency, and hence more potent amylin receptor agonists are explored.

Dual Amylin and Calcitonin Receptor Agonists (DACRAs) elicit activation not only of the amylin receptor, but also of the calcitonin receptor, and have been shown to possess superior activity in terms of activation of the amylin receptor, when compared to classical amylin receptor agonists (Andreassen et al., 2014). Interestingly, they also activate the receptors for an extended period of time, when compared to the classical agonists, which appears to increase the *in vivo* efficacy as well as reduce the dosing frequency (Gydesen et al., 2016).

In vivo studies of DACRAs have recently demonstrated a protection against diet induced weight gain, a reduction in overall adiposity, as well as adipocyte hypertrophy (Gydesen et al., 2016). Furthermore, DACRAs have been shown to improve glucose homeostasis in the diabetic ZDF rats, a phenomenon not observed with selective and less potent amylin receptor agonists (Mack et al., 2010, 2011; Andreassen et al., 2014; Hjuler et al., 2015), while alleviating obesity derived insulin resistance (Hjuler et al., 2016). Hence, the DACRAs

induce amylin receptor mediated responses *in vivo* – food intake reduction, weight reduction and suppression of glucagon levels (Roth et al., 2006), but also additional beneficial effects on fasting blood glucose and insulin sensitivity. With the limited number of DACRAs available, the search for highly potent molecules in this family was continued, resulting in the development of KBP-089.

In this study, we characterize the effects of KBP-089, on bodyweight, glucose homeostasis, and fatty acid accumulation in liver and muscle tissue. We then explore whether KBP-089 possesses beneficial effects in addition to a substantial weight loss, using a weight-matched group. Finally, we explore the potential effect of KBP-089 on food preference, by comparing intake of a highly palatable and energy dense diet (chocolate) to regular chow in the presence or absence of KBP-089.

Materials and methods

Peptide therapy

Synthetic KBP-089 (American Peptide Company, CA, USA) was dissolved in saline for subcutaneous delivery (s.c.). The doses chosen for peptide administration in the current investigations were based on previous comparable DACRA studies in animal models of obesity using potent DACRAs, KBP-042 and KBP-088 (Gydesen et al., 2016; Hjuler et al., 2016). The molecular target nomenclature conforms to the BJP's Concise Guide to Pharmacology <http://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=11> (Alexander et al., 2015).

Animal experiments

All animal procedures were performed in accordance with guidelines from the Animal Welfare Division of the Danish Ministry of Justice under the institutional license issued to Nordic Bioscience (2012-15-2934-00094). Male Sprague Dawley rats (Harlan, Venray, The Netherlands) and Zucker Diabetic Fatty ZDF-*Lepr*^{fa}/Cr1 (ZDF) (Kingston, NY, USA) were obtained at 6 weeks of age and housed (2 rats per cage, standard wood chips enriched with red-tinted huts, nest material and sticks) at the Nordic Bioscience animal facility (21-23 °C, 55-65% relative humidity, 12-h light/dark cycle) with *ad libitum* access to food and water.

Animals

From arrival and throughout the study periods, high fat-diet fed rats (HFD) were fed a 60 kcal% fat diet (#58Y1, TestDiet, London, UK), lean normal diet age-matched rats (ND) were fed a standard pelleted chow (#5002, LabDiet, St. Louis, MO, USA), and ZDF rats Purina Formulab diet (#5008, LabDiet, St. Louis, MO, USA). The rats received food and tap water *ad libitum*. The HFD and ND rats were non-blindedly assigned into experimental groups according body weight – ZDF rats non-blinded according to fasting plasma glucose (FPG), glycated haemoglobin (HbA1c) and body weight, ensuring an equal average value of body weight, FPG and HbA1c in the experimental groups at study start. Body weights are visualized as percentage of initial body weight for comparison to other drugs as previously described (Larsen et al., 2001; Mack et al., 2010). Lean and fat mass data as well as the weight of the different adipose tissues are normalized to the body weight of the individual animal.

Chronic *in vivo* studies

KBP-089 in HFD rats. After 10 weeks of high fat feeding, HFD rats were assigned into treatment groups receiving either vehicle (saline s.c.), KBP-089 (0.625, 1.25 and 2.5 $\mu\text{g}\cdot\text{kg}^{-1}$, s.c.) once daily in the afternoon with a food restricted pair-fed control group to the highest concentration of peptide (n = 10 rats per group in vehicle and KBP-089 treatment groups; n = 9 rats in pair-fed group due to loss of an animal). Pair-fed animals received an average of the daily intake of the 2.5 $\mu\text{g}\cdot\text{kg}^{-1}$ treatment group every day in the afternoon. Food intake and body weight were monitored daily the initial two weeks and once weekly throughout the entire study period. Following 6 and 7 weeks of treatment, oral and intravenous glucose tolerance tests (OGTT and IVGTT, respectively) were performed in overnight-fasted (12 h) rats with blood glucose measured and EDTA-plasma obtained for hormonal analysis. At the end of the study animals were euthanized; anesthetized by inhalation (isoflurane) followed by exsanguination and dissection. Retroperitoneal, epididymal and subcutaneous inguinal fat were surgically removed and weighed. Overnight-fasted blood samples were collected for basal plasma hormonal analyses.

KBP-089 in ZDF rats. The day prior to dosing initiation, 20 ZDF rats were assigned into two groups (n = 10 rat per group) receiving either vehicle (saline, s.c.) or KBP-089 (5 $\mu\text{g}\cdot\text{kg}^{-1}$ for 4 weeks, 20 $\mu\text{g}\cdot\text{kg}^{-1}$ for additional 4 weeks, s.c.) once daily. Oral glucose tolerance test was performed after 4 weeks, intraperitoneal insulin tolerance test (IPITT) was performed after 7

weeks. At the end of the study end FPG, HbA1c were measured and the homeostasis model assessment of insulin resistance (HOMA-IR) analysis was calculated using the formula; $\text{HOMA-IR} = \text{fasting insulin } (\mu\text{U/ml}) \times \text{fasting blood glucose (mmol/L)} / 22.5$ (Matthews et al., 1985). HOMA-IR was developed for humans; however, it can be used as a surrogate measurement for insulin resistance in rodents (Cacho et al., 2008; Mather, 2009).

Weight matched HFD rats. To address KBP-089 efficacy independent of weight loss we did a 6 week study in HFD rats (n = 12) with a weight-matched group to the $2.5 \mu\text{g}\cdot\text{kg}^{-1}$ KBP-089 group. Food intake and body weight were monitored daily throughout the study period and in order to match the body weights we estimated the needed food restriction to achieve comparable weight reductions based on pilot studies (data not shown) and adjusted estimations to body weight on a daily basis. The rats were subjected to an OGTT after 3 weeks of treatment. The rats were weighed and scanned for body composition (EchoMRI-4in1; EchoMRI, Houston, TX, USA) at study end, and euthanized as for the chronic *in vivo* studies.

Food preference in ND rats: To assess KBP-089 effect on the preference of diet ND rats were offered normal chow or chocolate (Milk chocolate with hazelnuts) (Marabou, Mondelez Danmark, Brøndby, Denmark). The animals were allowed to accustom to the chocolate for 1 week before injections with $2.5 \mu\text{g}\cdot\text{kg}^{-1}$ was initiated. Volunteer food and chocolate intake was monitored for 24 hours after treatment for 7 days.

Glucose tolerance tests

HFD rats received glucose per oral gavage (p.o.) ($2 \text{ g}\cdot\text{kg}^{-1}$) or intravenous (i.v.) in the lateral tail vein ($0.5 \text{ g}\cdot\text{kg}^{-1}$); ZDF p.o. ($1 \text{ g}\cdot\text{kg}^{-1}$). Blood samples were collected from the tail vein before glucose challenge (0 min) in both tests and 5, 15, 30, and 60 minutes post glucose challenge in the IVGTT, and 15, 30, 60, and 120 minutes post glucose challenge in the OGTT.

Insulin tolerance test

ZDF rats (fasted for 6 hours) were administered with KBP-089 at $t=-30$ and received intraperitoneal insulin ($1.0 \text{ U}\cdot\text{kg}^{-1}$) at $t=0$ and blood glucose was measured subsequently at $t=0, 30, 60$ and 120 minutes after insulin injection. The data is visualized as percentage of initial blood glucose for simplicity.

Fat accumulation in liver and muscle tissue

To address tissue fat accumulation, liver and gastrocnemius muscle were surgically removed for optimal cutting temperature compound (OCT) embedding, snap frozen on ice/ethanol, stored at -80°C until cryosectioning. Tissue sections were oil red O stained and images were captured with a light microscope (magnification of x40 for gastrocnemius and x20 for liver, 9 images per animal; 3 pictures per depth) and quantified using ImageJ capable of calculating the amount of red pixels in relation to μm^2 as previously described (Mehlem et al., 2013) and visualized as the fold-induction from lean rats for simplicity.

Biochemical analysis

Blood samples were collected in EDTA tubes and centrifuged at 5000 rpm for 10 min at 4°C . Blood glucose was monitored by Accu-Check® Avia monitoring system (Roche Diagnostics, Rotkreuz, Switzerland). HbA1c was measured using an automatized DCA Vantage Analyzer (Siemens AG, Erlangen, Germany). Plasma levels of insulin (Mercodia Rat Insulin ELISA, Mercodia AB, Uppsala, Sweden) was analysed according to manufacturer's instruction.

Statistical analysis

All data is presented as means \pm SEM. The statistical analysis of various drug effects were conducted using one-way ANOVA followed by Tukey's post test for multiple comparison for parametric data and Kruskal-Wallis test with Dunn's post test for non-parametric data if F achieved the necessary level of statistical significance ($p < 0.05$). Lean age-matched controls are compared to HFD vehicle, and ZDF vehicle to ZDF KBP-089, using Student's t-test. All analyses were performed using GraphPad Prism software (GraphPad Prism, San Diego, CA). A value of $p < 0.05$ was considered statistically significant. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2015).

Results

KBP-089 potently reduces appetite, body weight and fat depots

High-fat feeding resulted in a phenotype with significantly increased body weight (596 ± 12 g vs. 545 ± 8 g, $p < 0.01$), hyperinsulinemia (1.9 ± 0.1 ng·mL⁻¹ vs. 0.9 ± 0.1 ng·mL⁻¹, $p < 0.001$),

impaired glucose control without hyperglycaemia (OGTT tAUC 1343 ± 18.1 vs. 1259 ± 17.1 , $p < 0.01$), and impaired insulin sensitivity (HOMA-IR) (11.3 ± 0.3 vs. 4.3 ± 0.3 , $p < 0.001$), compared to the lean age-matched controls. Thus, the HFD rats resemble an obese and pre-diabetic phenotype as expected from previous studies (Hjuler et al., 2015; Gydesen et al., 2016).

To investigate the anti-obesity potential of KBP-089 *in vivo* we treated HFD rats for 8 weeks. Previously, DACRAs have shown to induce hypophagia (Hjuler et al., 2015; Gydesen et al., 2016) therefore, a pair-fed group to the highest concentration of KBP-089 was included to explore the impact of food restriction on body weight. KBP-089 was subcutaneously administered in three doses (0.625 , 1.25 and $2.5 \mu\text{g}\cdot\text{kg}^{-1}$) for 56 days. Food intake was transiently attenuated by KBP-089 (Figure 1A), albeit cumulative food intake after the initial 2 weeks of treatment was not significantly different in $2.5 \mu\text{g}\cdot\text{kg}^{-1}$ treated rats compared to vehicle rats (504 ± 35 g/animal vs. 408 ± 19 g/animal). 8 weeks of KBP-089 treatment resulted in a dose-dependent and sustained $17\% \pm 1.7$ weight loss in the $2.5 \mu\text{g}\cdot\text{kg}^{-1}$ (Figure 1B), while pair-feeding resulted in a $4\% \pm 2.0$ body weight reduction. Based on food intake and body weight change food efficiency was calculated. Expectedly, treatment with KBP-089 markedly attenuated the food efficiency compared to vehicle and the pair-fed group (Figure 1C).

Epididymal, inguinal and perirenal fat pads were weighed, and in conjunction with the significant body weight reduction, the weight of the adipose tissues was significantly reduced after treatment with KBP-089 (Figure 1D-F). This reduction was not observed in the pair-fed control rats.

KBP-089 enhances glucose tolerance and potentially insulin sensitivity

An OGTT was performed after 6 weeks of treatment, and followed by an IVGTT allowing circumvention of the influence of the gastrointestinal tract and thereby assessment of peripheral glucose tolerance after 7 weeks of treatment (Figure 2). In contrast to previous DACRA studies (Hjuler et al., 2015, 2016; Gydesen et al., 2016) the rats were not dosed 30 minutes prior to the glucose challenge to avoid the strong effect on gastric emptying. In both tests all treatment groups showed a trend towards lower blood glucose levels compared to vehicle and pair-fed controls 5 minutes (IVGTT) and 15 minutes (OGTT) after glucose administration (Figure 2A+D). However, tAUC was not significantly changed for either test when compared to vehicle or pair-fed controls (Figure 2B+E). Interestingly, the glucose-

induced insulin hyper secretion observed in vehicle and pair-fed groups was markedly and dose-dependently suppressed by KBP-089 during both OGTT and IVGTT, which resulted in significantly reduced insulin AUC values in KBP-089 treated rats (Figure 2C+F). Pair-feeding did not improve glucose tolerance or hyperinsulinemia in either test.

KBP-089 reduces accumulation of lipids in both muscle and liver

After treatment with KBP-089 for 56 days lipid accumulation was assessed in liver and muscle tissue. As seen in figure 3, high fat feeding led to increased lipid accumulation in both liver and muscle compared to lean age-matched controls. This inappropriate storage of lipids was completely eliminated by treatment with $2.5 \mu\text{g}\cdot\text{kg}^{-1}$ KBP-089, despite the rats having been on HFD for 10 weeks prior to initiation of therapy. Importantly, this effect was not obtained by pair-feeding.

KBP-089 lowers glycaemia and increases glucose tolerance and insulin action in ZDF rats

We tested the anti-hyperglycaemic efficacy of KBP-089 *in vivo* in ZDF rats for 8 weeks ($5 \mu\text{g}\cdot\text{kg}^{-1}$ for 4 weeks, $20 \mu\text{g}\cdot\text{kg}^{-1}$ for additional 4 weeks, s.c.). In ZDF rats, fasting blood glucose levels decreased significantly ($6.9 \pm 0.7 \text{ mM}$, $p < 0.001$) over 7 weeks by KBP-089 treatment compared to vehicle, resulting in HbA1c reduction by $\sim 2.5 \pm 0.2\%$ compared to vehicle at the end of the study (Figure 4A+B). Glucose tolerance was tested by an OGTT where treatment with KBP-089 resulted in a moderate glucose excursion compared to vehicle. The tAUC was lowered significantly ($\sim 30\%$, $p < 0.001$). Insulin action was assessed in an insulin tolerance test which manifested in significant larger drop in blood glucose in response to insulin in KBP-089 treated rats compared to vehicle (tAUC $\sim 19\%$, $p < 0.001$), supporting increased insulin sensitivity.

KBP-089 induces metabolic improvements in addition to those induced by weight loss through food restriction

In order to evaluate drug-induced metabolic improvements beyond what a weight loss can do, we performed a study with a weight-matched control in which weight reductions were induced either by KBP-089 administration (“KBP-089”) or by food restriction alone (“Pair-weighted”/“PW”). In order to match the body weights the pair-weighted rats received significantly less food compared to KBP-089 treated rats (Figure 5A). As in the previous study, body weight was significantly reduced by KBP-089 administration, and this was

matched during the study in the pair-weighed group (Figure 5B). There was no significant difference between the groups in body weight at study start (vehicle: 409 ± 3 g, KBP-089: 410 ± 3 g, PW: 408 ± 4 g). At study the body weight was significantly reduced in KBP-089 and pair-weighed rats, albeit there was no difference between KBP-089 treated rats and the pair-weighed rats (vehicle: 462 ± 6 g, KBP-089: 398 ± 4 g, PW: 403 ± 6 g). Interestingly, the epididymal and perirenal adipose tissues, which are directly associated with visceral adiposity and insulin resistance (Gabriely et al., 2002), were significantly lower in the KBP-089 and the pair-weighed group compared to vehicle (Figure 5C). There was no significant difference between the surgically removed adipose tissues in KBP-089 treated rats and the pair-weighed rats; however, there was a trend towards a more distinct reduction in adipose tissue in KBP-089 treated rats compared to pair-weighed rats. Using MR we found a slight increase in lean body mass in KBP-089 treated rats compared to vehicle rats, and reduced amount of whole body fat mass in KBP-089 treated and pair-weighed rats compared to vehicle (Figure 5D), underlining that the weight loss occurs in fat tissue primarily. As expected, KBP-089 again caused improved glucose tolerance with significantly lowered plasma insulin levels (Figure 5D,E+G,H) compared to vehicle. Surprisingly, the pair-weighed group did not markedly improve in glucose tolerance despite the significant weight reduction. Food restriction alone had significantly ameliorated hyperinsulinemia during OGTT (Figure 5H); however, it was still significantly higher compared to KBP-089. Finally, KBP-089 treated rats had a reduced rate of gastric emptying compared to vehicle and pair-weighed rats (data not shown) as previously observed with DACRA treatment (Hjuler et al., 2016).

KBP-089 induces changes in food preference

To examine the effect of the reduced food intake in detail, a food preference test was performed (Figure 6). When the rats had access to *ad libitum* chow and chocolate (as compared to chow alone) caloric intake was significantly increased. Furthermore, chow intake was significantly reduced as the rats preferred the chocolate and obtained 74% of their calories from chocolate (caloric intake vehicle/chow: 143 ± 3.0 kcal, caloric intake of vehicle/chow + chocolate: 173 ± 8.1 kcal (chow = 46.1 ± 2.9 kcal, chocolate = 127.3 ± 9.8 kcal). KBP-089 administration was associated with significantly reduced caloric intake – 34% compared to vehicle-treatment (caloric intake of KBP-089/chow + chocolate: 115 ± 10.7 kcal (chow = 74 ± 6.6 kcal, chocolate = 41.4 ± 9.4 kcal), accompanied by a relative increase in chow consumption and a drastic lowering in chocolate consumption (127.3 ± 9.8 kcal vs. 41.4 ± 9.4 kcal).

Discussion

The present study describes a novel dual amylin- and calcitonin receptor agonist, called KBP-089, which is able to induce and sustain a significant weight loss irrespective of food intake.

Importantly, KBP-089, possesses the ability to improve glucose tolerance above what can be obtained with weight loss alone.

Treatment with KBP-089 reduced food intake initially. However, this effect was reduced during the course of the study, and the effects obtained with KBP-089 treatment on weight, glucose tolerance, adipose tissue reduction, removal of ectopic lipid depositions in liver and muscle were not achieved with pair-feeding, clearly demonstrating effects of KBP-089 beyond appetite restriction. The weight reducing effect, as well as, the effect on food intake can most likely be attributed central amylin receptor activation. It has previously been described that amylin facilitates reduction in body weight that cannot only be attributed suppression of food intake (Isaksson et al., 2005; Roth et al., 2006). An interesting aspect of the reduction of fatty acid accumulation in the liver is the known relation between liver fat, insulin resistance and non-alcoholic steatohepatitis (NASH) (Cusi, 2009; Milić et al., 2014), and these data indicate that KBP-089 at least due to its weight reducing capacity could be a novel treatment candidate for liver steatosis.

The KBP-089-mediated changes in adiposity were confirmed in the pair-weight study where KBP-089-treated rats had significantly lower epididymal, inguinal, and perirenal adipose tissues compared to vehicle-treated rats, and trends towards lowered adiposity compared to pair-weighted adipose tissues – despite same body weight. This could indicate that KBP-089 treatment results in a loss of fat mass rather than lean body mass. Food restriction alone is however not sufficient to obtain the same reduction in adipose depots. As mentioned above, amylin agonism has long been associated with an increase in respiratory quotient which is associated with a preferential oxidation of fat (Wielinga et al., 2010). Moreover, other studies have also associated activation of amylin receptors with a specific reduction in fat mass rather than lean mass (Roth et al., 2006, 2007), whereas inhibiting amylin signalling centrally increases fat mass (Rushing et al., 2001), thus potentially explaining the difference in fat depots in this study. This was tested using MR-scanning. There was no difference in whole body fat mass between the KBP-089 treated and pair-weighted rats; however, in support of the limited loss of lean mass we found a slight – albeit significant – increase in lean mass in KBP-089 treated rats compared to vehicle rats, underscoring that the weight loss is primarily

mediated through reduction of adipose tissue weight. Furthermore, the slight increase in lean mass is greeted positively as heavy weight loss in many cases is associated with loss in lean body mass in humans (Garthe et al., 2011; Weiss et al., 2016).

In terms of hyperglycemia and insulin resistance, amylin analogues have shown promise (Ratner et al., 2004; Mack et al., 2011); however, they do not possess the intrinsic ability to reduce fasting plasma glucose levels and insulin tolerance, in contrast to KBP-089 as shown here in both ZDF and HFD rats, or other DACRAs (Hjuler et al., 2015). These data are further corroborated by the pair-weight study where substantially lower glucose and insulin levels were observed during glucose tolerance tests, in the KBP-089 treatment group when compared to the weight-matched group. The improvement in insulin levels manifested in improvement in glucose control too; however, it cannot be ruled out that the PW animals had an 'artificial' increase in glucose intolerance due to prolonged fasting (or significant food restriction), although they did not show any signs of malnutrition or ill behaviour. The lowering of insulin levels could attribute the lack of improvement in glucose tolerance when compared to the improvement observed in KBP-089 treated rats, which would have been likely after a significant weight loss (Horton and Hill, 2001; Lafontan and Langin, 2009; Karpe et al., 2011). The KBP-089 induced improvement in glucose tolerance is partly mediated through the lowering of gastric emptying rate, as previously observed with amylin agonism (Young et al., 1995; Young, 2005). In an oral glucose tolerance test without dosing prior to the glucose challenge (data not shown), glucose tolerance was slightly improved in KBP-089 treated rats and insulin levels were significantly lowered compared to vehicle rats, while PW animals mimic glucose tolerance and insulin levels as described in the illustrated OGTT. Data which underscore the strong insulinostatic effect of KBP-089.

Another important regulator of body weight could be to manipulate volunteer food intake/composition of food in the brain. This was hypothesized to be relevant for KBP-089 due to a known effect of amylin agonism on the release of dopamine in the hypothalamus (Brunetti et al., 2002) and alterations in the melanocortigenic system (Roth et al., 2012) both of which are mediators of the reward/pleasure circuits known to affect feeding patterns (Pandit et al., 2016). Normally, amylin does not produce conditioned taste aversion (Lutz et al., 1995; Rushing et al., 2002) hence this is not normally used to explain the alterations in food intake. Alternatively, the reduced impulse to consume sugar instead of normal chow could be explained in other ways. In humans, patients treated with pramlintide also experience a voluntary shift in eating behaviour and 'binge eating' (Smith et al., 2007). A

change of food intake towards a more healthy diet (less energy dense and sweet) is also observed in patients after surgical weight intervention (Mathes and Spector, 2012). The mechanisms behind this are not clearly described however alterations in food reward or taste functions have been suggested as possible explanations (Miras and le Roux, 2014). From the food preference study presented here it could be speculated that dosing with KBP-089 offers some of the effects obtained by surgical interventions, making KBP-089 a relevant option for treating severely obese patients and thereby aiding a significant weight loss along with a change in lifestyle which might improve the results even further.

In conclusion, novel DACRA KBP-089 induces and sustains a substantial weight loss in obese rats and reduces overall adiposity and ectopic lipid accumulation in the liver. In addition, KBP-089 improved glucose tolerance and implied improved insulin action independent of food intake and body weight, hence revealing the potential of KBP-089 as an anti-obesity agent with additional benefits on glucose control and liver steatosis.

Conflicts of interest

MAK and KH own stock in Nordic Bioscience. All other authors disclose no conflict of interest.

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Author contribution

S.G., S.T.H and K.H designed the study.

S.G., S.T.H. performed the study.

S.G., S.T.H., Z.F and N.S. analysed data.

S.G. and S.T.H. drafted the manuscript.

S.G., S.T.H., K.A.N., L.I.H., M.A.K. and K.H revised the manuscript.

S.G., S.T.H., Z.F., K.A.N., N.S., L.I.H., M.A.K., and K.H approved the final version of the manuscript.

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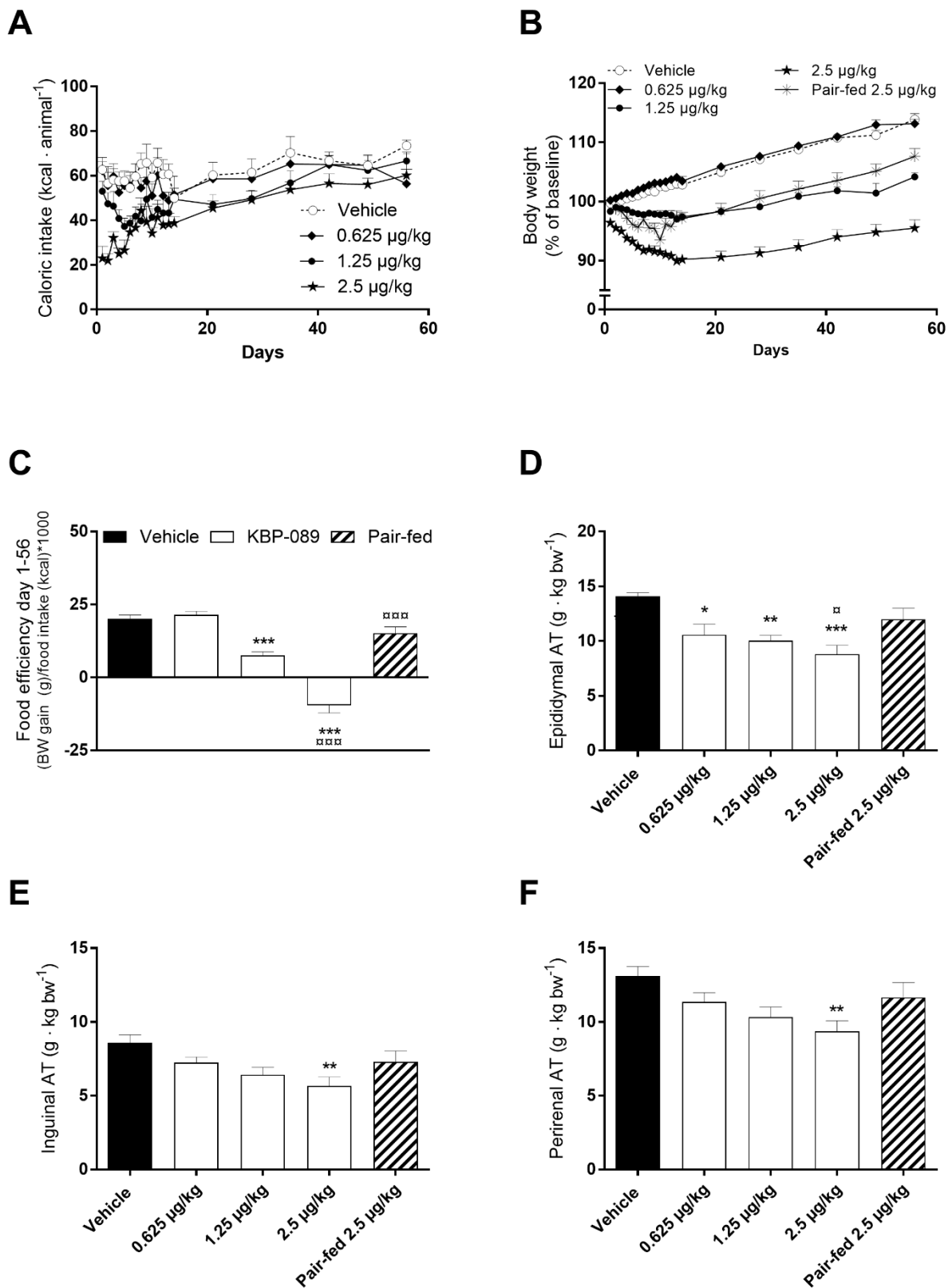
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Figures and Legends

Figure 1

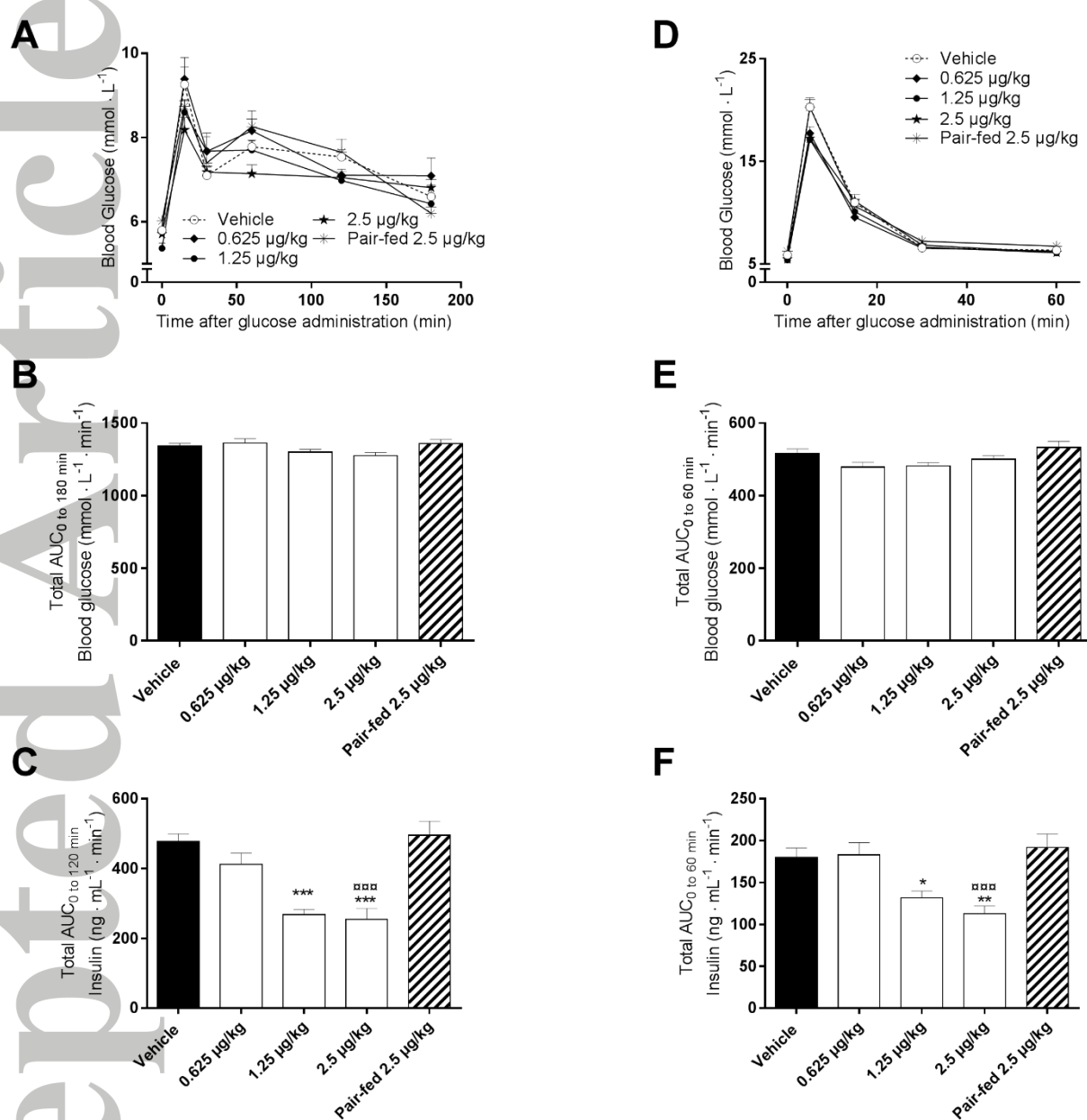


KBP-089 potently reduces appetite, body weight and fat depots in HFD rats

(A) Caloric intake monitored daily initially, then weekly in high fat diet fed rats. Expressed as daily intake per animal. (B) vehicle-corrected body weight. (C) Food efficiency day 1-56 in rats dosed with KBP-089 (0.625, 1.25 and 2.5 $\mu\text{g}\cdot\text{kg}^{-1}$). Relative weight of (D) epididymal, (E) inguinal and (F) peritoneal adipose tissue at study end. (n = 10 rats per group in vehicle and KBP-089 treatment groups; n = 9 rats in pair-fed group). AT, adipose tissue. Statistical analysis between groups was evaluated by an ordinary one-way ANOVA with Tukey's multiple comparisons test. $P < 0.05$. * compared to vehicle, α compared to pair-fed.

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Figure 2

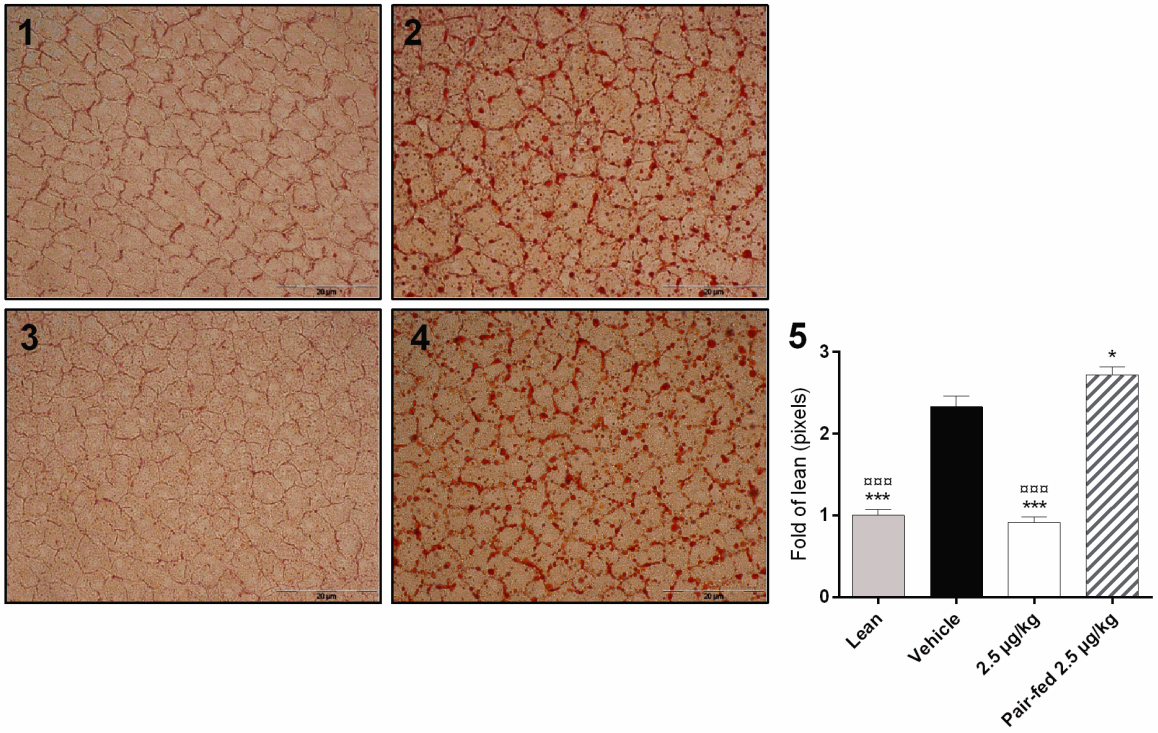


KBP-089 enhances glucose tolerance and potentially insulin sensitivity in HFD rats

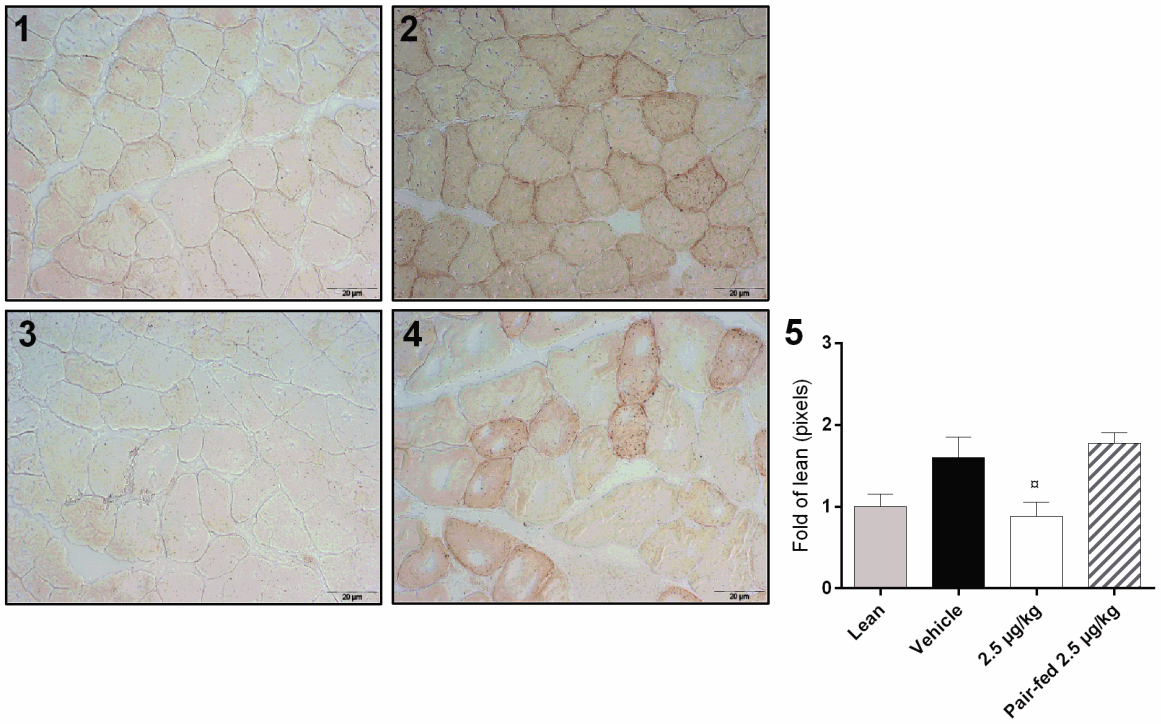
(A, D) Plasma glucose during oral glucose tolerance test (OGTT) and intravenous glucose tolerance test (IVGTT) in high fat diet fed rats treated with KBP-089 (0.625, 1.25 and 2.5 µg · kg⁻¹) for 6 and 7 weeks, respectively. Total AUC for (B, E) glucose and (C, F) plasma insulin during OGTT and IVGTT after 6 and 7 weeks, respectively. (n = 10 rats per group in vehicle and KBP-089 treatment groups; n = 9 rats in pair-fed group). Statistical analysis between groups was evaluated by (B, C, F) an ordinary one-way ANOVA with Tukey's multiple comparisons test and (E) Kruskal-Wallis test with Dunn's multiple comparisons test. P<0.05. * compared to vehicle, □ compared to pair-fed.

Figure 3

A



B



A

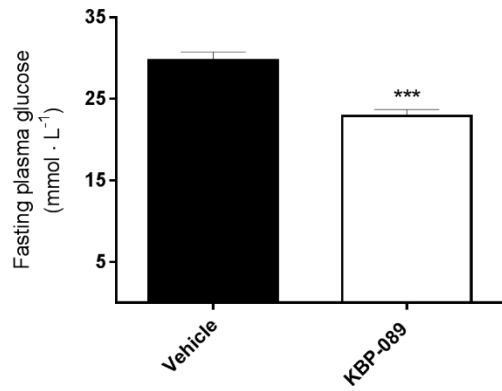
KBP-089 reduces accumulation of lipids in both muscle and liver in HFD rats

Oil Red O stained frozen (A) liver sections and (B) gastrocnemius muscle (magnification of x20 for liver and x40 for gastrocnemius, 9 images/ animal; 3/ depth) in (1) ND rats (2) vehicle HFD-rats (3) 2.5 $\mu\text{g}\cdot\text{kg}^{-1}$ KBP-089 (4) pair-fed to 2.5 $\mu\text{g}\cdot\text{kg}^{-1}$ KBP-089 and (5) quantification. (n = 10 rats per group in vehicle and KBP-089 treatment groups; n = 9 rats in pair-fed group). Data is expressed as fold of lean. Statistical analysis between groups was evaluated by a Kruskal-Wallis test with Dunn's multiple comparisons test. P<0.05. * compared to vehicle, α compared to pair-fed.

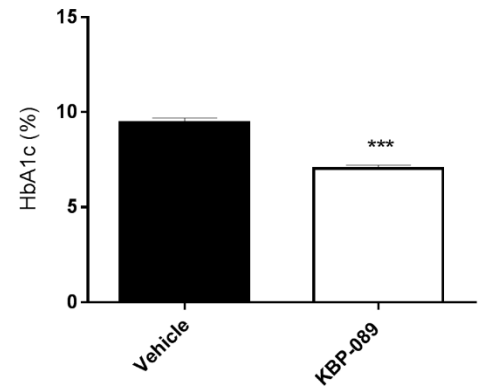
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Figure 4

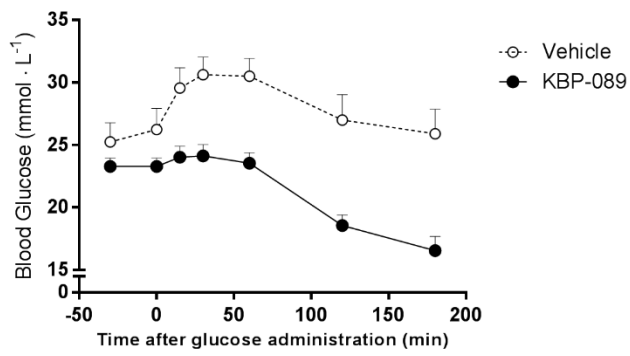
A



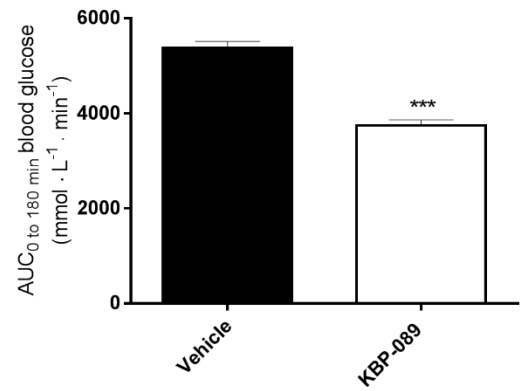
B



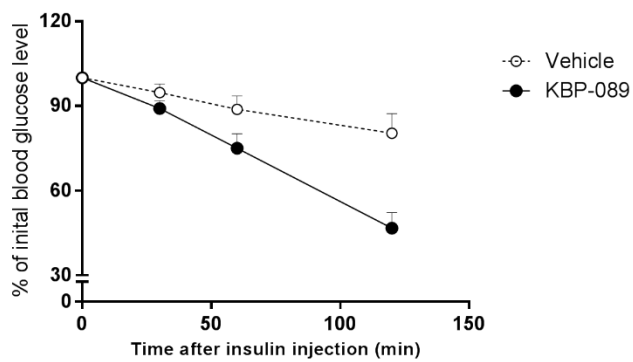
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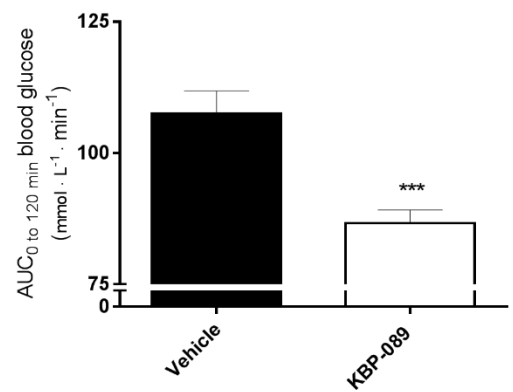
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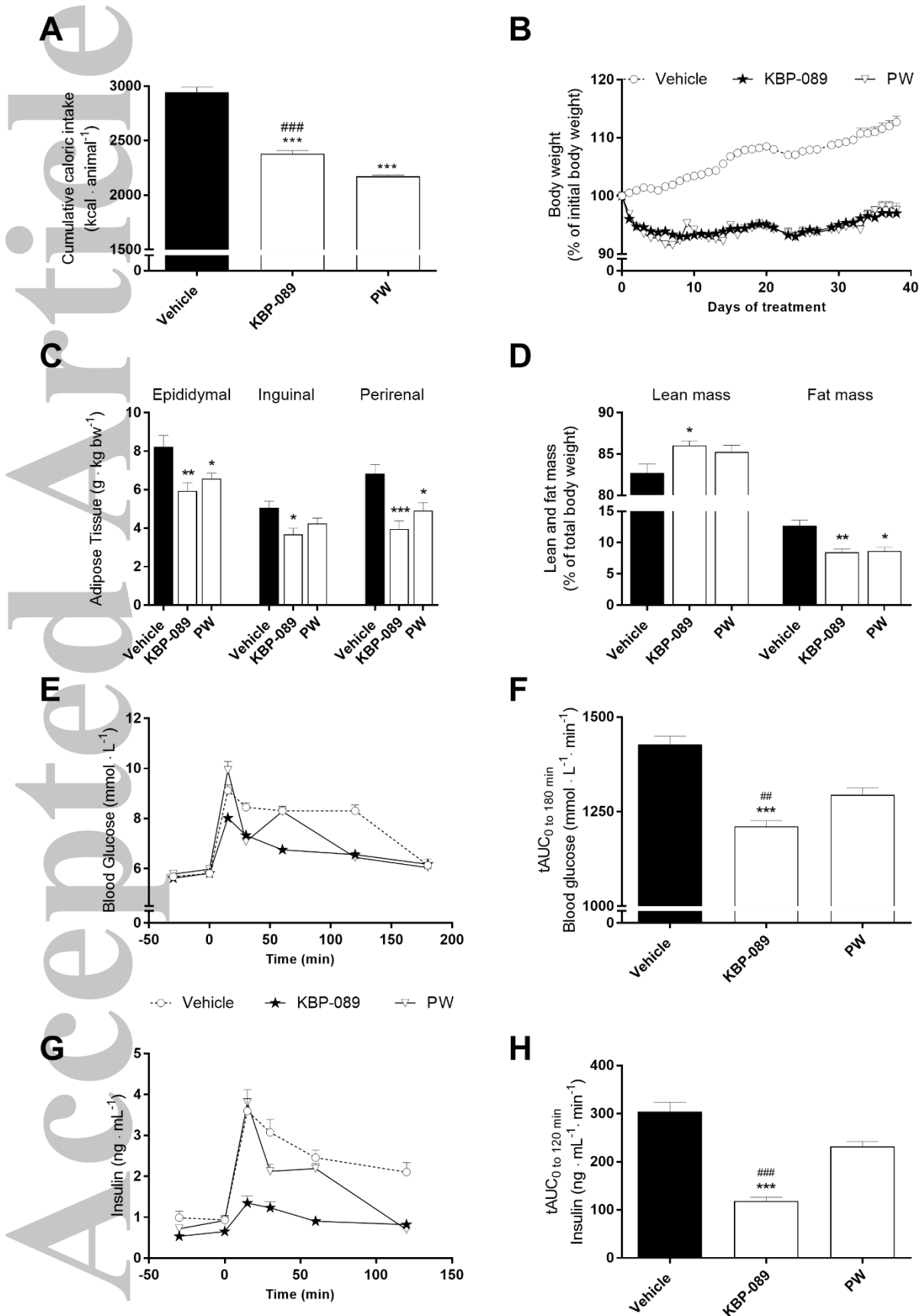


KBP-089 lowers glycaemia and increases glucose tolerance and insulin action in ZDF rats

(A,B) Fasting plasma glucose and HbA1c levels respectively in ZDF treated with KBP-089 or saline (vehicle) for 8 weeks. (C) Plasma glucose during oral glucose tolerance test (OGTT), (D) total AUC of the OGTT displayed in (C). (E) Plasma glucose during insulin tolerance test (ITT) displayed as % of initial blood glucose value. (F) Total AUC of the ITT displayed in (E). (n = 10 rats per group). Statistical analysis between groups was evaluated by t-test. $P < 0.05$.

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Figure 5

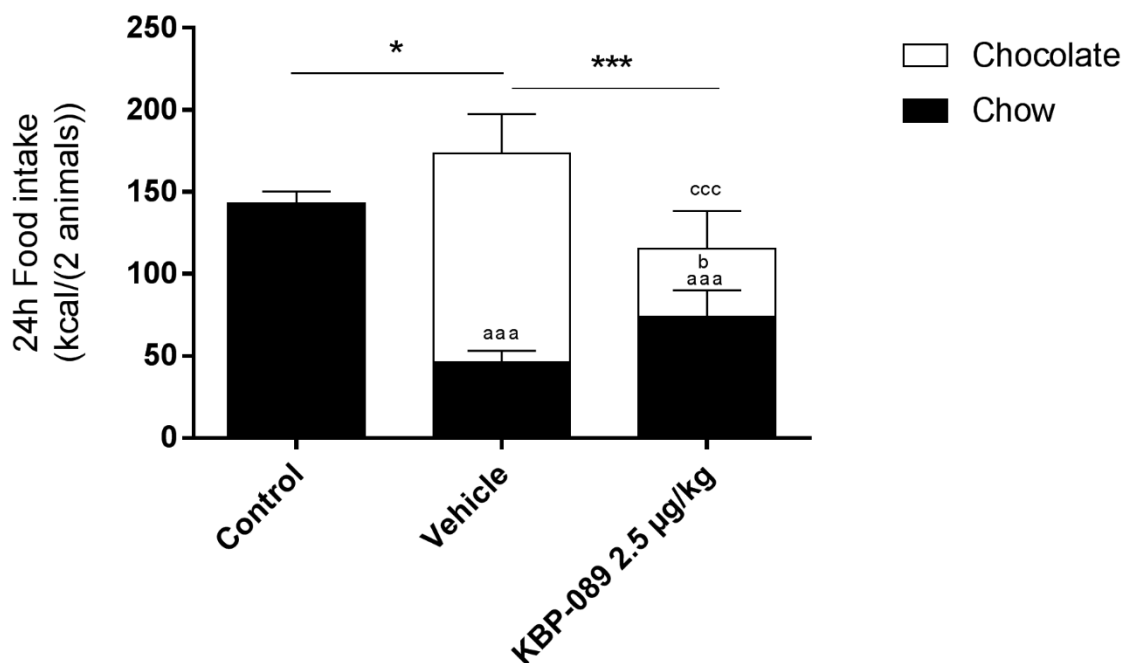


KBP-089 and weight-matched rats

(A) Cumulative caloric intake at study end. (B) Daily body weight and (C) relative weight of epididymal, inguinal and retroperitoneal adipose tissue at study end in HFD rats treated with KBP-089 ($2.5 \mu\text{g}\cdot\text{kg}^{-1}$) and weight-matched rats. (E, G) Plasma glucose and plasma insulin respectively (F, H) total AUC of glucose and insulin respectively during OGTT after 3 weeks. (n = 12 rats per group). Statistical analysis between the groups was evaluated by an ordinary one-way ANOVA with Tukey's multiple comparisons test (5C, F, and H) or (5D) Kruskal-Wallis with Dunn's multiple comparisons test. $P < 0.05$. *compared to vehicle, # compared to PW.

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Figure 6



KBP-089 induces changes in food preference

Volunteer food (chow, black bars) and chocolate (white bars) intake was monitored for 24 hours after 7 days of $2.5 \mu\text{g}\cdot\text{kg}^{-1}$ KBP-089 treatment. Statistical analysis between groups and diets was evaluated by two-way ANOVA with Tukey's multiple comparisons test. $P < 0.05$.

*treatment groups total caloric comparison; a, compared to control (chow); b, compared to vehicle (chow) and c, compared to vehicle (chocolate).