Dual Amylin and Calcitonin Receptor Agonists: A Novel Treatment for Obesity and Related Co-Morbidities

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A Novel Treatment for Obesity and Related Co-Morbidities

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NORDIC BIOSCIENCE

Preface

This thesis is submitted to the Technical University of Denmark (DTU), Department of Biotechnology and Biomedicine as part of the requirements to obtain the degree as doctor of philosophy (Ph.D.).

The work was conducted at DTU, Lyngby, Denmark and Nordic Bioscience, Herlev, Denmark from April 2014 to March 2017. Associate Professor Lars I. Hellgren, Ph.D. at DTU and Kim Henriksen, Ph.D. at Nordic Bioscience supervised the work.

In the results section, I present five papers – four published and one manuscript. A combined summary of the findings is presented in advance of the manuscripts and publications.
Besides the five papers, this thesis includes an introduction to the topics relevant for the presented data followed by a summarizing discussion of the most significant findings obtained during this thesis in relation to relevant literature.

Acknowledgements

First, I would like to thank to my supervisors, Kim Henriksen at Nordic Bioscience and Lars Hellgren at DTU. You have incredible scientific overview and an admirable enthusiasm for your specific fields of research. I am very thankful for the guidance, support and encouragement you have shown me during all my experimental work. I also extend my gratitude to Morten Karsdal for giving me the opportunity to perform my studies at Nordic Bioscience. Thank you.

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Thank you to my scientific colleagues at Nordic Bioscience in the diabetes group – especially, Sara, Kim, Katrine and Anna for both rewarding scientific discussions and good company at 'home' as well as on different Ph.D. courses around Denmark and our trips overseas.
Furthermore, I would like to thank all my colleagues, students and technical staff at Nordic Bioscience for providing technical help and meaningful discussions during this project. In particular, I would like to thank Christina, Mia, Julie and Majbrith for their work and delightful spirits in the animal facility.

Last, but not least, I am forever thankful to all of my friends and family for your endless love and support - even at the most stressful times. Thank you for your understanding and encouragement during the last three years. I really appreciate it.

All the best,

_____

Sofie Gydesen
Copenhagen, March 2017

Papers and Manuscripts

Papers and Manuscripts Included In This Thesis

Paper I: Gydesen, S., Hjuler, S.T., Freving, Z., Andreassen, K.V., Sonne, N., Hellgren, L.I., Karsdal, M.A., and Henriksen, K. (2017). 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. British Journal of Pharmacology.

Paper II: Hjuler, S.T., **Gydesen, S.**, Andreassen, K.V., Lund, S., Pedersen, K., Hellgren, L.I., Karsdal, M.A., and Henriksen, K. (2016). The Dual Amylin- and Calcitonin-Receptor Agonist KBP-042 Increases Insulin Sensitivity and Induces Weight Loss in Rats with Obesity. Obestiy (Silver Spring).

Paper III: Gydesen, S., Andreassen, K.V., Hjuler, S.T., Christensen, J.M., Karsdal, M.A., and Henriksen, K. (2016). KBP-088, a novel DACRA with prolonged receptor activation, is superior to davalintide in terms of efficacy on body weight. American Journal of Physiology – Endocrinology and Metabolism.

Paper IV: Gydesen, S., Andreassen, K.V., Hjuler, S.T., Karsdal, M.A., and Henriksen, K. (2017). Optimization of Tolerability and Efficacy of Dual Amylin and Calcitonin Receptor Agonist, KBP-089, through Dose Escalation and Combination with a GLP-1 Analogue. American Journal of – Physiology Endocrinology and Metabolism.

Paper V: Gydesen, S., Daniels, S.J., Larsen, A.T., Sonne, N., Karsdal, M.A., and Henriksen, K. (2017). The Dual Amylin and Calcitonin Receptor Agonist (DACRA) KBP-089 improves metabolic and hepatic features of non-alcoholic steatohepatitis in high fat, high cholesterol fed rats. Manuscript in preparation.

Papers and Manuscripts Not Included In This Thesis

Andreassen, K. V, Feigh, M., Hjuler, S.T., **Gydesen, S.**, Henriksen, J.E., Beck-Nielsen, H., Karsdal, M.A., and Henriksen, K. (2014). A novel oral dual amylin and calcitonin receptor agonist (KBP-042) exerts antiobesity and antidiabetic effects in rats. American Journal of Physiology – Endocrinology and Metabolism.

Feigh M, Hjuler ST, Andreassen KV, **Gydesen S**, Ottosen I, Henriksen JE, Beck-Nielsen H, Christiansen C, Karsdal MA, Henriksen K (2014). Oral salmon calcitonin enhances insulin action and glucose metabolism in diet-induced obese streptozotocin-diabetic rats. European Journal of Pharmacology.

Gydesen, S., Daniels, S.J., Karsdal, M.A., and Henriksen, K. (2017). Characterization of diet induced rat models of nonalcoholic fatty liver and nonalcoholic steatohepatitis. Manuscript in preparation.

Gydesen, S., Karsdal, M.A., and Henriksen, K. (2017). Dual Amylin and Calcitonin Receptor Agonist, KBP-042, directly reduces insulin and glucagon secretion from isolated islets, while protecting against glucolipotoxicity. Manuscript in preparation.

Hjuler, S.T., Andreassen, K. V., **Gydesen, S.**, Karsdal, M. A., and Henriksen, K. (2015). KBP-042 improves bodyweight and glucose homeostasis with indices of increased insulin sensitivity irrespective of route of administration. European Journal of Pharmacology.

Hjuler, S.T., **Gydesen, S.**, Andreassen, K.V., Karsdal, M. A., and Henriksen, K. (2017). The Dual Amylin- and Calcitonin Receptor Agonist KBP-042 works as Adjunct to Metformin on Fasting Hyperglycemia and HbA1c in a rat model of Type 2 Diabetes. Journal of Pharmacology and Experimental Therapeutics.

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List of Abbreviations

7TM	Seven Transmembrane
aa	Amino Acid
AC 187 / 253	Amylin Antagonist
ACC	Acetyl-Coenzyme A carboxylase
ALT	Alanine Transaminase
AMY-R	Amylin Receptor, Unspecified
ANOVA	Analysis of Variance
ASK1	Apoptosis Signal-Regulating Kinase 1
AST	Aspartate Transaminase
ATP	Adenosine Triphosphate
BMI	Body Mass Index
cAMP	Cyclic Adenosine Monophosphate
CCR2/5	Chemokine 2 and Chemokine 5 Receptors
CGRP	Calcitonin Gene-related Peptide
CLR	Calcitonin-like Receptor
CT	Calcitonin
CTR	Calcitonin Receptor, Unspecified
CVD	Cardio Vascular Disease
DACRA	Dual Amylin And Calcitonin Receptor Agonist
DIO	Diet-Induced Obese
DNL	*De novo* Lipogenesis
DPP-4	Dipeptidyl Peptidase-4
DTU	Technical University Of Denmark
FAS	Fatty Acid Synthase
FDA	Food And Drug Administration (American)
FFA	Free Fatty Acid
FLINT	Phase 2b Clinical Trial evaluating FXR Ligand Obeticholic Acid in NASH
FPG	Fasting Plasma Glucose
FXR	Farnesoid X Receptor
EDTA	Ethylenediaminetetraacetic Acid
EE	Energy Expenditure
EGP	Endogenous Glucose Production
ER	Endoplasmatic Reticulum
GE	Gastric Emptying
GABA	γ-Aminobutyric Acid
GIP	Gastric Inhibitory Polypeptide
GIR	Glucose Infusion Rate
GLP-1	Glucagon-Like Peptide-1
GLUT	Glucose Transporter
GOLDEN	Phase 2b Clinical Trial evaluating dual PPARα/δ agonist Elafibranor in NASH
GPCR	G-Protein Coupled Receptor
GSIS	Glucose Stimulated Insulin Secretion
HbA1c	Glycated Hemoglobin
HCC	Hepatocellular Carcinoma
hCT	Human Calcitonin
HDL	High-density Lipoproteins
HFCC	High Fat, High Cholesterol and Cholate diet

HFD	High Fat Diet
HGP	Hepatic Glucose Production
HIEC	Hyperinsulinemic-Euglycemic Clamp
HOMA-IR	Homeostasis Model Analysis For Insulin Resistance
HSC	Hepatic Stellate Cell
IAPP	Insulinoma Amyloid Peptide, Islet Amyloid Polypeptide, Amylin
IP	Intraperitoneal
IR	Insulin Resistance
IRS-PI3-Akt	Insulin Receptor Substrate, Phosphoinositide 3-kinase/ Protein Kinase B pathway
IV	Intravenous
IVGTT	Intravenous Glucose Tolerance Test
IPITT	Intraperitoneal Insulin Tolerance Test
KBP	Key Bioscience Peptide
LEAN	Phase 2 Clinical Trial Evaluating Liraglutide Efficacy and Action NASH
LDL	Low-density Lipoproteins
MS	The Metabolic Syndrome
MRI	Magnetic Resonance Imaging
mTOT	Mitochondrial Target of Thiazolidinedione Insulin Sensitizers
NAFL	Nonalcoholic Fatty Liver
NAFLD	Nonalcoholic Fatty Liver Disease
NAS	Nonalcoholic Fatty Liver Disease Activity Score
NASH	Nonalcoholic Steatohepatitis
OCA	Obeticholic Acid
OGTT	Oral Glucose Tolerance Test
PW	Pair-weighed / Weight-matched
Ph.D.	Doctor Of Philosophy
PPAR	Peroxisome Proliferator-Activated Receptor
PPG	Postprandial Plasma Glucose
q.a.d.	Quoque Alternis Die / Every Other Day
RAMP	Receptor Activity-Modifying-Protein
rAMY	Rat Amylin
ROS	Reactive Oxygen Species
RT-PCR	Real Time Polymerase Chain Reaction
s.c.	Subcutaneous
sCT	Salmon Calcitonin
SCD	Stearoyl-Coenzyme A Desaturase
SEM	Standard Error Of The Mean
SD	Sprague Dawley
SGLT2	Sodium Glucose Transporter 2
s.i.d.	Semel In Die / Once Daily
SREBP-1c	Sterol Response Element Binding Protein-1c
T1D	Type 1 Diabetes Mellitus
T2D	Type 2 Diabetes Mellitus
TG	Triglyceride
VLDL	Very-Low-Density Lipoprotein
ZDF	Zucker Diabetic Fatty

Summary

Amylin and/or calcitonin receptor agonists such as pramlintide and davalintide have shown promise on weight reduction in preclinical models and clinical settings, albeit with limited efficacy on glucose homeostasis.

The overall aim of this Ph.D. project was to investigate the metabolic effect of the dual amylin and calcitonin receptor agonists (DACRA), KBP-042, KBP-088, KBP-089, focusing on the weight reducing and glucoregulatory potential in preclinical animal models of obesity and related morbidities like type 2 diabetes (T2D) and nonalcoholic steatohepatitis (NASH). Both synthetic and naturally occurring DACRAs exert prolonged receptor activation and it is hypothesized that this prolonged receptor activation will improve the *in vivo* efficacy. Furthermore, it is hypothesized that DACRAs have beneficial metabolic effects beyond caloric intake and simple diet-induced weight loss.

In this series of studies, the focus was on metabolic effects of KBPs. Effects on body weight and adipose tissue as well as glucose metabolism were thoroughly explored in experimental rat models resembling the phenotypes of obesity, T2D and NASH, to address whether these beneficial effects were solely due to suppression of food intake and the subsequent weight loss. As amylin agonism induces a well-known anorexic effect at dose initiation, these studies also focused on different dosing regimens including dose escalation and dosing frequency. Finally, we compared KBPs to a second-generation amylinomimetic, davalintide, and combination of KBPs with the GLP-1 analogue, liraglutide.

KBPs potently activated both the amylin and calcitonin receptors *in vitro*, and demonstrated a prolonged receptor activation when compared to second-generation amylinomimetic, davalintide.

KBPs transiently suppressed caloric intake, and induced and sustained a dose-dependent weight loss compared to vehicle and pair-fed rats. Concomitantly, overall adiposity was decreased and obesity related adipocyte hypertrophy were improved – findings superior to the effects obtained with davalintide treatment. The inappropriate high fat diet-induced lipid accumulation was eliminated by KBP treatment, and interestingly, KBPs alleviated hyperinsulinemia and improved glucose tolerance even with significantly lower insulin levels. KBP treatment increased the glucose infusion rate during a hyperinsulinemic euglycemic clamp indicating enhanced insulin action. Importantly, KBPs also improved glucose homeostasis and enhanced insulin action in Zucker Diabetic Fatty rats.

To investigate beneficial effects beyond weight loss, a weight-matched group was implemented. Of interest, weight matching led to improved glucose homeostasis through lowered plasma insulin; however, these were inferior to the effect of KBPs.

KBPs were introduced using various dosing regimens and frequencies. Dosing every day and every second day resulted in an equal weight loss at study end; however, with a later onset of maximal weight loss. To optimize tolerability, KBPs were introduced by dose escalation. In a 4-fold dose escalation, KBPs induced a transient reduction in food intake at every escalation step – with reducing magnitude over time. Two-fold and linear escalations suppressed body weight evenly with no significant reduction in food intake at either escalation step; however, with a delayed onset of maximum efficacy.

Interestingly, when KBP and liraglutide were combined, the effect on acute food intake was superior to either of peptides as single-dose. Chronically, KBP-089 (1.25 μg/kg) and liraglutide (50 μg/kg) lowered body weight 8% and 2% in HFD rats, respectively, while the combination resulted in a 12% body weight reduction. Moreover, the combination improved glucose tolerance.

In a rat model resembling the phenotype of human NASH, KBP treatment led to a reduction of the high fat, high cholesterol and cholate diet induced increase in liver weight and circulating aspartate transaminase (AST) levels. Finally, at the histological level KBP treatment reduced hepatic steatosis, ballooning and inflammation, hence resulting in a reduced NAS score in combination with a lowered fibrosis stage.

In conclusion, KBPs induce and sustain weight loss, leading to improved metabolic parameters including food preference, and these are beyond those observed simply by diet-induced weight loss. Additionally, these peptides are well tolerated when introduced by dose escalation. Finally, KBPs reduce liver steatosis in both obese and NASH rats, and importantly reduced inflammation and fibrosis scores in NASH, hence underscoring the DACRA potential as an anti-obesity agent with benefits on glucose control and NASH.

Dansk Resume

Fedme og de associerede livsstilssygdomme, såsom type 2 diabetes (T2D), non-alkoholisk steatohepatitis (NASH), hjerte-karsygdomme og kræft er konsekvenser af den moderne stillesiddende livsstil. Behandlingen af fedme er begrænset til livsstilsinterventioner. I alvorlige tilfælde kan fedmekirurgi og nogle få farmakoterapier dog benyttes. Der er derfor intensiv søgen på nye lægemidler, der fokuserer på vægttab, insulinfølsomhed og lever patofysiologi. Amylin og/eller calcitonin receptor agonister såsom pramlintide og davalintide har vist potentiale i forbindelse med vægtreduktion i prækliniske modeller og kliniske forsøg, omend effekten på glukosemetabolismen var begrænset.

Det overordnede formål med dette Ph.D. projekt var at undersøge den metaboliske effekt af kombinerede amylin og calcitonin receptoragonister (DACRA) behandling, hvor fokus var på vægttab samt glukoseregulering i forskellige prækliniske modeller for fedme, T2D og NASH.

Hypotesen er, at en forlænget receptoraktivering vil forbedre effekten *in vivo,* og at de gavnlige effekter af DACRA behandling ikke kun er drevet af et reduceret fødeindtag samt vægttab.

I denne række undersøgelser, var der fokus på de metaboliske effekter af behandling med de tre DACRA, KBP-042, KBP-088 og KBP-089. Effekten på kropsvægt og fedtvæv samt på glukosemetabolismen blev undersøgt i eksperimentelle rottemodeller med fedme, T2D og NASH fænotyper, og ligeledes undersøgtes det, om de gavnlige effekter alene skyldtes et reduceret fødeindtag med efterfølgende vægttab. Det er velkendt, at amylin injektioner inducerer en anorektisk effekt ved doseringsstart, hvorfor disse forsøg ligeledes er fokuseret omkring forskellige doseringsregimer – herunder dosiseskalering samt variabel doseringsfrekvens. Endelig har vi sammenlignet KBP-behandling med en 2. generations amylinanalog, davalintide, og kombinationen af en KBP og GLP-1-analogen, liraglutide.

KBP peptiderne aktiverede både amylin og calcitonin receptorer *in vitro* meget potent, og ligeledes havde de en forlænget aktiveringen af receptorerne sammenlignet med davalintide.

KBP-behandling havde en transient effekt på kalorieindtaget, inducerede og vedligeholdte et dosisafhængigt vægttab sammenlignet med ubehandlede rotter. Samtidig reducerede behandlingen den samlede mængde kropsfedt og størrelsen af fedtcellerne. KBP-behandling eliminerede den ophobning af fedt, der var i leveren og ligeledes forbedrede KBP-behandling hyperinsulinæmien og glukosetolerance i rotterne selv med væsentligt lavere insulin niveauer. I en hyperinsulinæmisk euglykæmisk clamp øgede KBP-behandling glukoseinfusionsraten, hvilket indikerer øget insulinfølsomhed. Endeligt forbedrede KBP-behandling og glukosehomeostase og insulinvirkningen i T2D rotter.

For at undersøge om KBP-behandlingen havde effekter, der ikke kun var drevet af vægttabet, implementerede vi en vægt-matchet gruppe. Vægttabet i sig selv medført en forbedret glukosetolerance via sænkede insulinniveauer – disse var dog ringere end effekterne med KBP-behandling.

Vi testede dosering hver og hver anden dag. Begge doseringsformer resulterede i samme vægttab ved afslutning. Ligeledes opnåede rotterne, der dosiseskaleredes, et lignende vægttab dog med en knap så kraftig reducering af fødeindtaget.

Da vi kombinerede KBP og GLP-1 var effekten på akut fødeindtag overlegen sammenlignet med DACRA og GLP-1 hver for sig. I det kroniske behandlingsforsøg kombinerede vi KBP-089 (1,25 ug / kg) og liraglutide (50 ug / kg). De reducerede kropsvægten i fede rotter med henholdsvis 8% og 2%, hvorimod kombinationen resulterede i en reduktion på 12%. Ydermere forbedrede kombinationen glukosetolerancen.

I en rotte model med en NASH-lignende fænotype reducerede KBP-behandling levermarkører, leversteatosen samt ”ballooning” og inflammationen i leveren, hvilket resulterede i en sænket NAS score og et sænket fibrose niveau.

Konklusionen er, at KBP-behandling var i stand til at sænke samt vedligeholde et substantielt vægttab, der medførte en række forbedrede metaboliske parametre. Disse gavnlige effekter var større end effekterne set med almindeligt vægttab. Ydermere var peptiderne tolerable selv i høje doser, når de introduceredes ved dosiseskalering. Endeligt forbedrede KBP-behandling leverstatussen, hvilket understreger potentialet af KBP-behandling som pharmakoterapi til fedme og eventuelt T2D og NASH.

CHAPTER I

I. Introduction

Obesity

Obesity and the development of the metabolic syndrome (MS) are major health concerns. The associated morbidities, such as cardio vascular disease (CVD), type 2 diabetes mellitus (T2D), metabolic disorders, certain cancers (i.e., endometrial, breast, colon)[1], depression[2,3], osteoarthritis, and infertility[4] are among this century's greatest health challenges[5–8]. Worldwide obesity has more than doubled since 1980. In 2014, 39% of the adult population were overweight, 13% obese, and most people worldwide live in countries where overweight kills more people than underweight. Unfortunately, adults are not the only affected by the cheap and easily accessible calories – 41 million children under the age of 5 were overweight or obese in 2014[9], underpinning the necessity for both prevention, management, and treatment of this pandemic.

The Pathophysiology of Obesity

Obesity is a multifactorial disorder. Due to an imbalance in energy consumption and expenditure, excessive fat accumulates and the pathogenesis involves both genetic, environmental, and behavioral factors. The most easily accessible measurement of overweight and obesity is the body mass index (BMI). Although not all phenotypes fit this index, it provides an apt surrogate for overweight and additionally for total body fat. In adults, except for those of Asian heritage[10], a BMI from 25 to 29.9 kg/m^2 indicates overweight, whereas a BMI >30 kg/m^2 defines obesity[9].
The main role of adipose tissue is to store energy in the form of lipids and modulates the metabolism by releasing free fatty acids (FFA), pro-inflammatory cytokines and hormones[11–13]. In obese individuals many of these mediators are increased such as the inflammatory cytokines IL-6 and TNF-α, while the anti-inflammatory adipokine, adiponectin is diminished[14,15]. Interestingly, adiponectin has been found to protect against insulin resistance (IR) and CVD[16], while FFA and pro-inflammatory cytokines promote the development of IR[11,17,18].

Impaired glucose uptake and Insulin Resistance

Glucose is an essential substrate for the metabolism and homeostasis in eukaryotic cells and cannot passively diffuse through the cell membrane but requires facilitated transport

by glucose transporters (GLUT). 14 different GLUTs are known; however, the most well characterized are GLUT1-4[19]. GLUT1 and GLUT3 are located in plasma membranes and facilitate maintenance of the basal rate of glucose uptake from the bloodstream[20]. GLUT2 is expressed in hepatocytes and ß-cells and the rate of glucose uptake is proportional to blood glucose concentrations. GLUT4 transporters are situated in skeletal muscle and adipose tissue and under normal conditions highly insulin sensitive[19–21]. Glucose is storage is facilitated mainly by skeletal muscle and adipose tissue; hence, GLUT4 is important in clearance of excess glucose in the bloodstream[22].

When insulin is secreted in response to nutrient ingestion, the binding of insulin to the receptors activates the IRS-PI3K-Akt pathway initiating glucose uptake. A phosphorylation inactivates the protein that prevents GLUT4 translocation, and thereby the cytoplasmic vesicles storing GLUT4 moves towards the cell surface and are fused with the membrane. Thus, insulin promotes the GLUT4 translocation from inner vesicles, resulting in increased GLUT4 expression on the cell surface, and thereby induce glucose uptake (Figure 1A)[21,23,24].

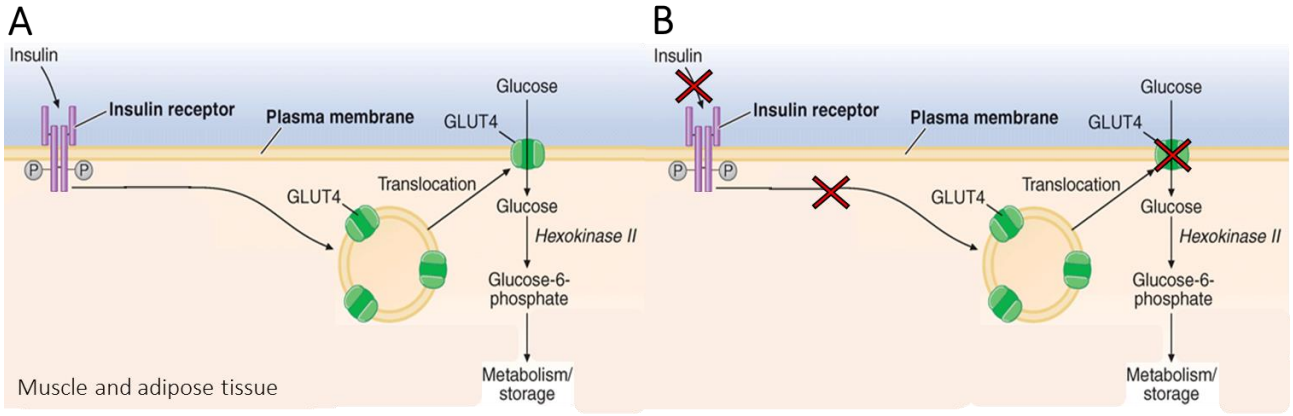

Figure 1. Simplified schematic illustration of insulin stimulated glucose uptake in peripheral tissues (A) under normal circumstances and (B) in insulin resistant peripheral tissues, where GLUT4 translocation fail. Figure is modified from Harrison's Principles of Internal Medicine, 17th Edition.

Generally, the term insulin sensitivity is the ability of insulin to regulate the circulating FFA and glucose uptake, by stimulating disposal into skeletal muscle, inhibiting hepatic gluconeogenesis and the ability to suppress lipolysis in adipose tissue. In healthy individuals, there is a feedback regulation between the insulin sensitive tissues and the insulin producing ß-cells as they increase insulin levels in response to the demand from the muscles, liver, and adipose tissue [25,26]. When the feedback loop is damaged it will result in a deviating glucose tolerance[27]. IR is the reduced ability of insulin to promote glucose uptake in peripheral tissues[28,29]. Adipose and skeletal muscle cells require insulin to absorb glucose, and when these cells respond insufficiently to circulating insulin, GLUT4 will not translocate to the cell membrane resulting in rising glycemia (Figure 1B). The impaired

translocation of GLUT4 transporters is suggested to be linked to receptor desensitizing and deficiency in IRS-1 phosphorylation[30].
Under normal conditions, the liver is part of maintaining glucose homeostasis. In the presence of insulin, hepatic glucose production (HGP) is reduced – a reduction that may not occur in insulin resistant individuals[29]. This feedback loop regulating the interaction between the insulin-sensitive tissues and ß-cells, as well as the relationship between insulin sensitivity and insulin secretion might explain the hyperinsulinemia in insulin resistant individuals compared to insulin-sensitive individuals[28]. IR reduces insulin-mediated suppression of lipolysis, hence elevating the FFA release into the circulation which are taken up by striated muscle where they inhibit insulin signaling and impair glucose uptake – as well as in the liver where increased FFA concentrations lead to an increase of the hepatic glucose output, and consequently to an increase in glucose load. The pancreatic ß-cells responds by increasing the insulin output thus further inducing hyperinsulinemia. Over time, the pancreatic ß-cells re unable to meet the increased demand for insulin. This is partly due to induction of pancreatic islet ß-cell apoptosis by prolonged exposure to FFAs. Subsequently diabetes will occur[31].
Metabolic overload will either increase the number of adipocytes (hyperplasia) or induce adipocyte enlargement (hypertrophy). Adipocyte hypertrophy is associated with macrophage infiltration in adipose tissue and development of inflammation impairing triglyceride deposition and increasing lipolysis. When the circulating amounts of lipids exceeds the adipose tissue uptake capability, fatty acids accumulate in tissues with confined capacity for lipid storage[32,33]. Lipids accumulate in the liver as lipid droplets and excessive lipid droplet accumulation will lead to cellular dysfunction. This observation was the basis of the lipotoxicity concept. The mechanisms are not fully understood, albeit the inhibition of glycolysis and insulin signaling pathway together with lipid accumulation, increased ROS, and mitochondrial dysfunction is known to affect IR negatively[29,34].

The Metabolic Syndrome

Nearly 30 years ago Gerald Reaven suggested the existence of *syndrome X* that described the co-occurrence of a number of metabolic disorders such as hypertriglyceridemia, hypertension, reduced HDL-cholesterol, and hyperglycemia[35] and IR was thought as the link connecting these metabolic abnormalities, hence triggered the name, *the insulin resistance syndrome*. Later on, the term metabolic syndrome was applied as further knowledge was obtained regarding the disproportionate FFA flux from excess adipose tissue, which was now believed to be a central the development of the syndrome. MS is a tool to help identify subjects in risk of developing T2D and CVD. MS is defined as a cluster of risk factors including central obesity, hypertension, dyslipidemia (elevated triglycerides and reduced high density lipids (HDL) cholesterol), and hyperglycemia (Figure 2), which

increases the risk of CVD and T2D and ultimately death[36–39]. Clinically, the presence of three of the clinical signs would signify the presence of MS[36,40]. The definition of MS was designed to be applicable in clinical practice – a simplified structure with no requirements of advanced testing such oral glucose tolerance test (OGTT) or IR measurement using a hyperinsulinemic-euglycemic clamp technique.

Metabolic Syndrome
is diagnosed in patients with presence of 3 out of 5 risk factors

- Increased waist circumference (population specific,
  European values are > 94 cm in men, > 80 cm in women)
- Elevated triglycerides (> 1.7 mmol/L),
- Reduced HDL cholesterol
  (> 1.03 mmol/L in men, > 1.29 mmol/L in women)
- Elevated blood pressure (systolic ≥ 130 mm Hg, diastolic ≥ 85 mm Hg)
- Elevated fasting plasma glucose (> 5.6 mmol/L)

Figure 2. Cut-off values for the five risk factors that constitutes the metabolic syndrome[36,40].

Interestingly, the waist circumference ratio is population specific, and it is speculated that the genetic variation among ethnic groups might be the reason for the increased risk of developing T2D at lower BMI in Asian people compared to Caucasians[41,42]. Of note, a heavily increased waist circumference is associated with increased visceral fat[43], and central obesity and intra-abdominal adiposity – independent of BMI – are profound risk factors for CVD and metabolic derangements[44].

Type 2 Diabetes Mellitus

Diabetes is currently affecting the lives of more than 400 million people. Numbers are increasing and have doubled globally the past 30 years[45]. There are several forms of Diabetes Mellitus: type 1 Diabetes Mellitus (T1D) and T2D, which account for 5-10% and 90-95%, respectively. Moreover, a minority of diabetic patients have gestational diabetes, and other types caused by genetic defects of the ß-cell and insulin secretion etc.[46]. T1D, gestational diabetes and other types of diabetes are not further discussed in this thesis.

Type 2 diabetes is on the rise worldwide. Unfortunately, it is believed that around 50% of people with pre-diabetes or T2D are undiagnosed[47]. T2D was previously thought to be a disorder of the elderly population, albeit with the increasing prevalence of obese individuals in all age groups, T2D is today diagnosed in children and adolescents[46]. T2D is a major

cause of morbidity and mortality and is one of the most challenging public-health problems worldwide; as many as 50% of all T2D patients are affected by cardiovascular complications such as heart attack and stroke that ultimately can be fatal [45,48]. Diagnosis is established by documentation of abnormal glycemia. Diabetic patients are characterized by having both fasting and postprandial hyperglycemia. Previously, the diagnosis of T2D was based on plasma glucose criteria; the two-hour value in an oral glucose tolerance test (OGTT) or fasting plasma glucose (FPG) measurement. These were replaced by glycated hemoglobin (HbA1c) as the gold standard[49–51]. Cut off values are found in figure 3. However, a combination of HbA1c, FPG and OGTT test values for diagnosing and monitoring of T2D is still recommended.

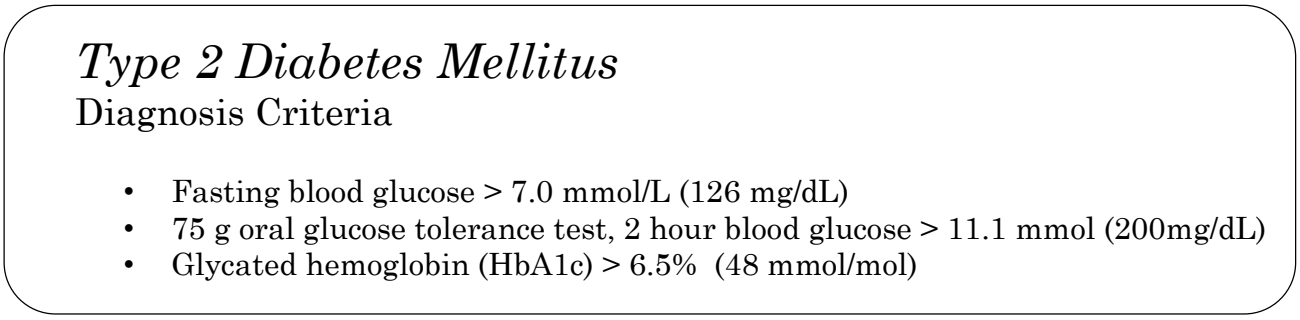

Figure 3. Cut-off values for Type 2 Diabetes (T2D) diagnosis. The diagnosis of T2D was previously based on the two-hour value in an oral glucose tolerance test (OGTT) or fasting plasma glucose (FPG) measurement, albeit presently glycated hemoglobin (HbA1c) is the gold standard for diagnosis[45].

Pathogenesis of Type 2 Diabetes Mellitus

T2D is a complex polygenic disorder characterized by hyperglycemia caused by IR, loss of pancreatic ß-cell secretory function and elevated HGP[52]. Furthermore, T2D is highly associated with obesity, impaired insulin action, hyperglucagonemia, and abnormalities of lipoprotein metabolism[34,53,54]. Due to the slow progression of T2D, it can be divided into a pre-diabetic state and a diabetic state; the pre-diabetic state is characterized by glucose intolerance and hyperinsulinemia. The diabetic state occurs when the secretory capacity of pancreatic ß-cells is unable to compensate for the IR resulting in hyperglycemia[55]. The impaired secretory function of the ß-cells and massive IR in peripheral tissues, such as muscle, liver and adipose tissue, cause increased fasting and postprandial glycemia and glucose intolerance[56]. Furthermore, the hyperglucagonemia in T2D patients will further lower the rate of glucose clearance[57,58].
Two different versions of T2D pathophysiology are described. One includes the preliminary development of IR followed by compensatory hyperinsulinemia and then progressive loss of pancreatic ß-cell mass and function, resulting in hypoinsulinemia and chronic hyperglycemia[59]. The second enrolls ß-cell dysfunction and ß-cell death as initiating factors of T2D[28,60]. Usually, the circulating levels of insulin are elevated prior to the diagnosis of

T2D; however, insulin levels will eventually decline as chronic glucotoxicity will cause pancreatic ß-cell dysfunction and death[61].

Chronic hyperglycemia is associated with long-term damage, dysfunction, and failure of different organs resulting in increasing disability, reduced life expectancy and enormous health costs. Pancreatic ß-cell destruction, liver lesions, neuropathy, muscle atrophy, nephropathy, damaged micro vasculature, and atherosclerosis are some of the complications[56,62–64].

Pancreatic hormones

The human pancreas consists of both an endocrine and an exocrine gland. The exocrine secretes enzymes and bicarbonate, neutralizing gastric acid, into the duodenum aid digestion of consumed nutrients. The endocrine multi cellular mini-organs, islets of Langerhans, are highly vascularized and innervated and make up 1-2% of the pancreatic mass[65]. Each of the Islets of Langerhans includes at least five hormone secreting cell types: α-cells, ß-cells, δ-cells, PP cells and ε-cells producing glucagon, insulin and amylin, somatostatin, pancreatic polypeptide, and ghrelin, respectively. These multi cellular structures are responsible for the regulation of glucose homeostasis, most dominantly insulin and glucagon (Table 1). Pancreatic islets are regulated by paracrine, autocrine, and endocrine signaling and by sympathetic, parasympathetic and sensory nerves placed deeply into the islets. Thus, multiple regulation factors determines hormone release from the islets of Langerhans[60,66,67].

Insulin is an anabolic hormone and the principle hormone capable of lowering glycemia[68]. Insulin synthesis and secretion from ß-cells are mainly regulated by circulating concentrations of glucose – hyperglycemia stimulates insulin release[69]. Intracellular glucose enters glycolysis and glycogenesis and the produced adenosine triphosphate (ATP) from glycolysis blocks the ATP-sensitive potassium channels resulting in a decreased K^+ efflux that will depolarize the membrane opening the voltage-dependent Ca^{2+} channels. The increased cytoplasmic Ca^{2+} triggers insulin secretion by exocytosis in a biphasic response[70,71]. Insulin contributes to the maintenance of glucose homeostasis by stimulating glucose uptake in peripheral tissues and by inducing glycolysis, glycogenesis and lipogenesis, while suppressing HGP[72].

Hormone	Cell	Target tissue	In response to	Main effects in healthy subjects
Insulin	ß-cells	Skeletal muscle, adipose tissue, liver and kidney	Hyperglycemia	Anabolic effect, blood glucose ↓, glycolysis ↑, glycogenesis ↑, lipogenesis ↑
Glucagon	α-cells	Liver	Hypoglycemia	Catabolic effect, blood glucose ↑, gluconeogenesis ↑, glycogenolysis ↑
Amylin	ß-cells (co-secreted with insulin)	Skeletal muscle, brain and kidney	Hyperglycemia	Gastric acid secretion and gastric emptying↓ Induces anorexia, glucose release and hepatic glucose production in the postprandial period ↓

Table 1. Overview of important pancreatic glucoregulatory hormones. References [67,73–76]

Glucagon is a catabolic hormone synthesized by the α-cells, which maintain glucose homeostasis during fasting and exercise[72]. α-cells are highly sensitive to glucose levels and hypoglycemia stimulates glucagon release[67,69]. As with insulin, the increased intracellular ATP blocks the ATP-sensitive potassium channels resulting in a decreased K^+ efflux and depolarization of the membrane opening the voltage-dependent Ca^{2+} channels and glucagon is released[69]. Glucagon is suppressed by hyperglycemia hence per se inhibited by insulin[67,77]. Finally, glucagon inhibits glucose uptake, stimulates lipolysis and provokes the release of FFA from adipose tissue as well as increases gluconeogenesis and glycogenolysis are stimulated while glycolysis in liver is suppressed[78].

Amylin is synthesized in the ß-cells and co-secreted with insulin in response to nutrients exerting complementary prandial actions to insulin[79,80]. Amylin contributes in the control of carbohydrate metabolism and has several biological effects and high affinity sites are located in the brain, kidney and skeletal muscle[75]. The pharmacological and physiological actions of amylin are described in the section of target receptors and ligands.

Nonalcoholic Fatty Liver Disease

Nonalcoholic fatty liver disease (NAFLD) is believed to be the hepatic manifestation of MS[81]. NAFLD is characterized by excessive fat accumulation in the liver in the absence of excessive alcohol consumption or any other specific causes of hepatic steatosis[82]. A lipid accumulation of at 5-10% of the liver weight is classified as NAFLD[83].

NAFLD is closely related to IR, and thus frequently occurs at the initial part of MS[84]. The pathologic spectrum of NAFLD comprises four different stages: non-alcoholic fatty liver (NAFL), NASH, fatty fibrosis and cirrhosis (Figure 4).

The mild NAFL is characterized by the presence of fat with or without mild local necrosis and inflammation. While this harmless first stage is not correlated with increased short-term morbidity or mortality, it can progress into NASH dramatically increasing the risks of

cirrhosis, liver failure, and hepatocellular carcinoma[82,85,86]. Besides excessive liver steatosis, NASH is characterized by hepatocellular ballooning and inflammation, and may progress more rapidly to hepatic fibrosis including steatohepatitis and portal fibrosis. Finally, cirrhosis is described as steatohepatitis with fibrotic septum around regenerative nodules of parenchyma[87].

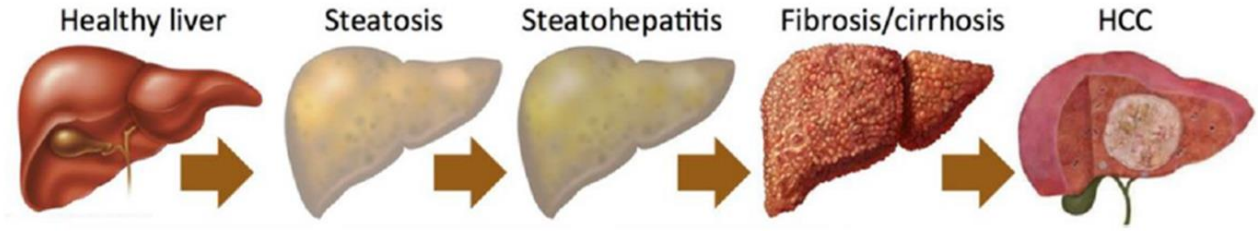

Figure 4. Spectrum of nonalcoholic fatty liver disease (NAFLD). Schematic illustration of the development of NAFLD. A healthy liver becomes steatotic when fat accumulates. When inflammation occurs the steatotic liver progresses to the next stage, steatohepatitis (NASH) that can progress into fatty fibrosis and ultimately cirrhosis and hepatocellular carcinoma (HCC). Figure from[88].

NAFLD and NASH are the number one cause of liver disease in the western countries[89]. The incidence of NAFLD has doubled during the last 20 years corresponding to the increase in obese individuals, while the prevalence of other chronic liver diseases has remained stable or even decreased[90].

Pathogenesis of Nonalcoholic Steatohepatitis

When the balance between lipid uptake and utilization is impaired, lipids accumulate in the liver. NAFLD patients have an increased hepatic *de novo* lipogenesis and secretion of very low density lipoproteins from the liver resulting in dyslipidemia characterized by increased circulating levels of triglycerides and cholesterol[91]. Ectopic lipid accumulation in the liver is both a consequence of and a marker for systemic IR and MS[92]. Metabolic overload; however, is not the only cause of hepatic lipid accumulation. Since multiple metabolic pathways have limited capacity, intermediate lipid products can also cause lipid accumulation. The mechanisms include: reduced oxidation of FFAs secondary to decreased expression of peroxisome proliferators activated receptor (PPAR)-α; impaired mitochondrial ß-oxidation; increased *de novo* lipogenesis mediated via PPAR-γ and sterol responsive element binding protein (SREBP)-1c; and increased hepatic lipolysis[93–98]. Nevertheless, when the limit of mitochondrial oxidative capacity is reached peroxisomal ß-oxidation will increase, hence creating ROS. Peroxisomal ß-oxidation does not degrade fatty acids completely but only acts chain-shortening creating toxic lipids[99–101] and palmitate that are routed into other pathways e.g. ceramide synthesis.

IR, oxidative stress, hepatic stellate cell (HSC) activation, inflammation and genetics are key factors in the pathogenesis and development of NASH (Figure 5)[102,103]. ROS production causes apoptosis and combined with the accumulated lipid by-products, inflammation is induced and HSC are activated potentially leading to NASH and possibly fibrosis[104]. In a normal liver, Kupffer cells[105], dendritic cells and regulatory T cells[106] maintain the healthy non-inflamed phenotype of the liver. During NASH development macrophages and neutrophils[107] are recruited to the liver and in conjunction with high lipid content dendritic cells among others these immune cells are contributing to the development of inflamed liver phenotype. IR coupled with oxidative stress may be the underlying mechanism responsible for lipid accumulation and disease progression.

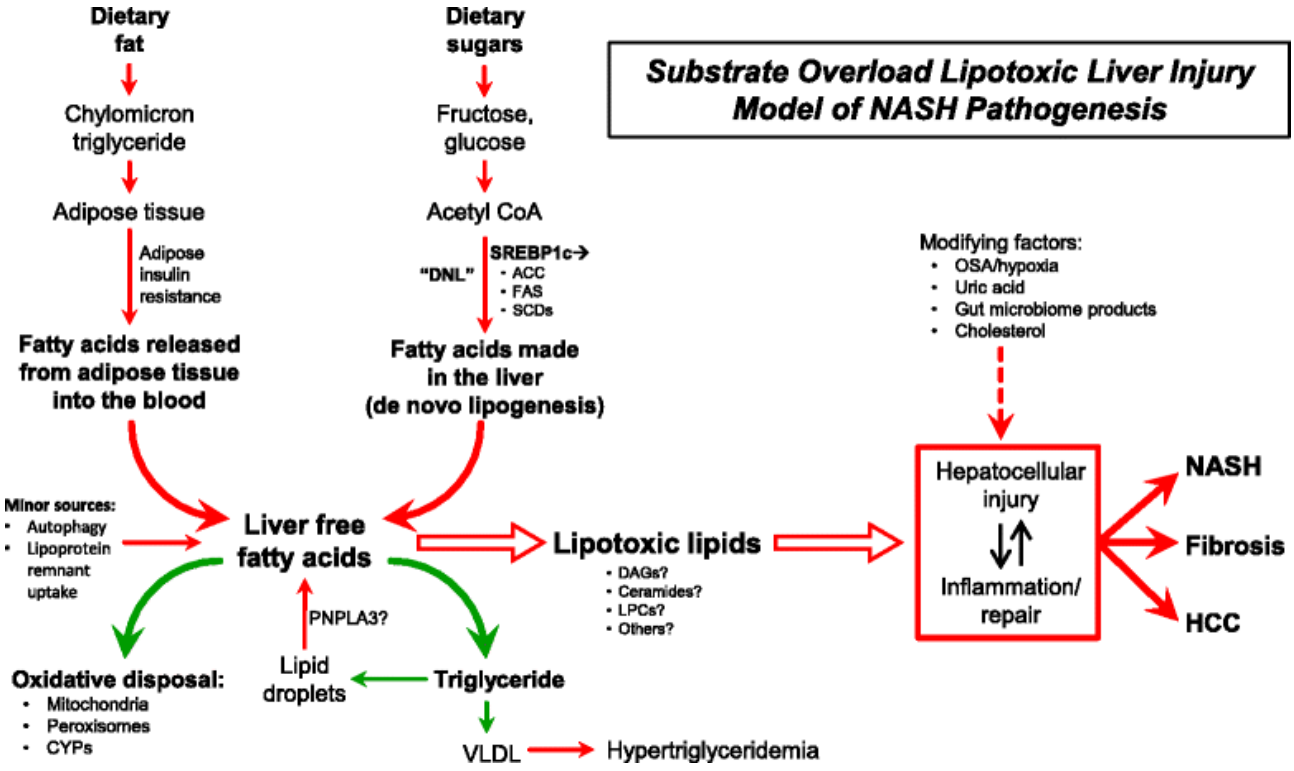

Figure 5. Fatty acids are delivered to the liver from adipose tissue and dietary sugars are turned into fatty acids in the liver. Excessive supply of dietary sugars and fatty acids are involved in the pathogenesis of nonalcoholic fatty liver disease. Insulin resistance, substrate overload, oxidative stress, hepatocellular injury, inflammation, and apoptosis produce the histological phenotype called nonalcoholic steatohepatitis that can evolve into fibrosis and possibly cancer. Figure from Neuschwander-Tetri, 2017[108]. ACC, acetyl-Coenzyme A carboxylase; DNL, de novo lipogenesis; FAS, fatty acid synthetase; HCC, hepatocellular carcinoma; OSA, obstructive sleep apnea; PNPLA3, patatin like phospholipase domain containing 3; SCD, stearoyl-Coenzyme A desaturase; SREBP1c, sterol response element binding protein-1c; VLDL, very low density lipoprotein.

Previously, insulin-sensitizing agents such as Thiazolidinediones have therefore been the most promising drugs for NAFL and NASH as these therapeutics have shown convincing reductions in aminotransferase levels, hepatic steatosis and improved histology[109–112]. Both cytoprotective agents and antioxidants have been considered as treatments; however, they

exhibit limited efficacy. Furthermore, in selected patients, lipid lowering drugs and iron depletion may be appropriate options[113]. The fact remains that there is no optimal treatment for these diseases, and thus novel anti-steatotic treatments that relieves IR and reduces body weight are intensively sought.

Current Treatment Possibilities

The following section describes the current treatment possibilities for obesity, T2D and NAFLD. Treatment of obesity is in most cases limited to lifestyle interventions, which is also first line therapy for T2D and NAFLD/NASH. Currently, there are no pharmacotherapies available for NAFLD and the search for relevant anti-steatotic drugs with effects on hepatic fatty fibrosis is intense.

Obesity

Obesity is a prevalent health challenge irrespective of gender, ethnicity, and age, affecting millions worldwide. Treatment of obesity is in most cases limited to lifestyle interventions; the comprehensive lifestyle consists of hypocaloric dieting, exercise, and behavioral strategies[114]. Many different diets and exercise styles are suggested worldwide and humans are constantly reminded of the healthy lifestyle they are supposed to live. Actually, a Mediterranean diet has been shown to be a proper alternative to a western diet[115] and furthermore, early studies suggest that weight loss is easier obtainable in groups[116]. Despite this, the fact remains that the number of obese people is increasing, and even though exercise and a healthy diet form the first line of therapy to prevent obesity and related morbidities, we need additional tools to fight obesity for obvious health and life quality reasons as well as economic reasons. The obesity epidemic has a marked impact on the global socioeconomic state that can be translated into enormous direct and indirect annual costs including sick days, and lack of productivity. Besides economy, there are social implications of obesity e.g. discrimination, inequality, and psychological burdens[117].

When lifestyle interventions fail, bariatric surgery and a few pharmacotherapies are available although these are only used in cases of severe obesity[118]. Table 2 summarizes the pharmacotherapies currently available. The obesity pandemic and the numerous co-morbidities have turned the focus on more invasive and aggressive therapies. The most effective treatment of obesity and T2D is bariatric surgery[119–121], hence this method is trending as a therapeutic option. However, considering the peri- and postoperative hazards, less invasive treatments could be favorable.

Class	Agent	Route of Administration	Mechanism Of Action
Lipase inhibitors	orlistat	Oral	Lipid degradation and uptake ↓
Amphetamine derivative/ GABA inhibitor	phentermine/ topiramate	Oral	Food intake ↓ Possibly energy expenditure ↑
Dopamine reuptake inhibitors/ Opioid antagonists	bupropion/ naltrexone	Oral	Food intake ↓
Subtype-selective serotonin receptor agonist	lorcaserin	Oral	Food intake ↓
GLP-1 analogue	liraglutide	Subcutaneous injection	Food intake ↓ gastric emptying rate ↓

Table 2. Current approved pharmaceutical treatments against obesity. Inspired by[122]. GABA, γ-aminobutyric acid; GLP-1, glucagon-like peptide-1.

Anti-obesity pharmacotherapy has been disappointing in terms of safety, efficacy, and long-term maintenance profile. Several promising drugs have been developed with pronounced efficacy *in vitro,* and in animal models and humans, albeit only a few of these are presently available on the market. Several candidates such as the serotonin releasers, fenfluramine and dexfenfluramine[123], and the selective serotonin and norepinephrine reuptake inhibitor, sibutramine[124], were associated with unacceptable side effects and withdrawn. Furthermore, cannabinoid receptor antagonist rimonabant was also removed as it was associated mood-related side effects[125]. Lorcaserin (Belviq)[126,127] and phentermine/ topiramate combination (Qsymia)[126,128] are both approved in US; however, they are not approved in Europe also due to safety concerns. As of current, orlistat, phentermine/ topiramate, bupropion/naltrexone, lorcaserin, and liraglutide are available in the United States, whereas only orlistat, bupropion/naltrexone, and liraglutide are available as anti-obesity pharmacotherapies in Europe, hence novel drug targets with an improved safety profiled are sought. Ideally, an anti-obesity treatment should not only reduce body weight but preferably also target some of the other MS risk factors such as fasting and postprandial hyperglycemia, IR and dyslipidemia, hence making KBPs a potential candidate.

Type 2 Diabetes Mellitus

Table 3 summarizes the pharmacological treatment possibilities for T2D including drug class, route of administration, mechanisms of action, and the associated side effects. There are multiple insulin therapies available – short acting, long acting and rapid acting as well as various premixed combination, and not all the different agents are listed. First line therapy is focused on treating the underlying obesity and IR with life style changes including weight loss, dieting, and exercise as described for obesity above[129–133]. As life style interventions become insufficient, drug therapy is applied[134,135]. An ideal anti-diabetic therapeutic approach should not only improve fating and postprandial hyperglycemia, but

also reduce body weight and change eating behavior. Traditionally, anti-diabetic treatments have been focused on correcting insulin deficiency with exogenous insulin and stimulating the insulin secretion from the ß-cells. Presently, several oral anti-diabetic agents are available, which are able to improve glycemic control[136–138]. The major classes of oral therapeutics include Sulfonylureas, Meglitinides, Biguanides, Thiazolidinedione, α-glucosidase inhibitors, dipeptidyl peptidase-4 (DPP-4) inhibitors, and sodium-glucose co-transporter (SGLT2) inhibitors. Sulfonylureas and Meglitinides enhance insulin secretion[136,139,140], while both Thiazolidinediones and Metformin improve peripheral insulin sensitivity. Thiazolidinediones stimulate adipogenesis hence increasing the number of insulin sensitive cells and thus glucose transport activity[138], and Metformin decreases the endogenous glucose production thus reducing hyperglycemia[141]. Additionally, SGLT2 inhibitors provide an insulin-independent reduction in blood glucose by blocking renal reabsorption of glucose. Unfortunately, some of these oral agents are associated with unwanted side effects like hypoglycemia, weight gain and even heart failure[142].

Another approach for treating T2D is to resemble mechanisms of action of the gut-derived hormone, glucagon-like peptide-1 (GLP-1), and the pancreatic glucoregulatory hormone, amylin[143–145]. These agents control food intake and induce weight loss. Interestingly, amylin and mimetics are unable to improve FPG alone and are therefore administrated in combination with insulin analogs[146–148]. Finally, DPP-4 inhibitors indirectly upregulate the concentration of GLP-1 by inhibiting the breakdown of GLP-1 by DPP-4[149]. These agents have no markedly adverse effects such as hypoglycemia or weight gain; some even stimulate body weight loss[150,151].

Class	Agents	Mechanism of action	Side effects
Sulfonylureas (oral)	Glyburide Glipizide Gliquidone Glyclopyramide Glimepiride Gliclazide	Insulin secretion ↑	Risk of hypoglycemia, occasionally severe
Meglitinides (oral)	Repaglinide Nateglinide	Insulin secretion ↑ (more rapid onset and shorter duration of action than sulfonylureas)	Lower risk of severe hypoglycemia than sulfonylureas
Biguanide (oral)	Metformin	Counters insulin resistance, EGP ↓	Lactic acidosis (rare)
Thiazolidinediones	Rosiglitazone Pioglitazone	Insulin sensitivity ↑ (peripheral glucose utilization ↑)	Edema, heart failure, weight gain, bladder cancer, fractures
α-Glucosidase inhibitors (oral)	Acabose Miglitol	Carbohydrate digestion rate ↓	Gastrointestinal adverse effects
DPP-4 inhibitors (oral)	Sitagliptin Saxagliptin Vidagliptin Linagliptin Alogliptin	Glucose-dependent insulin secretion ↑, glucagon secretion ↓	Nausea, drug interactions dependent on hepatic metabolism of individual agents
SGLT-2 inhibitors (oral)	Canagliflozin Dapagliflozin Empagliflozin	Glucosuria ↑ blocking (90%) of renal glucose reabsorption	Ketoacidosis (rare) Genital mycosis Bone fractures
Insulin (injection)	Short-Acting Long-Acting Rapid-Acting Pre-Mixed Combinations	Peripheral glucose utilization ↑, hepatic glucose output and lipolysis ↓	Risk of episodes of severe hypoglycemia, especially when achieving glycemic target, weight gain
GLP-1 analogues (injection)	Liraglutide Exenatide Dulaglutide Lixisenatide	Glucose-dependent insulin secretion ↑, glucagon secretion ↓, satiety ↑, gastric emptying rate ↓ body weight ↓	Nausea
Amylin analogue (injection)	Pramlintide	Glucagon secretion ↓, satiety ↑, gastric emptying ↓, only approved with simultaneous insulin treatment, body weight ↓	Possible reaction at injection site. Risk of hypoglycemia, nausea

Table 3. Available anti-diabetic treatments. Route of administration are in brackets. DPP-4, dipeptidyl peptidase-4; SGLT2, sodium-glucose co-transporter; GLP-1, glucagon-like peptide-1; EGP, endogenous glucose production. Inspired by[136–138].

Nonalcoholic Steatohepatitis

NAFLD is increasingly becoming common in parallel with the prevalence of obesity and MS[83,152]. The harmless and often undetected NAFL progresses into NASH and further to fibrosis and possibly cirrhosis, and NAFLD is projected to be the leading cause of liver transplants in the future[153]. Lifestyle modifications focusing on healthy eating, weight loss and regular exercise is also the cornerstone of NAFLD therapy in adults[154–156] and children[157]. Bariatric surgery has been shown to reverse NASH and even substantial fibrosis[158,159]; however, surgery is only performed in a minority of the patients and is associated with peri- and postoperative hazards, hence there is clearly a need for pharmacological therapies to treat NASH[160,161]. Recent trial results were reviewed by Brent Neuschwander-Tetri and figure 6 is an organized overview of the multiple potential points of attack in NASH treatment[108].

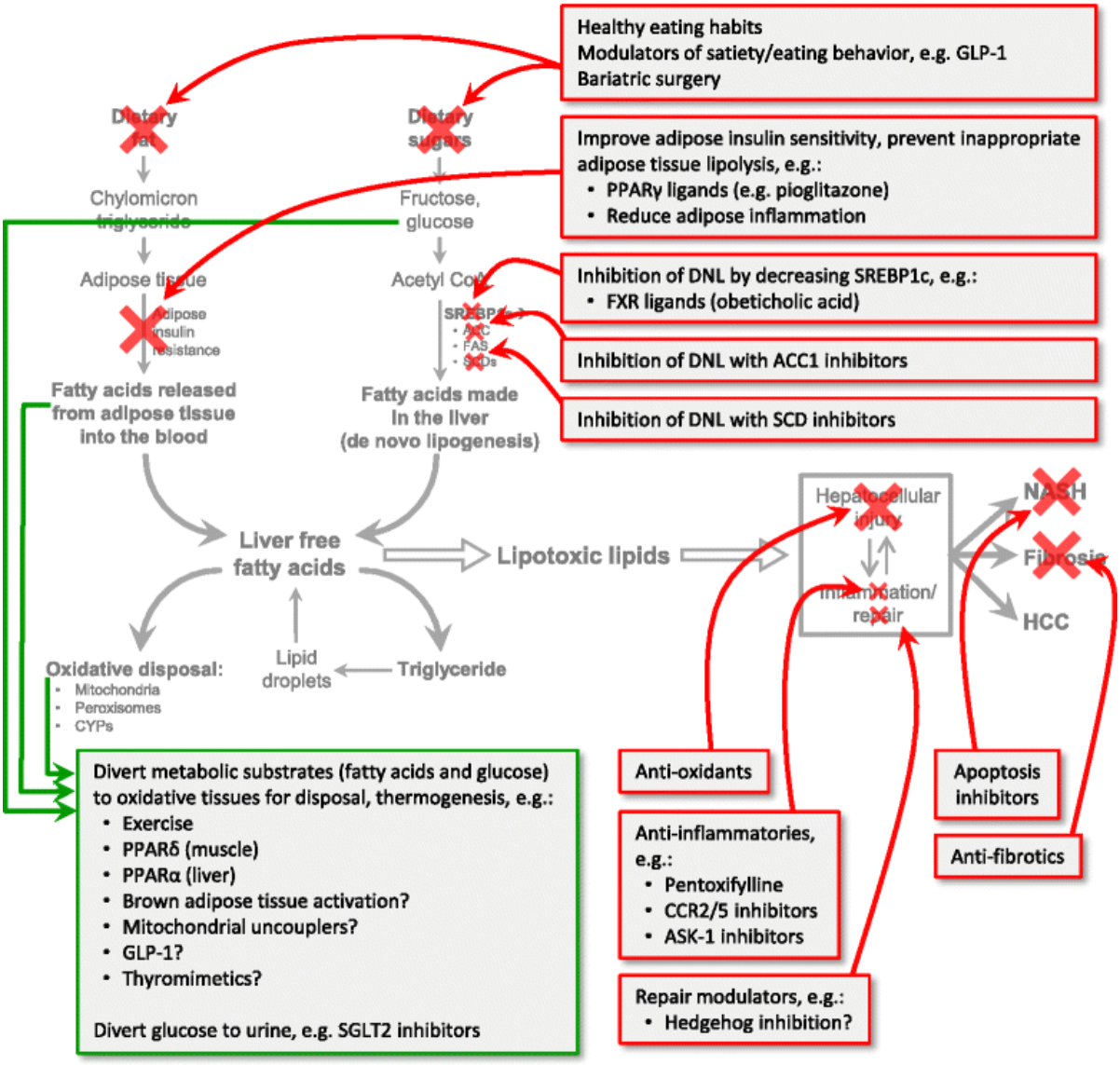

Figure 6. A schematic model of potential therapy targets. The red arrows are inhibitory approaches; green arrows are possible advantageous redirection of metabolic substrates. Figure created by Neuschwander-Tetri, 2017[108]. ACC, acetyl-Coenzyme A carboxylase; CCR2/5, chemokine 2 and chemokine 5 receptors; DNL, de novo lipogenesis; FXR, farnesoid X receptor; GLP-1, glucagon-like peptide-1; PPAR, peroxisome proliferator-activated receptor; SREBP1c, sterol response element binding protein-1c; FAS, fatty acid synthetase; SCD, stearoyl-Coenzyme A desaturase.

There are no approved pharmacotherapies for NASH. Clinical trials have addressed modulation of several pathways, and currently, there are anti-NASH candidates undergoing pivotal phase 3 clinical trials. Table 4 is a simple overview of drug candidates targeting NASH, which are most advanced in the clinical evaluation. As there are numerous candidates/drug classes under investigation, not all of them are described in detail in this thesis.

The PPAR family senses present lipophilic molecules and regulate gene expression accordingly. PPARα upregulates hepatic oxidative metabolism and PPARδ does that in muscle. Elafibranor, a PPARα/δ ligand, was evaluated in a phase 2 clinical trial (GOLDEN)

and appeared to relieve NASH and fibrosis[162], and these findings led to initiation of a phase 3 trial. Further, PPAR-γ agonists pioglitazone[109,110] and rosiglitazone[111] have shown beneficial effects on the histologic features of livers in patients with NASH.

A different approach to modulate metabolism was evaluated in the FLINT trial namely the farnesoid X receptor (FXR) ligand, obeticholic acid (OCA). The bile-acid-activated nuclear receptor, FXR, plays a pivotal role in the regulation of bile acid, lipid and glucose homeostasis as well as the regulation of inflammatory responses[163]. Recently, OCA was approved to treat primary biliary cholangitis[164]; however, at a lower dose compared to doses used in the FLINT trial where treated patients improved the composite NAFLD activity score (NAS) and their hepatic fibrosis[165]. This drug is also moved into phase 3.

A phase 2 clinical trial evaluating the inhibitor of apoptosis signal-regulating kinase 1 (ASK1), a protein that promotes inflammation, apoptosis and fibrosis during oxidative stress, selonsertib, was recently completed, and the preliminary analysis showed that patients receiving selonsertib demonstrated decreased hepatic steatosis and decreased fibrotic stage[166–168] and the drug is initiating phase 3.

GLP-1 analogue, liraglutide, is used to treat obesity and T2D but has also been evaluated in a small trial (LEAN) with NASH patients[169] where they found histological resolution of NASH. The DPP-4 inhibitor, sitagliptin, is used in T2D management; however, it did not significantly improve liver histology or transaminase levels in NAFLD patients[170]. Preliminary studies investigating cenicriviroc – an antagonist of the chemokine 2 and chemokine 5 receptors – suggest improvements in fibrosis[171,172] and other anti-inflammatory agents have been under investigation[173,174].

Class	Agent	Clinical trial advancement
FXR agonist	Obeticholic acid, Ocaliva	Phase 3, recruiting, FDA fast track status
FXR agonist	GS-9679	Phase 2b, recruiting, FDA fast track status
PPARα/δ agonist	GFT-505, Elafibranor	Phase 3, recruiting, FDA fast track status
CCR2/5 antagonist	Cenicriviroc	Phase 3, initiating, FDA fast track status
ASK1 inhibitor	GS-4997, Selonsertib	Phase 3, initiating
Galectin inhibitor	GR-MD-02	Phase 2b, ongoing, FDA fast track status
FGF21	BMS-986036	Phase 2b, ongoing
Fatty acid bile acid conjugate	Aramchol	Phase 2b, ongoing
Caspase inhibitor	IDN-6556, Emricasan	Phase 2b, recruiting
mTOT insulin sensitizer	MSDC-0602	Phase 2b, recruiting
ASBT inhibitor	Volixibat	Phase 2b, ongoing, FDA fast track status
GLP-1 analogue	Semaglutide	Phase 2b, recruiting
GLP-1 analogue	Liraglutide	Phase 2
Anti-LPS antibody	Imm124-E	Phase 2b, recruiting
PPARα/γ agonist	Saroglitazar	Phase 2b, initiating
PPARα/δ/γ agonist	IVA-337	Phase 2b, initiating

Table 4: Schematic presentation of some of the drug candidates targeting nonalcoholic steatohepatitis (NASH). Only drugs in phase 2b or farther clinical trial are mentioned. ASBT, apical sodium-dependent bile acid transporter; ASK1, apoptosis signal-regulating kinase 1; CCR2/5, chemokine 2 and chemokine 5 receptors; FGF21, fibroblast growth factor-21; GLP-1, glucagon-like peptide-1; mTOT, mitochondrial target of thiazolidinedione insulin sensitizers; PPAR, peroxisome proliferator-activated receptor. References[175,176,166]

Currently, therapies are focused on downstream events of liver injury such as inflammation and fibrogenesis. It perhaps would be of interest to target upstream events such as weight loss, control of satiety mechanisms, energy efficiency[177,178], hence possibly preventing the prevalence of progression into fibrosis and cirrhosis and targeting the core of MS. This makes liraglutide and potentially KBPs relevant for the treatment of NASH.

Target Receptors

The Key Bioscience peptides (KBP) are dual agonists that activate both the calcitonin receptor (CTR) and the amylin receptors (AMY-R).

The CTR receptor is located in various tissues. It is found in bone and has been demonstrated transcribed in rat brain, skeletal muscle, kidney and lung using RT-PCR technique[179]. The CTR is a seven transmembrane G-protein coupled receptor (GPCR) from the family B (Secretin family of 7 transmembrane receptors) of GPCRs. These receptors activate adenylyl cyclase and the phosphatidyl-inositol-calcium pathway[180]. In general, GPCRs sense extracellular ligands followed by an activation of the inside signaling pathways initiating a cellular response. When activated by an extracellular ligand, the GPCR undergoes a conformational change allowing the GPCR to act as a guanine nucleotide exchange factor, thus activating the G protein. The α-subunit dissociates from the ß and γ-subunits to further affect intracellular signaling. GPCRs are found only in eukaryotes[181]. The CTRs increase intracellular cyclic adenosine monophosphate (cAMP) concentration for

signaling, hence activating the adenylate cyclase[182]. Additionally, the CTR can induce intracellular Ca^{2+} levels activating phospholipase C for signaling[180,182]. Finally, the CTR can activate the mitogen-activated protein kinase pathway, which is important in the regulation of cellular differentiation, proliferation and transformation[183].

The AMY-R is formed when the receptor activating modifying proteins (RAMPs) associate with the CTR (Figure 7), and high affinity sites for amylin are localized in the brain, kidney and skeletal muscle[75]. The RAMP modifies the properties of the CTR to a high-affinity receptor for amylin[184,185]. The RAMP family consists of three members – three single transmembrane domains (RAMP1, RAMP2 and RAMP3)[75,180,186]. RAMPs form complexes with GPCRs altering their trafficking, ligand affinity, pharmacology, and/or signaling capabilities, and thereby, providing a mechanism diversity in the calcitonin peptide family receptors[187–190]. RAMP1 and RAMP3 creates a high-affinity AMY-R when associating with the CTR[75,183,185]. The CTR can associate with RAMP2; however, the physiological relevance of this complex is not that well described.

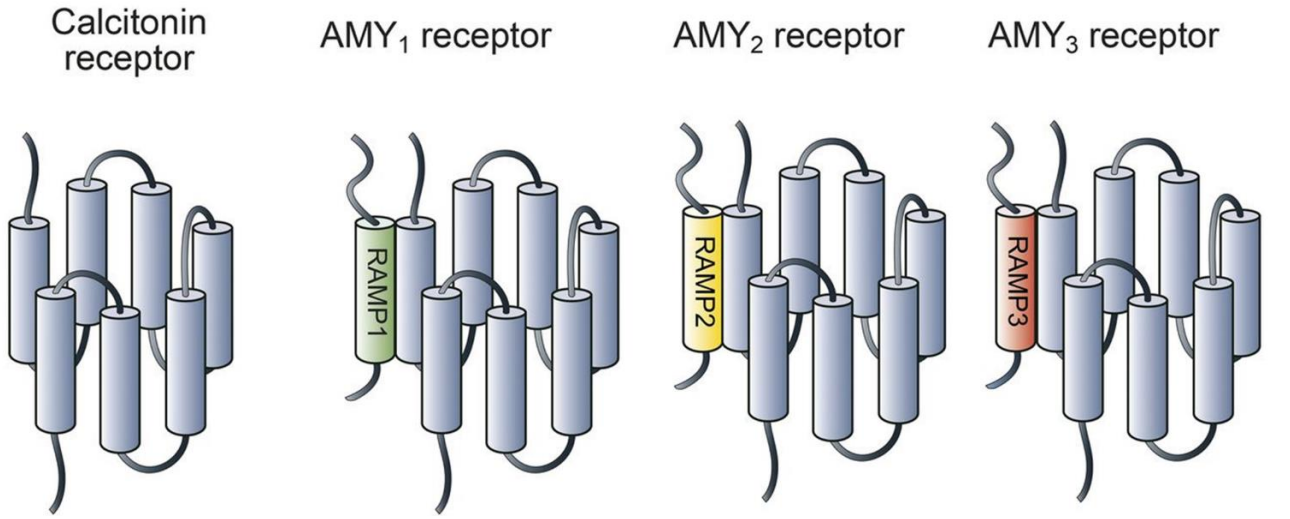

Figure 7. A schematic illustration of the calcitonin and the amylin receptors, which are formed by the interaction of the calcitonin receptor with RAMP1, RAMP2, or RAMP3 to generate the AMY1, AMY2, or AMY3 receptors. AMY, amylin; RAMP, receptor activity-modifying protein. Figure from[186].

The Calcitonin Peptide Family

The calcitonin peptide family is a family of proteins, including amylin, adrenomedullin, calcitonin gene-related peptide (CGRP) and intermedin[191,192]. Table 5 is a schematic overview of the calcitonin peptide family. This family of peptides share some structural homology; however, only little amino acid sequence homology is shared[180]. Adrenomedullin, CGRP and intermedin that do not target the CTR/AMY-R are not discussed in detail in thesis.

Peptide	Length	Secreted by	Receptor	Pharmacological effects in humans
hCT	32 aa	C-cells in the thyroid gland	CTR	Bone resorption ↓
sCT	32 aa	Ultimopharyngeal body	CTR AMY-R	Bone resorption ↓ gastric acid secretion ↓ gastric emptying↓ renin secretion, plasma lactate↑
Amylin	37 aa	Co-secreted with insulin by the pancreatic ß-cells	AMY-R (CTR + RAMP1 or RAMP3)	Gluconeogenesis in skeletal muscle ↓ renin secretion ↑ glucagon secretion ↓ gastric emptying ↓ plasma lactate↑
α-/β CGRP	37 aa	Peripheral neurons, central neurons and C-cells in the thyroid gland	CLR + RAMP1	Vasodilatory, renin secretion ↑ neuro-transmission, elevated during migraine
Adrenomedullin	52 aa	Adrenal medulla, vascular endothelial cells, smooth muscle cells, cardiomyocytes, fibroblasts, macrophages, neurons, glial cells and retinal pigment epithelial cells	CLR + RAMP2 or RAMP3	Vasodilatory, diuretic, bronchodilatory
Intermedin	47 aa	Pituitary gland and the digestive tract	CLR + RAMP1 or RAMP3	Food intake↓, gastric emptying ↓

Table 5. Schematic overview of the calcitonin peptide family. Inspired by following papers[75,76,136,180,182,191–198]. hCT, human calcitonin; sCT, salmon calcitonin; aa, amino acids; AMY-R, amylin receptor; CTR, calcitonin receptor; RAMP, receptor activity-modeling protein; CGRP, calcitonin-gene related peptide; CLR, calcitonin like receptor.

Calcitonin

Calcitonin is a 32-amino acid linear polypeptide hormone that is produced in C-cells of the thyroid in response to increased plasma Ca^{2+} concentration[199]. Copp and colleagues first discovered calcitonin in 1961, and its effect in calcium homeostasis was elucidated one year later[200]. Calcitonin was classified as a hypocalcemia agent reducing Ca^{2+} concentrations in

the blood, opposing the parathyroid hormone effect[199–201]. Calcitonin has been discovered in fish, reptiles, birds and mammals; however, the physiologic role of calcitonin is not completely known[194] – known pharmacological and physiological properties of calcitonin are presented in table 6.

Body function	Pharmacological effect	Physiological effect in humans
Calcium homeostasis	Alleviate hypercalcemia	Alleviate hypercalcemia
Bone resorption	Inhibition	?
Pain	Analgesic	?
Insulin secretion	Insulinostatic	?

Table 6. Overview of pharmacological and physiological effects of calcitonin. References [201–215]

Salmon calcitonin (sCT) – a teleost member of the calcitonin family – was isolated in 1968[216,217]. The teleost/avian calcitonins are the class with most interest due to their high potencies and ability to induce a prolonged effect in contrast with mammal calcitonins. sCT was found to have superior potency compared to other known types of calcitonin[218]. The amino acid sequence similarity to human calcitonin (hCT) is approximately 50%. sCT and hCT share physical/chemical properties depending on the setup and the duration of the experiment. Interestingly, the intrinsic potency of sCT is significantly higher compared to hCT[219]. Moreover, activation of the CTR by sCT induces a prolonged response able to persist for up to 72 hours *in vitro*[220].

Since its characterization, calcitonin has been implicated in a wide range of biological and pharmacological activities, and its pharmacological properties have been used in humans to treat osteoporosis since more than 40 years ago[201,221,222] due to preservation of bone mineral density [194,223], and suppression of kidney calcium secretion[179]. Osteoporosis is a common disease in post-menopausal women caused by decreased estrogen levels causing altered bone remodeling and increased risk of fragility fractures. Bone remodeling is the equilibrium between resorption by osteoclasts and formation by osteoblasts[224]. Furthermore, sCT is used for treatment of Paget's disease, phantom limb pain, and bone associated pain due to its powerful analgesic effect[194,225], which has also been shown for human and porcine calcitonin[211,212,226].

In contrast to calcitonin pharmacology, determining calcitonin physiology has been more challenging. Several reviews have tried to elucidate the physiological role of calcitonin; however, with limited success. Using antagonist and knockout models have only yielded modest conclusions and perhaps the sparse knowledge of physiological roles is due to the fact that no syndromes, conditions or diseases occurs exclusively from excess calcitonin or deficiency[227–229]. What is suggested is that during pregnancy and following lactation period, calcitonin is elevated in rodent and human[205,230], and thus a physiological role has been proposed[231]. Moreover, CTR knockout mice had increased calcium levels for a prolonged period following calcitriol induced hypercalcemia, hence suggesting a physiological role of calcitonin during calcium stress[203,204]. Hypercalcitoninemia has been associated with

thyroid carcinomas[232], and further, calcitonin has been suggested to centrally regulate bone metabolism through as the CTR is highly expressed in the hypothalamus[221].

Amylin

Amylin is a 37-amino acid hormone synthesized in the ß-cells and co-secreted with insulin in response to nutrients exerting complementary prandial actions to insulin[79,80]. Amylin was initially identified with two different names: Insulinoma amyloid peptide (IAPP)[233] and amylin[234]. Currently, IAPP is used to describe the fibrillous plaques formed in the ß-cell during the pathogenesis of T2D, whereas amylin is the designation for the circulating peptide hormone. In contrast to rodent amylin, primate and feline amylin can aggregate and forms fibril-like structures known as amylin plaques[235]. These fibrils are believed to have an impact on islet death and T2D[236].

The physiological and pharmacological effects of amylin are not always easily distinguished. Table 7 is an overview of pharmacological and physiological properties of amylin. Most pharmacological effects of amylin are associated with the control of eating and influx of nutrients into circulation as amylin suppresses food intake, gastric acid secretion, delays gastric emptying and diminishes hyperglucagonemia and digestive enzyme secretion and thus controlling nutrient appearance and prandial glycemia[237–240]. Amylin actions are thought to be centrally mediated by receptors in the area postrema of the brain[241,242], which appear to be responsible for the direct anorectic effect[243], albeit also other brain areas are suggested to be facilitating the neuronal response of amylin[240,244,245]. Additionally, the amylin actions in the area postrema is modulated by glucose – increased glucose concentrations potentiate the neuronal response[246] and decreased glucose concentrations suppressed amylin effect on gastric emptying[247]. Besides the high affinity sites in the brain, kidney and skeletal muscle also have high affinity sites for amylin[75]. In muscles, amylin opposes glycogen synthesis and activates glycogenolysis[79]. Furthermore, amylin likely stimulates lactate flux, and thus is suggested to have an effect of transposing carbon from peripheral stores to the liver, making it available for glucose, glycogen and lipid synthesis[79,198].

Acutely, amylin induces IR in skeletal muscle, but does not alter insulin action in fat and might favor energy deposition in adipose tissue[79,248]. Amylin inhibits insulin secretion from ß-cells[76,249] and this reduced insulin secretion in combination with IR in skeletal muscle, relatively preserved insulin sensitivity in adipose tissue, increased lactate turnover, and increased HGP are features in IR and early T2D[79]. Like fasting insulinemia, fasting plasma levels of amylin are elevated in obese individuals, and high plasma concentrations of amylin in early T2D might contribute to increasing IR[76]. In late T2D, amylin is comparatively deficient, depending on the severity of the ß-cell dysfunction and as for insulin, resistance to amylin action has been suggested[76,250].

Body function	Pharmacological effect	Physiological effect in humans
Food intake	Anorectic	Anorectic
Gastric emptying rate	Delayed	Delayed
Energy expenditure	Enhanced	?
Insulin secretion	Insulinostatic	Insulinostatic
Glucagon secretion	Glucagonostatic	Glucagonostatic

Table 7. Overview of pharmacological and physiological effects of amylin and amylin analogues. References[251–265]

The understanding of the physiological role of amylin has been evaluated using amylin antagonists. The physiologic role for amylin in glucose homeostasis via mechanisms that include regulation of food intake, glucagon secretion and gastric emptying was evaluated using amylin antagonist, AC 187. The inhibition of amylin signaling increased food intake, glucagon concentration and accelerated gastric emptying in rats[262,266]. In humans, amylin antagonist AC 253 increased glucose stimulated insulin secretion[261] and in rats, AC 187 increased plasma concentrations of glucagon[262], hence indicating a physiological role of amylin in the regulation of insulin and glucagon secretion. Furthermore, using AC 187 it has been demonstrated that amylin is involved in regulation of energy expenditure (EE) in rats[267]; however, this amylin mediated increase in EE remains to be seen in man.

The importance of amylin in energy homeostasis and food intake as well as the insulinostatic and glucagonostatic properties has led to an increased interest in research investigating amylinomimetics as potential treatments for obesity and T2D.

Dual Amylin and Calcitonin Receptor Agonists

The Key Bioscience peptides (KBP) are DACRAs. They are based on the peptide backbone of sCT and as sCT, KBPs are dual agonists targeting both the CTR and the AMY-R, albeit with no activation of the CGRP receptor (Figure 8). DACRAs have higher affinity for the two receptors than the native ligands, hCT and amylin, that target the CTR and AMY-R, respectively[220,268]. Thus, sCT and KBPs mimic the effects of both hCT and amylin, albeit considerably more potent.

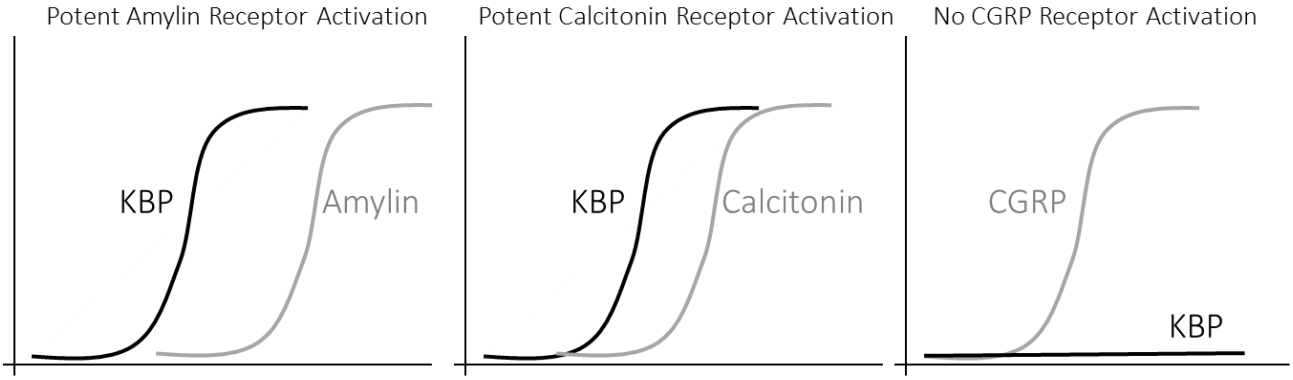

Figure 8. Schematic illustration of the relative potencies of KBPs and the endogenous ligands on the amylin, calcitonin and calcitonin gene-related peptide (CGRP) receptor. KBPs potently activate the amylin and calcitonin receptor, albeit not the CGRP receptor[268–270], which has been associated with undesirable side effects such as migraine[193,271,272].

This thesis addresses three different DACRAs, KBP-042, KBP-088 and KBP-089. The knowledge obtained in previous studies with sCT encouraged the development of novel dual agonists targeting both receptors. KBP-042 was the first peptide discovered and it was first presented in an oral form in 2014[268]. Later on, an injectable form of KBP-042 showed reduced variations on bioavailability[273] and similar peptides like KBP-088[269] and KBP-089[270,274] were developed. The sequences of the three KBPs as well as sCT, davalintide and the native ligands are schematically presented in table 8.

Name	N-term	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	C-term
hAMY	–	K	C	N	T	A	T	C	A	T	Q	R	L	A	N	F	L	V	H	S	S	N	N	F	G	A	I	L	S	S	T	N	V	G	S	N	T	Y	$-NH_2$
rAMY	–	K	C	N	T	A	T	C	A	T	Q	R	L	A	N	F	L	V	R	S	S	N	N	L	G	P	V	L	P	P	T	N	V	G	S	N	T	Y	$-NH_2$
hCT	–	C	G	N	L	S	T	C	M	L	G	T	Y	T	Q	D	F	N	K	F	H	T	F	P	Q	T	A	I	G	V	G	A	P						$-NH_2$
sCT	–	C	S	N	L	S	T	C	V	L	G	K	L	S	Q	E	L	H	K	L	Q	T	Y	P	R	T	N	T	G	S	G	T	P						$-NH_2$
KBP-042	Ac–	C	S	N	L	S	T	C	V	L	G	K	L	S	Q	E	L	H	K	L	Q	T	Y	P	R	T	D	V	G	A	N	A	P						$-NH_2$
KBP-088	Ac–	C	S	N	L	S	T	C	M	L	G	R	L	S	Q	E	L	H	R	L	Q	T	F	P	K	T	D	V	G	A	N	A	P						$-NH_2$
KBP-089	Ac–	C	S	N	L	S	T	C	M	L	G	R	L	S	Q	D	L	H	R	L	Q	T	Y	P	K	T	D	V	G	A	N	A	P						$-NH_2$
Davalintide	–	K	C	N	T	A	T	C	V	L	G	R	L	S	Q	E	L	H	R	L	Q	T	Y	P	R	T	N	T	G	S	N	T	Y						$-NH_2$

Table 8. Amino acid sequence of human and rat amylin (hAMY and rAMY), human calcitonin (hCT), salmon calcitonin (sCT), KBP-042, KBP-088, KBP-089 and davalintide. Modifications in KBPs consist of an N-terminal acetyl group and a C-terminal amid group[268–270,275].

Interestingly, the natural DACRA, sCT, as well as the synthetic peptides have shown anti-obesity and anti-diabetic potential in obese and diabetic animal models[195,276,277]. All the effects of the dual agonists are listed in figure 9.
DACRAs activate the AMY-R, induce similar effects as amylin and are partly amylinomimetics. Amylin induces weight loss, delays gastric emptying rate and reduces hyperglucagonemia in T2D patients, thus improving postprandial glycemic control[237,263,278]. In preclinical models of obesity and T2D, KBPs have demonstrated beneficial effects on body weight, fasting and postprandial glucose control and HbA1c levels[269,273,274,279], and interestingly, sCT was found to preserve pancreatic function and ß-cell area in ZDF rats[276].

Like amylin, KBPs delay the rate of gastric emptying and attenuate inappropriate hyperglucagonemia, thereby influencing postprandial plasma glucose (PPG) levels[273,279]. Interestingly, KBP treatment reduces obesity related hyperleptinemia[279,280] improves insulin and leptin action[273], which might be involved in the improved glucoregulation. The profound DACRA effect on gastric emptying and food intake is most like centrally mediated via AMY-R and/or CTRs located in area postrema in the brain, as it has been found with sCT treatment that prolong the excitation of these neurons compared to amylin[243,266,281,282].

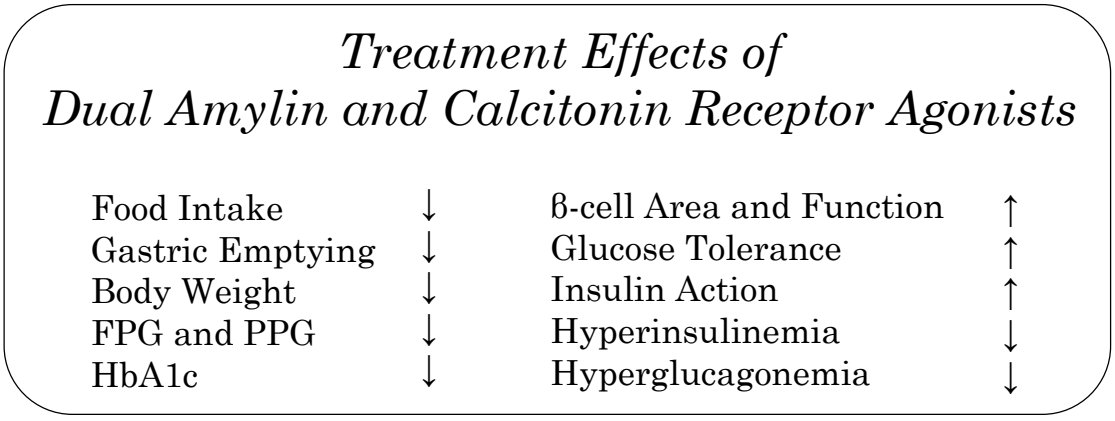

Figure 9. Schematic illustration of the treatment effects of dual amylin and calcitonin receptor agonists in rat models of obesity, type 2 diabetes and nonalcoholic steatohepatitis. FPG, fasting plasma glucose; PPG, postprandial plasma glucose. HbA1c, glycated hemoglobin. ↓ reduction, ↑ improvement.

DACRAs induce an amylin attributable hyperglycemic response in healthy people and lean rats[260,283,284]. Neither insulin nor glucagon is the promoter, but lactate is the suggested candidate as it is elevated after acute dosing of DACRAs[285]. Lactate is substrate for hepatic gluconeogenesis and therefore increases EGP resulting in hyperglycemia in non-diabetic rats[285]. These hyperglycemic and diabetogenic effects were observed as result of a single injection of DACRAs. The difference in physiological outputs could lie in single dose administration or a chronic treatment, and state of disease, as no hyperglycemic effect of sCT or any of the synthetic DACRAs have been observed in obese and diabetic rat models[269,274,276,279,286,287].

In summary, both natural and synthetic DACRAs potently activate both the AMY-R and CTR with prolonged duration. They regulate appetite and induce a substantial weight loss, which is associated with various metabolic improvements. However, whether the prolonged receptor activation manifests in enhanced *in vivo* efficacy, and whether the metabolic changes and suggested increase in EE is solely due to weight loss remains to be elucidated.

CHAPTER II

II. Hypothesis and Aim

Amylin and/or calcitonin receptor agonists such as pramlintide and davalintide have shown promise on weight reduction in preclinical models and clinical settings, albeit with limited efficacy on glucose homeostasis. The overall aim of this Ph.D. project was to investigate the pharmacological effect of the KBP peptides focusing on the anti-obesity potential in preclinical animal models of obesity and related morbidities like type 2 diabetes and NASH.

It was hypothesized that a prolonged receptor activation would improve the *in vivo* efficacy and that KBPs would show beneficial metabolic effects beyond caloric intake and simple diet-induced weight loss.
The more specific objectives for these studies were:

1) To explore whether the reduced food intake is the primary cause of weight loss, and whether the weight loss is the main reason for improved metabolic health.
2) To elucidate the KBP effect on lipid accumulation in key tissues, such as liver and muscle, as well as other hepatic features of NASH like ballooning and inflammation, and fibrosis.
3) To evaluate KBP tolerability using different dose escalation regimes and dosing frequencies.
4) To compare the *in vitro* and *in vivo* efficacy of KBP to second-generation amylinomimetic, davalintide, and to investigate whether KBP works complementary with GLP-1 receptor agonist, liraglutide.

CHAPTER III

III. The Major Findings

Summary of Results

Obesity and associated morbidities, such as diabetes, nonalcoholic fatty liver diseases and cardiovascular disease are consequences of the modern day lifestyle, and the number of obese people is increasing. Treatment of obesity is limited to lifestyle interventions; however, in severe cases, bariatric surgery and a few pharmacotherapies are available as tools against morbid obesity, hence novel candidates focusing on weight loss, insulin sensitivity and liver pathophysiology are intensely sought.

In this series of studies, the focus was on the metabolic effects of the KBPs. Effects on body weight, adiposity and glucose metabolism were thoroughly explored in experimental models of obesity, T2D and NASH. To address whether the beneficial effects were solely due to suppression of food intake and the subsequent weight loss we included pair-fed and weight-matched controls. As amylin agonism induces a well-known anorexic effect at dose initiation, these studies also focused on different dosing regimens including dose escalation and dosing frequency. Finally, we compared KBP to a second-generation amylinomimetic, davalintide, and investigated the combination of KBP and GLP-1 analogue, liraglutide.

The findings show that KBPs potently activated both the amylin and calcitonin receptors *in vitro*, and not the CGRP receptor, and demonstrated a prolonged receptor activation *in vitro* and *in vivo* when compared to second-generation amylinomimetic, davalintide. Thus, KBPs elicit prolonged activation supporting the relevance of long-term *in vivo* investigations.

The anti-obesity potential of KBPs was tested in high fat diet induced obese (HFD) rats. KBP transiently suppressed caloric intake, and induced and sustained a dose-dependent weight loss compared to vehicle rats and pair-fed rats. Concomitantly, the overall adiposity was decreased and obesity related adipocyte hypertrophy was reduced. The high fat feeding resulted in increased accumulation of ectopic lipids in liver and muscle tissue. Importantly, this inappropriate lipid accumulation was completely eliminated by KBP treatment even in an interventive setup. Plasma adiponectin was increased and plasma leptin levels were decreased following long-term treatment with KBP compared to vehicle rats. Interestingly, KBPs alleviated hyperinsulinemia and improved glucose tolerance even with significantly lower insulin levels. Insulin sensitivity was formally assessed in obese rats using the

hyperinsulinemic–euglycemic clamp. KBP increased the glucose infusion rate indicating enhanced insulin action. Importantly, KBP also improved glucose homeostasis and enhanced insulin action in Zucker Diabetic Fatty rats. To investigate beneficial effects beyond weight loss in HFD rats, a weight-matched group was included. Interestingly, weight matching led to improved glucose homeostasis through lowered plasma insulin; however, these effects were inferior to the effects of KBP. In a food preference test, KBP changed the food preference of normal diet rats. The rats that had *ad libitum* access to chow and chocolate obtained 74% of their calories from chocolate. Of interest, KBP administration reduced total caloric intake, and induced a relative increase in chow consumption while drastically lowering the chocolate compared to vehicle. Thus, KBPs have anti-obesity potential and beneficial effects on glucose metabolism and IR independent of food consumption and weight loss.

KBP was introduced using various dosing regimens and frequencies. Dosing every day and every second day resulted in an equal weight loss at study end, albeit with an uneven reduction in both food intake and body weight in the HFD rats dosed every second day. In a 4-fold dose escalation, KBP induced a transient reduction in food intake at every escalation step – with reducing magnitude over time. Two-fold and linear escalations suppressed body weight evenly with no significant reduction in food intake at either escalation step. Thus, KBP is well tolerated in high concentrations when introduced by dose escalation, and importantly, similar weight loss is obtained.

For the first time, a KBP and a GLP-1 receptor agonist were combined. Interestingly, when the two peptides were combined the effect on acute food intake was superior to the effect of either single-dosed peptide. Chronically, KBP-089 (1.25 μg/kg) and liraglutide (50 μg/kg) lowered body weight 8% and 2% in HFD rats, respectively, while the combination resulted in a 12% body weight reduction. Moreover, the combination improved glucose tolerance. Thus, KBP acts complementary with GLP-1, indicating the potential of an add-on therapy causing additional weight loss.

The effects of KBP on metabolic and hepatic features were tested in a rat model resembling the phenotype of human NASH – an obese model with excessive steatosis, and inflammation and mild fibrosis. In line with aforementioned results, KBP lowered body weight, reduced overall adiposity and improved insulin action. Furthermore, KBP treatment led to a reduction of the high fat, high cholesterol and cholate diet induced increase in liver weight and circulating AST levels. Finally, KBP reduced hepatic steatosis, ballooning and inflammation, hence resulting in a reduced NAS score in combination with a lowered fibrosis stage supporting an anti-NASH potential of KBP.

In summary, KBPs induce and sustain weight loss, leading to improved metabolic parameters including food preference, and these are beyond those observed simply by diet-induced weight loss. Additionally, these peptides are well tolerated when introduced by dose escalation and equal weight loss is obtained. Finally, KBPs reduce liver steatosis in both

obese and NASH rats as well as inflammation and fibrosis scores, hence underscoring the potential as an anti-obesity agent with benefits on glucose control and liver health.

Paper I:

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

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RESEARCH PAPER

A novel dual amylin and calcitonin receptor agonist, KBP-089, induces weight loss through a reduction in fat, but not lean mass, while improving food preference

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BACKGROUND AND PURPOSE

Obesity and associated co-morbidities, such as type 2 diabetes and non-alcoholic fatty liver disease, are major health challenges. Hence, there is an important need to develop weight loss therapies with the ability to reduce the co-morbidities.

EXPERIMENTAL APPROACH

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

KEY RESULTS

Rats fed a high-fat diet were treated, s.c., with KBP-089 0.625, 1.25, 2.5 $\mu g \cdot kg^{-1}$ or vehicle. KB-089 induced in a dose-dependent and sustained weight loss (~17% by 2.5 $\mu g \cdot kg^{-1}$). 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 by reducing plasma insulin; however, these effect were inferior compared to those of KBP-089. In the food preference test, rats fed a normal diet obtained 74% of their calories from chocolate. KBP-089 reduced total caloric intake and induced a relative increase in chow consumption while drastically reducing chocolate consumption compared with vehicle.

CONCLUSIONS AND IMPLICATIONS

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.

Abbreviations

DACRA, dual amylin and calcitonin receptor agonist; FPG, fasting plasma glucose; HbA1c, glycated haemoglobin; HFD, high-fat diet; HOMA-IR, homeostatic model assessment for insulin resistance; IVGTT, i.v. glucose tolerance test; ND, normal diet; OGTT, p.o. glucose tolerance test; PW, pair weighed; ZDF, Zucker Diabetic Fatty ZDF-Lepr^{fa}/Crl

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Tables of Links

TARGETS
Amylin receptors
Calcitonin receptors

LIGANDS	
Amylin	Insulin
Calcitonin	Pramlintide

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015).

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 the 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 being 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 with classical amylin receptor agonists (Andreassen *et al.*, 2014). Interestingly, they also activate the receptors for an extended period of time, when compared with the classical agonists, which appears to increase the *in vivo* efficacy as well as reducing 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 Zucker Diabetic Fatty ZDF-Lepr^{fa}/Crl (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* – reduce food intake, results in weight reduction and suppression of glucagon levels (Roth *et al.*, 2006), and also have 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 has continued, resulting in the development of KBP-089.

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

Methods

Peptide therapy

Synthetic KBP-089 (American Peptide Company, CA, USA) was dissolved in saline for s.c. delivery. 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).

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 ZDF (Kingston, NY, USA) were obtained at 6 weeks of age and housed (two 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. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath & Lilley, 2015).

Animals

From arrival and throughout the study periods, the high-fat diet (HFD) rats were fed a 60 kcal% fat diet (#58Y1, TestDiet, London, UK), lean normal diet (ND) age-matched rats were fed a standard pelleted chow (#5002, LabDiet, St. Louis, MO, USA) and ZDF rats were fed a 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-blindly assigned into experimental groups according to body weight. ZDF rats were non-blindly assigned into experimental groups 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 the start of the study. 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 on a high-fat diet, HFD rats were assigned to treatment groups receiving either vehicle (saline s.c.) or KBP-089 (0.625, 1.25 and 2.5 $\mu g \cdot kg^{-1}$, s.c.) once daily in the afternoon with a food restricted pair-fed control group for 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 the loss of an animal). Pair-fed animals received an average of the daily intake of food of the 2.5 $\mu g \cdot kg^{-1}$ treatment group every day in the afternoon. Food intake and body weight were monitored daily during the initial 2 weeks and once weekly throughout the entire study period. Following 6 and 7 weeks of treatment, p.o. and i.v. 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 killed by being anaesthetized with isoflurane (administered by inhalation) followed by exsanguination and dissection. Retroperitoneal, epididymal and s.c. 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 to two groups (n = 10 rats per group) receiving either vehicle (saline, s.c.) or KBP-089 (5 $\mu g \cdot kg^{-1}$ for 4 weeks, 20 $\mu g \cdot kg^{-1}$ for an additional 4 weeks, s.c.) once daily. The p.o. glucose tolerance test (OGTT) was performed after 4 weeks and the i.p. insulin tolerance test 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; HOMA-IR = fasting insulin ($\mu U \cdot mL^{-1}$) × fasting blood glucose ($mmol \cdot L^{-1}$)/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 $\mu g \cdot kg^{-1}$ 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 food restriction needed to achieve a comparable weight reduction 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 killed as for the chronic *in vivo* studies.

Food preference in ND rats. To assess the effect of KBP-089 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 $\mu g \cdot kg^{-1}$ KBP-809 were initiated. Voluntary food and chocolate intake were monitored for 24 h after treatment for 7 days.

Glucose tolerance tests

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

Insulin tolerance test

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

Fat accumulation in liver and muscle tissue

To address tissue fat accumulation, the liver and gastrocnemius muscle were surgically removed for optimal cutting temperature compound embedding, snap frozen on ice/ethanol, stored at −80°C until cryosectioning. Tissue sections were stained with oil red O stain, and images were captured with a light microscope (magnification of ×40 for gastrocnemius and ×20 for liver, nine images per animal; three 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, for simplicity, were visualized as the fold-induction from lean rats.

Biochemical analysis

Blood samples were collected in EDTA tubes and centrifuged at 1850 × g 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) were analysed according to the manufacturer's instruction.

Statistical analysis

All data are presented as means ± 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 with HFD vehicle, and ZDF vehicle with ZDF KBP-089, using Student's *t*-test. All analyses were performed using GraphPad Prism software (GraphPad Prism, San Diego, CA, USA). 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 vs. 545 ± 8 g, $P < 0.05$), hyperinsulinaemia (1.9 ± 0.1 vs. 0.9 ± 0.1 ng·mL^{-1}, $P < 0.05$), impaired glucose control without hyperglycaemia (OGTT tAUC 1343 ± 18.1 vs. 1259 ± 17.1, $P < 0.05$) and impaired insulin sensitivity (HOMA-IR) (11.3 ± 0.3 vs. 4.3 ± 0.3, $P < 0.05$), compared with the lean age-matched controls. Thus, the HFD rats resembled an obese and prediabetic 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 been 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 s.c. administered in three doses (0.625, 1.25 and 2.5 μg·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 μg·kg^{-1} treated rats compared with vehicle rats (504 ± 35 vs. 408 ± 19 g per animal). 8 weeks of KBP-089 treatment resulted in a dose-dependent and sustained 17 ± 1.7 % weight loss in the 2.5 μg·kg^{-1} group (Figure 1B), while pair-feeding resulted in a 4 ± 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 with 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 min 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 with vehicle and pair-fed controls 5 min (IVGTT) and 15 min (OGTT) after glucose administration (Figure 2A, D). However, tAUC was not significantly changed for either test when compared with 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 hyperinsulinaemia in either test.

KBP-089 reduces the 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 with lean age-matched controls. This inappropriate storage of lipids was completely eliminated by treatment with 2.5 μg·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 μg·kg^{-1} for 4 weeks, 20 μg·kg^{-1} for additional 4 weeks, s.c.). In ZDF rats, fasting blood glucose levels were decreased significantly (6.9 ± 0.7 mM, $P < 0.05$) over 7 weeks by KBP-089 treatment compared with vehicle, resulting in HbA1c reduction by ~2.5 ± 0.2% compared with 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 reduction compared with vehicle. The tAUC was lowered significantly (~30%, $P < 0.05$). Insulin action was assessed in an insulin tolerance test which manifested in a significant larger drop in blood glucose in response to insulin in KBP-089-treated rats compared with vehicle (tAUC ~19%, $P < 0.05$), 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 weighed'/'PW'). In order to match the body weights, the pair-weighed rats received significantly less food compared with the KBP-089-treated rats (Figure 5A). As in the previous study, body weight was

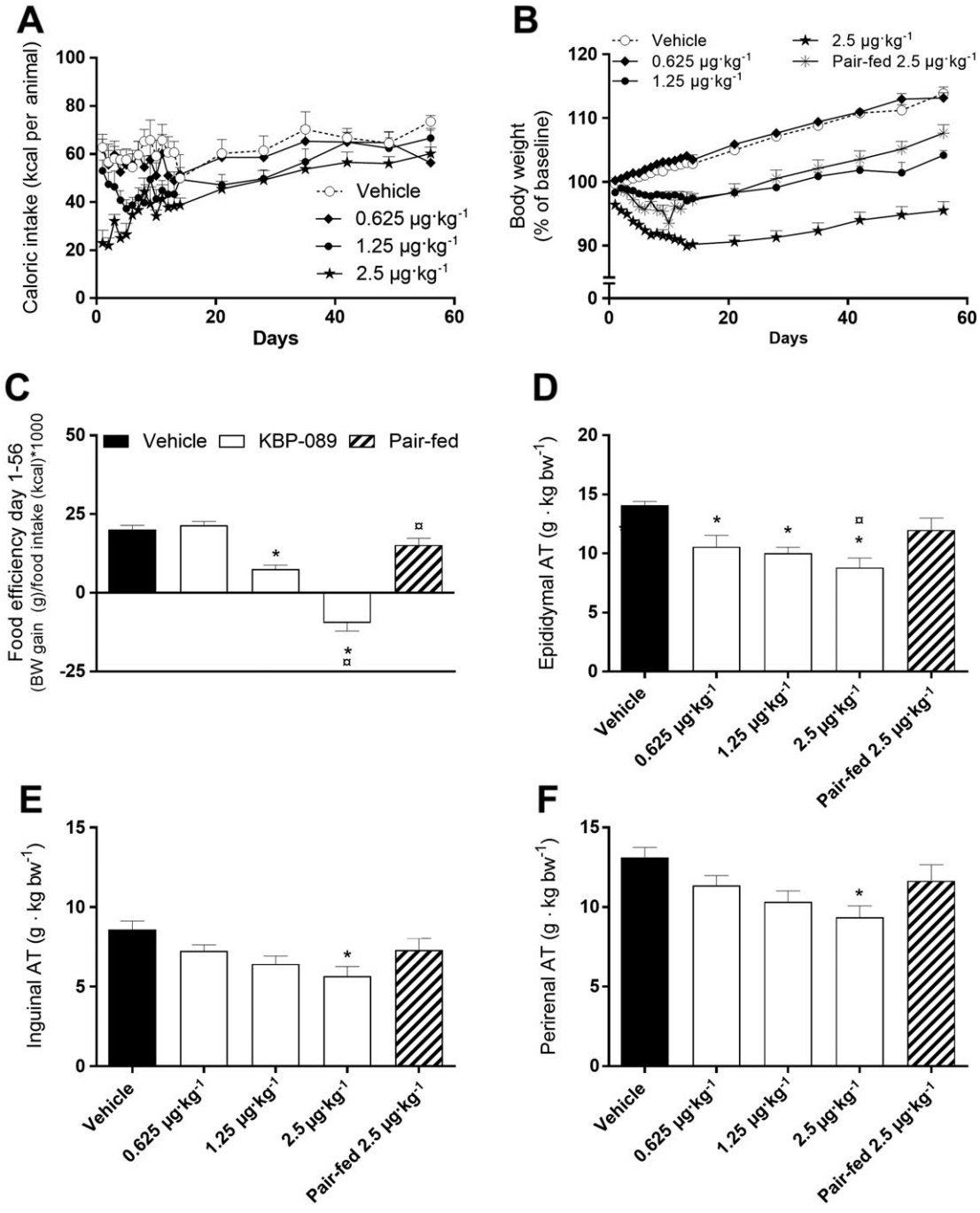

Figure 1

KBP-089 potently reduces appetite, body weight and fat depots in HFD rats. (A) Caloric intake monitored daily initially, and then weekly in high-fat diet fed rats. Expressed as daily intake per animal. (B) Body weight expressed as percentage from baseline. (C) Food efficiency day 1–56 in rats dosed with KBP-089 (0.625, 1.25 and 2.5 µg·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 with vehicle, ¤P<0.05 compared with pair-fed.

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 and PW: 408 ± 4 g). During the 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 and 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

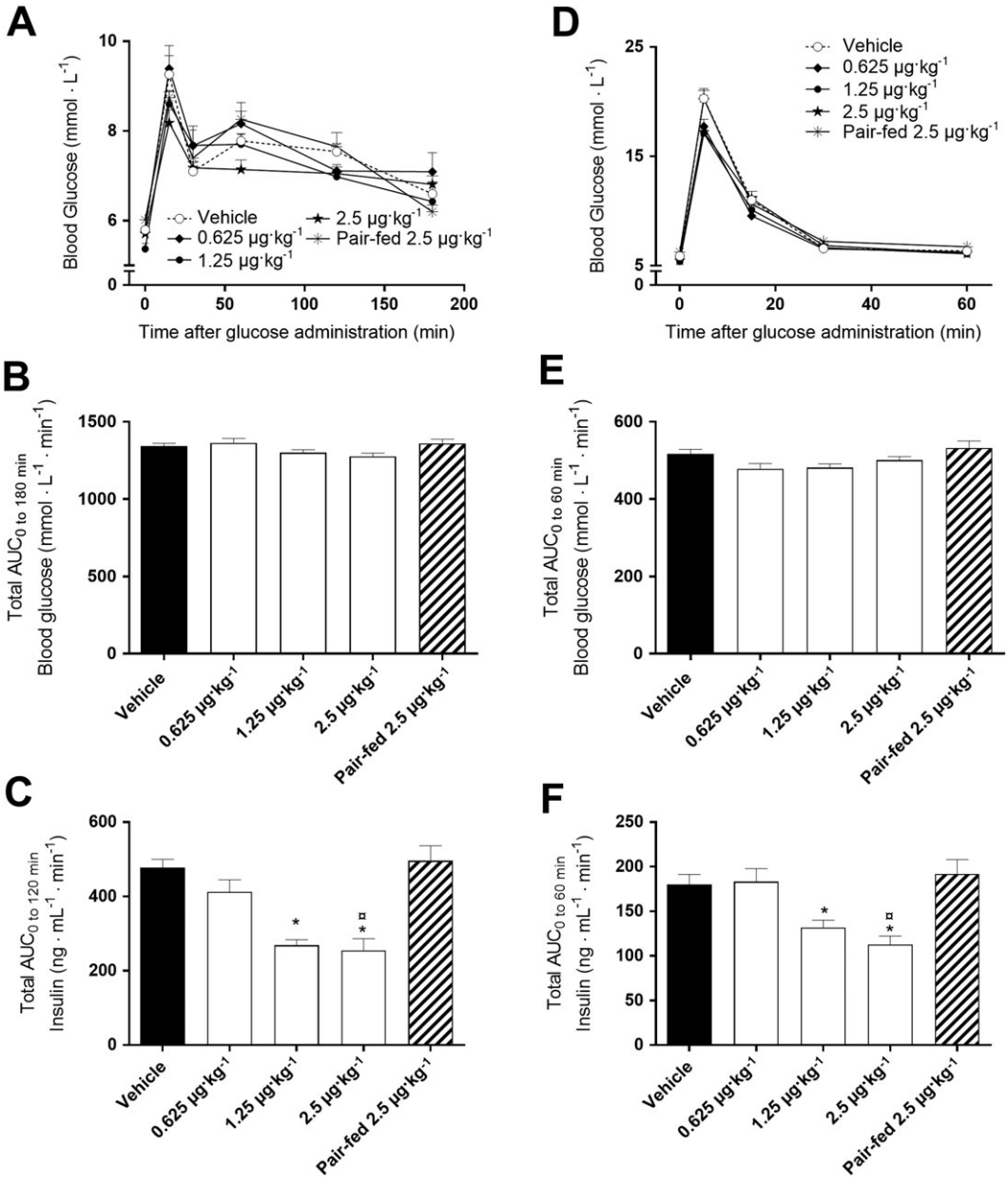

Figure 2

KBP-089 enhances glucose tolerance and potentially insulin sensitivity in HFD rats. (A, D) Plasma glucose during OGTT and IVGTT in high-fat diet fed rats treated with KBP-089 (0.625, 1.25 and 2.5 $\mu g \cdot kg^{-1}$) 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 with vehicle, ¤$P < 0.05$compared with pair-fed.

KBP-089 and the pair-weighed group compared with 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 with pair-weighed rats. Using MR, we found a slight increase in lean body mass in KBP-089-treated rats compared with vehicle rats, and a reduced amount of whole body fat mass in KBP-089-treated and pair-weighed rats compared with vehicle (Figure 5D), indicating that the weight loss occurs primarily in fat tissue. As expected, KBP-089 again caused improved glucose tolerance with significantly lowered plasma insulin levels (Figure 5D, E, G, H) compared with vehicle. Surprisingly, the pair-weighed group did not show a marked improvement in glucose tolerance despite the significant weight reduction. Food restriction alone had significantly ameliorated hyperinsulinaemia during OGTT (Figure 5H); however, it was still significantly higher compared with KBP-089. Finally, KBP-089-treated rats had a reduced rate of gastric emptying compared with vehicle and

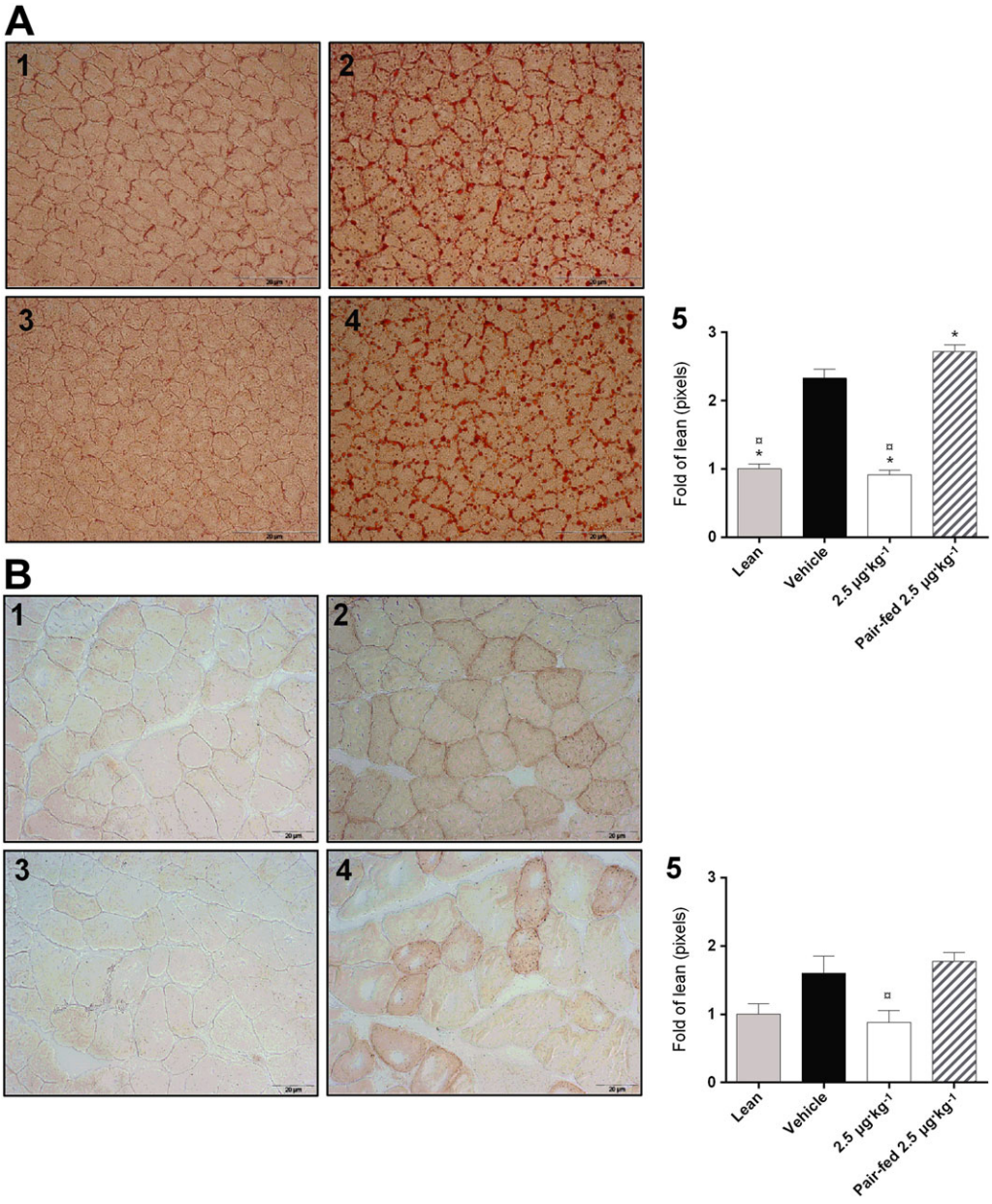

Figure 3

KBP-089 reduces the 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 ×20 for liver and ×40 for gastrocnemius, nine images per animal; three pictures per depth) in (1) ND rats, (2) vehicle HFD rats, (3) 2.5 $\mu g \cdot kg^{-1}$ KBP-089, (4) pair-fed to 2.5 $\mu g \cdot kg^{-1}$ KBP-089 and (5) quantification of the results ($n = 10$ rats per group in vehicle and KBP-089 treatment groups; $n = 9$ rats in pair-fed group). Data are 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 with vehicle, ¤$P < 0.05$ compared with pair-fed.

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 with 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 and chocolate = 127.3 ± 9.8 kcal)]. KBP-089 administration was associated with a significantly reduced caloric intake, – 34% compared with 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 reduction in chocolate consumption (127.3 ± 9.8 kcal vs. 41.4 ± 9.4 kcal).

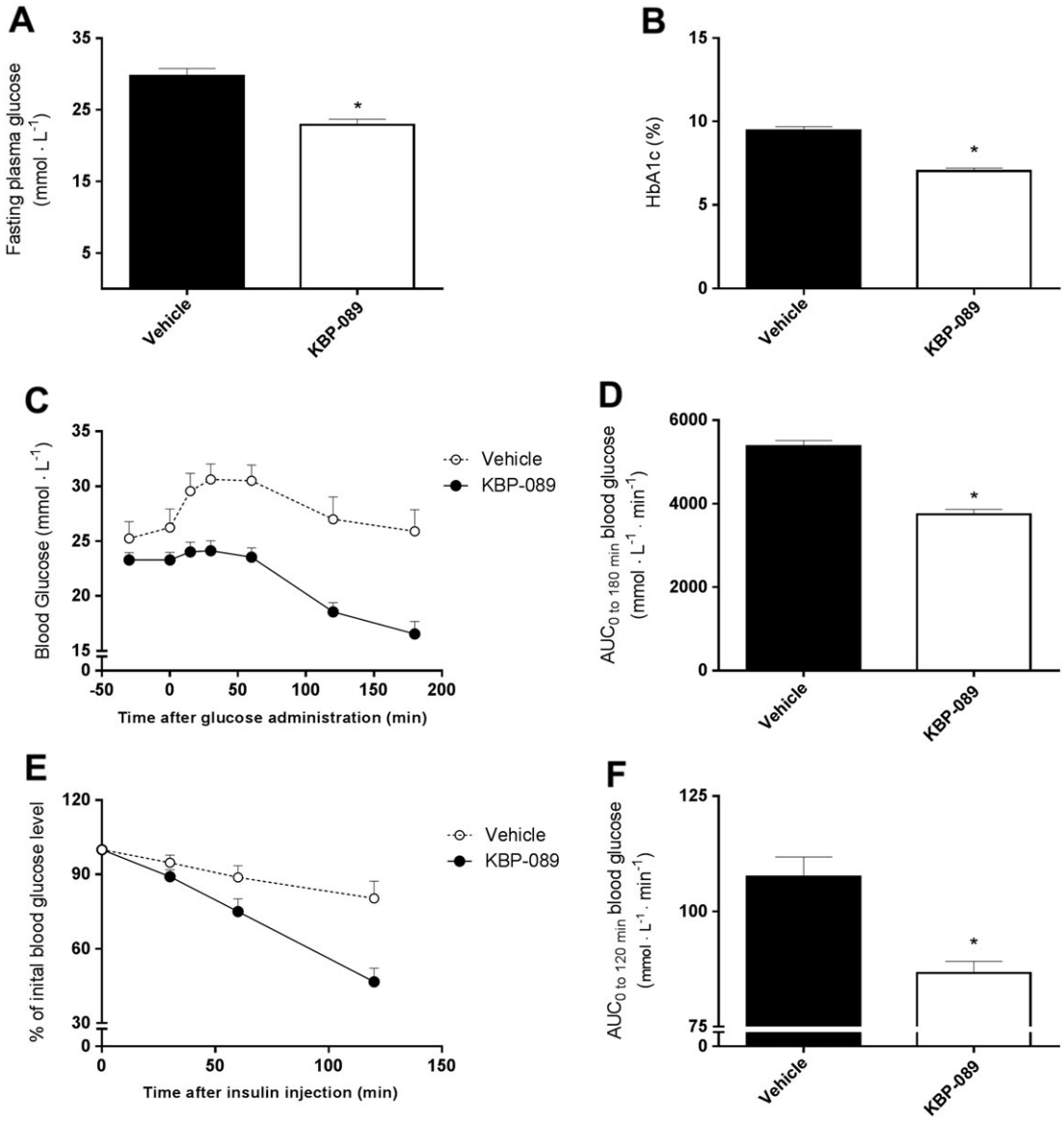

Figure 4

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 OGTT, (D) total AUC of the OGTT displayed in (C). (E) Plasma glucose during insulin tolerance test displayed as % of initial blood glucose value. (F) Total AUC of the insulin tolerance test displayed in (E). (n = 10 rats per group). Statistical analysis between groups was evaluated by t-test. $P < 0.05$.

Discussion

The present study describes a novel DACRA, 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 and 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 and the effect on food intake can most likely be attributed to central amylin receptor activation. It has previously been demonstrated that amylin facilitates a reduction in body weight that cannot only be attributed to suppression of food intake (Isaksson *et al.*, 2005; Roth *et al.*, 2006). An interesting aspect of the reduction in fatty acid accumulation in the liver is the known relationship between liver fat, insulin resistance and non-alcoholic steatohepatitis (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.

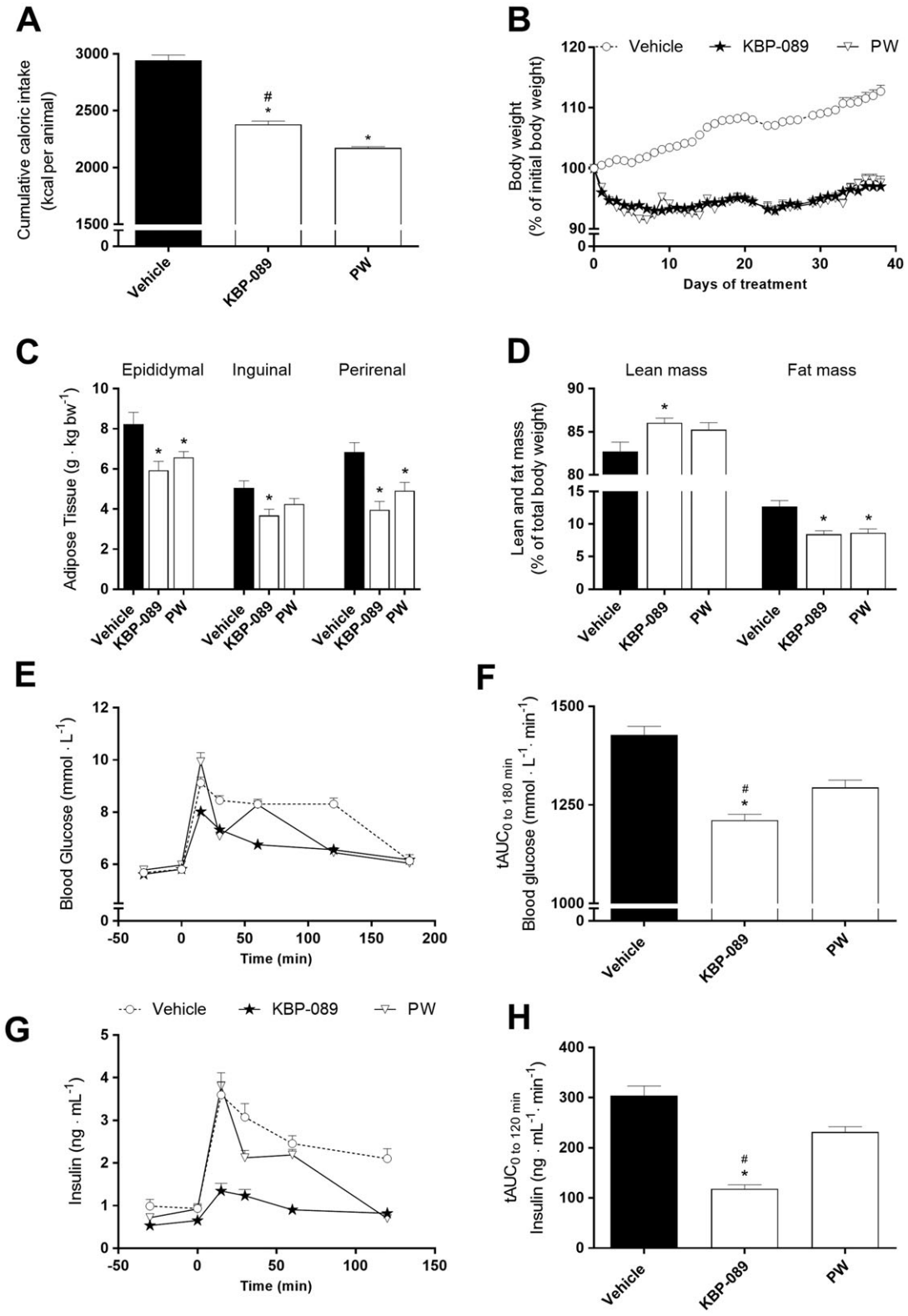

Figure 5

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 $\mu g \cdot kg^{-1}$) and weight-matched rats. (E, G) Plasma glucose and plasma insulin respectively and (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 (C, F, H) or (D) Kruskal–Wallis with Dunn's multiple comparisons test. $^{*}P < 0.05$ compared with vehicle, $^{\#}P < 0.05$ compared with PW.

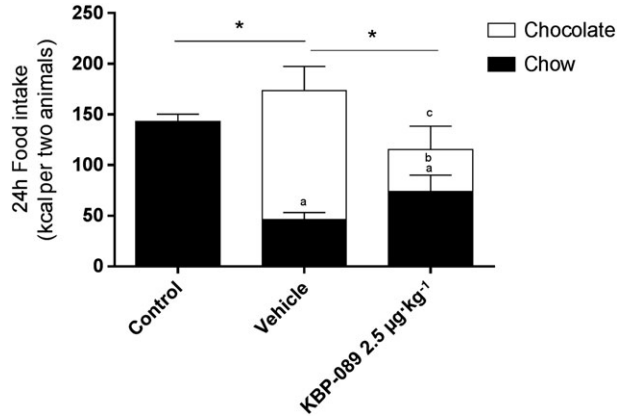

Figure 6

KBP-089 induces changes in food preference. Voluntary food (chow) and chocolate intake was monitored for 24 h after 7 days of 2.5 $\mu g \cdot kg^{-1}$ 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; $^{a}P < 0.05$ compared with control (chow); $^{b}P < 0.05$ compared with vehicle (chow); and $^{c}P < 0.05$ compared with vehicle (chocolate).

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 with vehicle-treated rats, and trends towards lowered adiposity compared with pair-weighed adipose tissues – despite the 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-weighed 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 with vehicle rats, underscoring that the weight loss is primarily mediated through a reduction in adipose tissue weight. Furthermore, the slight increase in lean mass is a positive effect, 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 hyperglycaemia 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 with the weight-matched group. The improvement in insulin levels also manifested as an improvement in glucose control too; however, the possibility that the PW animals had an 'artificial' increase in glucose intolerance due to prolonged fasting (or significant food restriction) cannot be out ruled, although they did not show any signs of malnutrition or ill behaviour. The lowering of insulin levels could be attributed to the lack of improvement in glucose tolerance when compared with 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 p.o. 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 with vehicle rats, while PW animals mimic glucose tolerance and insulin levels as demonstrated in the OGTT. These data further indicate the strong insulinostatic effect of KBP-089.

Another important way to regulate 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 clear; 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, the 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 indirectly 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.

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Author contributions

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 the 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.

Conflict of interest

M.A.K. and K.H. own stock in Nordic Bioscience. All other authors disclose no conflict of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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Paper II:

The Dual Amylin- and Calcitonin-Receptor Agonist KBP-042 Increases Insulin Sensitivity and Induces Weight Loss in Rats with Obesity

Original Article
OBESITY BIOLOGY AND INTEGRATED PHYSIOLOGY

Obesity

The Dual Amylin- and Calcitonin-Receptor Agonist KBP-042 Increases Insulin Sensitivity and Induces Weight Loss in Rats with Obesity

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Objective: In this study, KBP-042, a dual amylin- and calcitonin-receptor agonist, was investigated as a treatment of obesity and insulin resistance in five different doses (0.625 μg/kg–10 μg/kg) compared with saline-treated and pair-fed controls.

Methods: Rats with obesity received daily s.c. administrations for 56 days, and glucose tolerance was assessed after one acute injection, 3 weeks of treatment, and again after 7 weeks of treatment. To assess the effect on insulin sensitivity, rats received 5 μg/kg KBP-042 for 21 days before hyperinsulinemic–euglycemic clamp.

Results: KBP-042 induced a sustained weight loss of up to 20% without any significant weight reduction in the pair-fed groups. Decreases in adipose tissues and lipid deposition in the liver were observed, while plasma adiponectin was increased and plasma leptin levels were decreased. Acute administration of KBP-042 led to impaired glucose tolerance and increased plasma lactate, while this diabetogenic effect was reversed by chronic treatment. Finally, assessment of insulin sensitivity using the hyperinsulinemic–euglycemic clamp showed that KBP-042 increased the glucose infusion rate.

Conclusions: The study indicates that KBP-042 combines two highly relevant features, namely weight loss and insulin sensitivity, and is thus an excellent candidate for chronic treatment of obesity and insulin resistance.

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Introduction

Obesity is one of the greatest public health challenges of the 21st century (1). Obesity can lead to insulin resistance and type 2 diabetes (2), which are associated with a range of metabolic dysfunctions (3,4). Weight loss, improved glycemic control, and increased insulin action to reduce strain on the β cells are key points for improving disease status. This can be achieved by different interventions (exercise, diet, medication, surgery) which all cause improvements in metabolic profiles and increase of insulin sensitivity and β-cell function (5,6). However, as lifestyle changes often result in only minor weight reductions followed by a rapid regain of weight (7), there is a need for treatments targeting multiple factors of the obesity-related diseases. These include insulin resistance and β-cell failure to avoid development of type 2 diabetes, as well as diabetic complications.

Activation of amylin receptors has already been linked with reduction of food intake (8), increased responsiveness to leptin (9-11), weight loss (12,13), and indications of increased energy expenditure (11,13-16). However, amylin is a short-lasting agonist *in vivo*, and there is a need for improved ligands. KBP-042 is a dual amylin- and calcitonin-receptor agonist with highly potent antiobesity and antidiabetic effects (17), although a long-term chronic treatment has not yet been tested.

In this study, KBP-042 was tested in a long-term treatment of prediabetic rats with obesity, in order to evaluate KBP-042's potential as a chronic treatment of obesity. We further examined whether the beneficial effects on glucose homeostasis were maintained throughout the study, and finally we investigated whether treatment with KBP-042 could increase insulin sensitivity and reduce hepatic steatosis.

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Additional Supporting Information may be found in the online version of this article.
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Methods

Peptide therapy

Recombinant KBP-042 peptide (Unigene Laboratories, Boonton, NJ) was dissolved in saline for subcutaneous (s.c.) delivery. The doses for KBP-042 administration in the studies were based on previous studies in animal models of obesity and type 2 diabetes and ranged from 10 μg/kg to 0.625 μg/kg (~2.87–0.18 nmol/kg/day) (17,18).

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). All male Sprague Dawley rats were obtained at 6 weeks of age and housed under controlled temperature (20°C ± 2) on a normal 12-h light–dark cycle with *ad libitum* access to water and food. Normal diet control rats (ND) were fed rodent chow (5002, LabDiet, St. Louis, MO) and high-fat diet (HFD) rats a 60% fat kcal diet (#D12495, Research Diets Inc., NJ). After 10 weeks of high-fat feeding rats were assigned into groups ($n = 10$) and controlled for equal mean body weight.

Acute study. Food was removed in the afternoon (4 p.m.). After 16 to 18 h of fasting an oral glucose tolerance test (OGTT) was performed. Rats received a single dose of saline (vehicle) or peptide (10 μg/kg, 5 μg/kg, 2.5 μg/kg, 1.25 μg/kg, 0.625 μg/kg). After 30 min, a glucose bolus (2 g/kg, Sigma-Aldrich, Copenhagen, Denmark) was administered by oral gavage. Blood glucose was monitored by Accu-Check® Avia monitoring system (Roche Diagnostics, Rotkreuz, Switzerland) and EDTA-plasma was obtained from the lateral tail vein at $t = 0$, 15, 30, 60, and 120 min.

Pica test. Fasted animals were administered s.c. with 5, 10, or 50 μg/kg KBP-042 or vehicle (saline). After dosing, animals had free access to normal chow or kaolin pellets (5TBP, Test diet, MO) and food and kaolin intake was monitored after 4 and 24 h.

Chronic study. Each rat was dosed once daily with either saline (vehicle, pair-fed 5 μg/kg, pair-fed 10 μg/kg) or KBP-042 (10 μg/kg, 5 μg/kg, 2.5 μg/kg, 1.25 μg/kg, 0.625 μg/kg) in the afternoon for 8 weeks. The two pair-fed groups were food restricted to match the daily food intake of their corresponding treatment groups (5 μg/kg or 10 μg/kg). Pair-fed animals received an average of the daily intake of their treated paired group every day in the afternoon. Food intake and body weight were monitored daily for the first 6 days, then weekly. OGTT, performed as in the acute study and intravenous glucose tolerance tests (IVGTT) were performed after 3 and 7 weeks of treatment. IVGTT was performed in the morning after 18 h of fasting. Each rat received a single dose of either saline (vehicle, pair-fed 5 μg/kg, pair-fed 10 μg/kg) or peptide (10 μg/kg, 5 μg/kg, 2.5 μg/kg, 1.25 μg/kg, 0.625 μg/kg), after 30 min glucose (0.5 g/kg, Sigma-Aldrich, Copenhagen, Denmark) was administered in the lateral tail vein and blood glucose was monitored and EDTA-plasma was obtained at $t = 0$, 5, 15, 30, 60, and 120 min, as described above. To assess effect on gastric emptying, overnight-fasted rats received s.c. KBP-042 injection, were administered 40 mg/kg acetaminophen by oral gavage (4 mL/kg) after 30 min and the appearance of acetaminophen in plasma was monitored (19). Blood was collected 30 min after administration from the tail vein and acetaminophen levels were measured in EDTA-plasma (Acetaminophen Direct ELISA Kit, Immuneanalysis, Pomona, CA). Gastric emptying was calculated as % change relative to ND rats.

After 8 weeks, EDTA-Aprotinin plasma samples were collected for hormonal analyses after 3 h fasting. Animals were euthanized under isoflurane inhalation followed by exsanguination. Excised tissue was snap-frozen in liquid nitrogen and stored at −80°C, and plasma was stored at −20°C samples until further analysis.

Hyperinsulinemic–euglycemic clamp

Insulin-mediated whole body glucose uptake was estimated in rats fed either HFD or ND (as described above). The HFD rats were stratified into HFD vehicle or HFD-KBP-042 groups ($n = 5$–7). ND vehicle and HFD vehicle rats received saline injections while HFD-KBP-042 received 5 μg/kg of KBP-042 s.c. for 21 days. After the treatment period, animals were subjected to a hyperinsulinemic–euglycemic clamp experiment explained in details in the Supporting Information.

Plasma analysis

Plasma levels of lactate (L-lactate colorimetric assay, Abcam, Cambridge, UK), insulin (Mercodia Rat Insulin ELISA, Mercodia AB, Uppsala, Sweden), leptin (Rat Leptin ELISA, Millipore Corporation, Billerica, MA), glucose-dependent insulinotropic peptide (GIP) (Rat/Mouse GIP (Total) ELISA, Merck Millipore, Billerica, MA), and adiponectin (Rat Adiponectin ELISA, Millipore Corporation, Billerica, MA) were analyzed according to manufacturer's instruction.

Tissue analysis

Lipids were extracted from liver samples with addition of internal standards and triacylglycerol (TAG) was isolated from the total lipid extract using aminopropyl solid-phase extraction cartridges, *trans*-methylated, and quantified using Gas Chromatography–Flame Ionization Detector as previously described (20).

Statistical analysis

Data were statistically analyzed by one-way ANOVA multiple comparison followed by Tukey's test. In Supporting Information Table S1, ND controls were compared with HFD vehicle using Student's *t*-test. Values of $P < 0.05$ were considered to be significant.

Results

KBP-042 mediated substantial and sustained reductions in body weight

The baseline characteristics of HFD rats and lean controls confirmed the obese and prediabetic status of the HFD rats (Supporting Information Table S1).

After treatment with KBP-042 for 8 weeks, a dose-dependent and sustained reduction of body weight was observed. A large weight loss was observed in the initial phase of the study (Figure 1A, B) in the three highest treatment groups (2.5 μg/kg, 5 μg/kg, and 10 μg/kg), as well as the two corresponding pair-fed groups (pair-fed 5 μg/kg and pair-fed 10 μg/kg). This corresponds well with the large reduction in food intake in the first 6 days of treatment (Figure 1D). Due to the drastic reduction in food intake, pica behavior was tested as a surrogate for nausea in rats. The two highest doses, 5 and 10 μg/kg KBP-042 did not give rise to kaolin intake whereas a high dose of KBP-042 not used in this study (50 μg/kg) provoked pica behavior

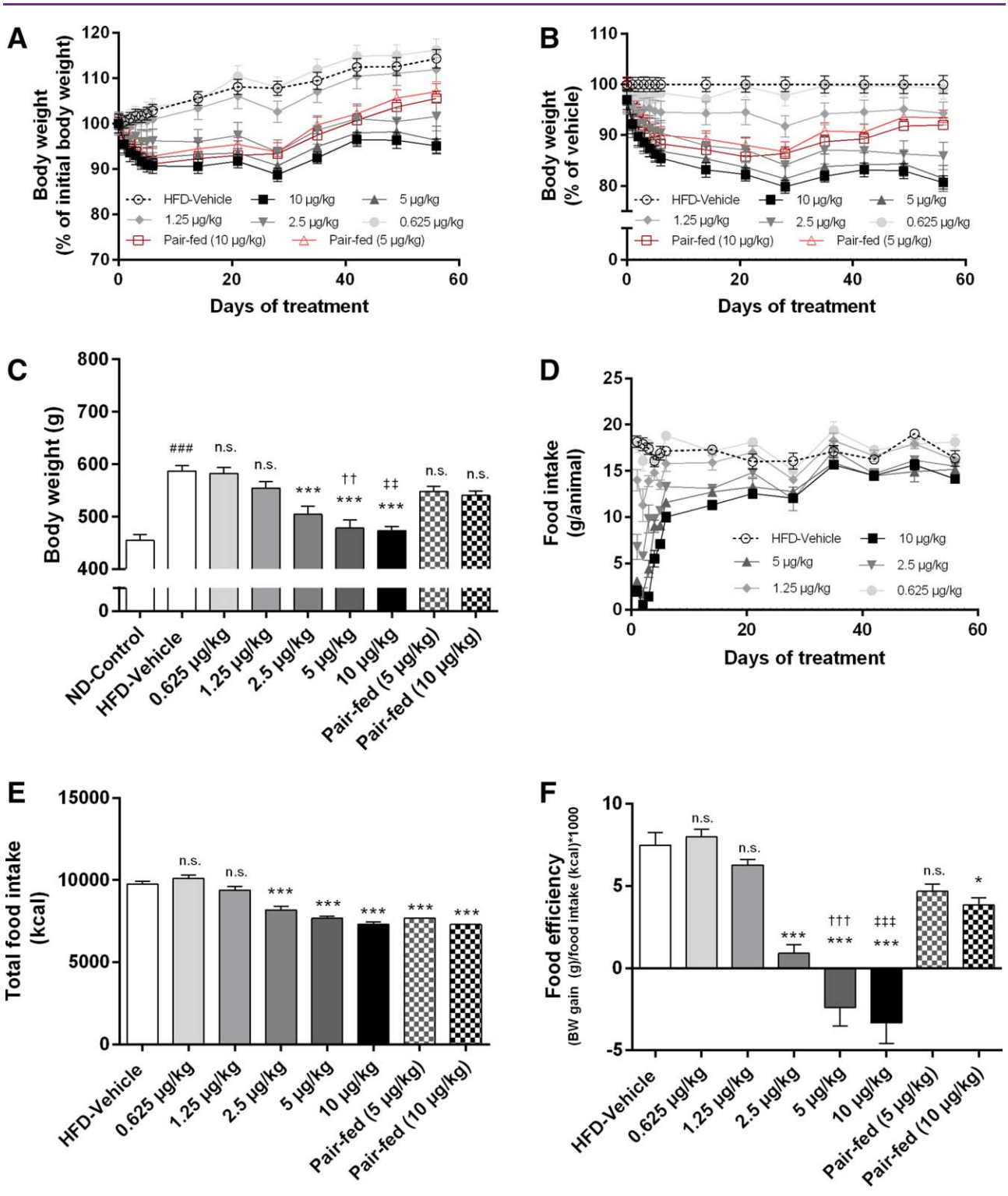


Figure 1 (**A**) Body weight progression in % of initial body weight during the study from randomization at day 0 to last day of treatment, day 56. (**B**) Vehicle-corrected body weights. (**C**) End point body weights. (**D**) Food intake of all treatment groups during the entire study. Food intake was monitored every day for the first 6 days followed by weekly monitoring. Pair-fed groups were fed the same as the average for their corresponding treatment group (5 µg/kg or 10 µg/kg). (**E**) Accumulated food intake for the entire duration of the study expressed in kcal/2 animals. (**F**) Calculated food efficiency. n = 10 for all groups except vehicle (n = 12). Statistical analysis between groups for panels C, E, and F performed as a one-way ANOVA followed by Tukey's post hoc test with the following annotations: ###$P < 0.001$ vs. normal diet control (ND). *$P < 0.05$, ***$P < 0.001$ vs. high-fat diet (HFD) vehicle. ††$P < 0.01$, †††$P < 0.001$ vs. pair-fed 5 µg/kg. ‡‡$P < 0.01$, ‡‡‡$P < 0.001$ vs. pair-fed 10 µg/kg. Data are expressed as mean ± SEM. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(Supporting Information Figure S1). After the transient reduction in feeding, food intake increased during the study. The pair-fed groups gained weight again after feeding increased; inversely, treatment with KBP-042 sustained the initial weight reduction throughout the 56 days, with significant reductions in the 2.5 μg/kg, 5 μg/kg, and 10 μg/kg groups compared with the HFD vehicle (Figure 1C). The accumulated food intake corresponds well with the weight change for the three highest treatment groups (2.5 μg/kg, 5 μg/kg, and 10 μg/kg) (Figure 1E), although the pair-fed groups which received the same amount of food as their corresponding treatment group did not lose significant weight. Accordingly, treatment with 2.5, 5, and 10 μg/kg KBP-042 resulted in drastic and significant reduction in food efficiency compared with pair-fed (Figure 1F), suggesting increased energy expenditure.

KBP-042 reduced adipose tissue and ectopic lipid accumulation

After treatment three different adipose tissues were isolated and as seen in Figure 2A–C, the weights of isolated epididymal and perirenal adipose tissues were significantly reduced after treatment with 10 μg/kg of KBP-042. The perirenal adipose tissue in the 2.5, 5, and 10 μg/kg groups was reduced significantly while inguinal fat was not. The same reduction was not seen in the pair-fed controls.

Lipid accumulation in liver was assessed as hepatic TAG concentration (Figure 2D). As expected the HFD vehicle group had dramatically higher TAG levels compared with ND group. This accumulation was significantly reduced after treatment with KBP-042 (10 μg/kg), while the corresponding pair-fed control group did not show a significant reduction in liver TAG. In order to assess the treatment effect on fatty acid metabolism in selective ways (e.g. metabolism of saturated vs. monounsaturated vs. polyunsaturated), the fatty acid composition of hepatic TAG was analyzed. The results showed that there was no difference in the relative distribution, i.e., the treatment caused a general reduction in TAG without effecting the metabolism of specific fatty acid types (Supporting Information Table S2).

Finally, adiponectin and leptin levels were measured after 56 days of treatment (Figure 2E, F). Adiponectin was significantly increased in response to treatment with all doses of KBP-042 except 0.625 μg/kg. For plasma leptin a statistically significant reduction was seen when comparing 10 μg/kg KBP-042 with the corresponding pair-fed control.

In summary, fat depots, lipid, and adipokine data support a strongly improved metabolic status as a function of treatment with KBP-042.

Chronic treatment with KBP-042 improved glucose tolerance with reduced insulin levels

OGTT was performed after the first injection, as well as after 3 and 7 weeks of treatment.

The acute OGTT showed a slightly impaired glucose tolerance for the 10 μg/kg group compared with HFD vehicle (Figure 3A, D). A hyperglycemic effect was observed 30 min after s.c. administration of KBP-042 at $t = 0$ compared with vehicle (5.9 mM) for 5 μg/kg (6.8 mM, $P = 0.033$) and for 10 μg/kg (7.4 mM, $P < 0.001$) groups. The total area under the curve (tAUC) was significantly increased after injection of 10 μg/kg KBP-042 (Figure 3D). However, the insulin levels during the first 60 min after glucose administration were reduced in animals dosed with KBP-042 (Figure 3G, J).

After 3 weeks of treatment with KBP-042 or saline, the three highest doses of KBP-042 resulted in a significantly lowered tAUC (Figure 3B, E). Insulin levels were lowered by KBP-042 except in the 0.625 μg/kg group (Figure 3H, K). Pair-fed 10 μg/kg group also had a reduced insulin response (Figure 3K).

During OGTT after week 7 (Figure 3C) the two highest dose groups had improved glucose tolerance when tAUC was considered (Figure 3F). The two highest dose groups showed increased glucose tolerance, while drastically reduced insulin levels were observed within the first 60 min after glucose administration (Figure 3I, L). Pair-feeding did not change glucose handling compared with HFD vehicle.

After administration of KBP-042, plasma lactate was dose-dependently increased in treatment of naive animals (Supporting Information Figure S2A) and resulted in a 1.5 mM increase in plasma lactate 30 min after s.c. administration of 10 μg/kg KBP-042. Interestingly, the KBP-042-provoked lactate response was completely blunted by chronic treatment (Supporting Information Figure S2B, C).

KBP-042 reduced gastrointestinal mobility and plasma levels of the gut hormone GIP

The rate of gastric emptying during OGTT was assessed in response to acute dosing with KBP-042, after treatment for 3 weeks, or after 7 weeks (Figure 4A, C, E, respectively). Acute s.c. administration of KBP-042 resulted in a significant reduction of gastric emptying 30 min after acetaminophen administration for the three highest treatment groups (2.5 μg/kg, 5 μg/kg, and 10 μg/kg) (Figure 4A). In animals treated for 3 weeks with KBP-042, gastric emptying was reduced for all treatment groups. The two pair-fed groups displayed a slower rate of gastric emptying due to food restriction; however, they still have significantly higher rates of gastric emptying compared with 5 μg/kg and 10 μg/kg groups of KBP-042 (Figure 4C).

After 7 weeks of treatment the reduced gastric emptying was still significant at most doses compared with HFD vehicle. The pair-fed groups were no longer different from the HFD controls (Figure 4E).

GIP levels in plasma were quantified 0 to 30 min after acute glucose administration, and after 3 and 7 weeks of treatment (Figure 4B, D, F). After acute administration of KBP-042, GIP levels were significantly lower in the groups treated with 2.5 to 10 μg/kg KBP-042 (Figure 4B). After treatment for 3 weeks, all groups displayed a drastic reduction in plasma GIP. The two pair-fed groups demonstrated significantly lowered GIP levels compared with HFD vehicle probably due to food restriction. They were still significantly higher than their corresponding treatment controls (5 μg/kg and 10 μg/kg) (Figure 4D). After 7 weeks of treatment, plasma GIP levels were reduced; however, the changes were only significant in the three highest treatment groups. The reductions in pair-fed groups were no longer present after 7 weeks of treatment (Figure 4F).

KBP-042 maintained peripheral glucose tolerance with lower insulin levels irrespective of altered gastric emptying

To circumvent the gastrointestinal tract and assess peripheral glucose tolerance, IVGTTs were performed after 3 and 7 weeks of treatment (Figure 5). In both tests, all KBP-042 groups showed a trend towards lower blood glucose compared with vehicle and pair-

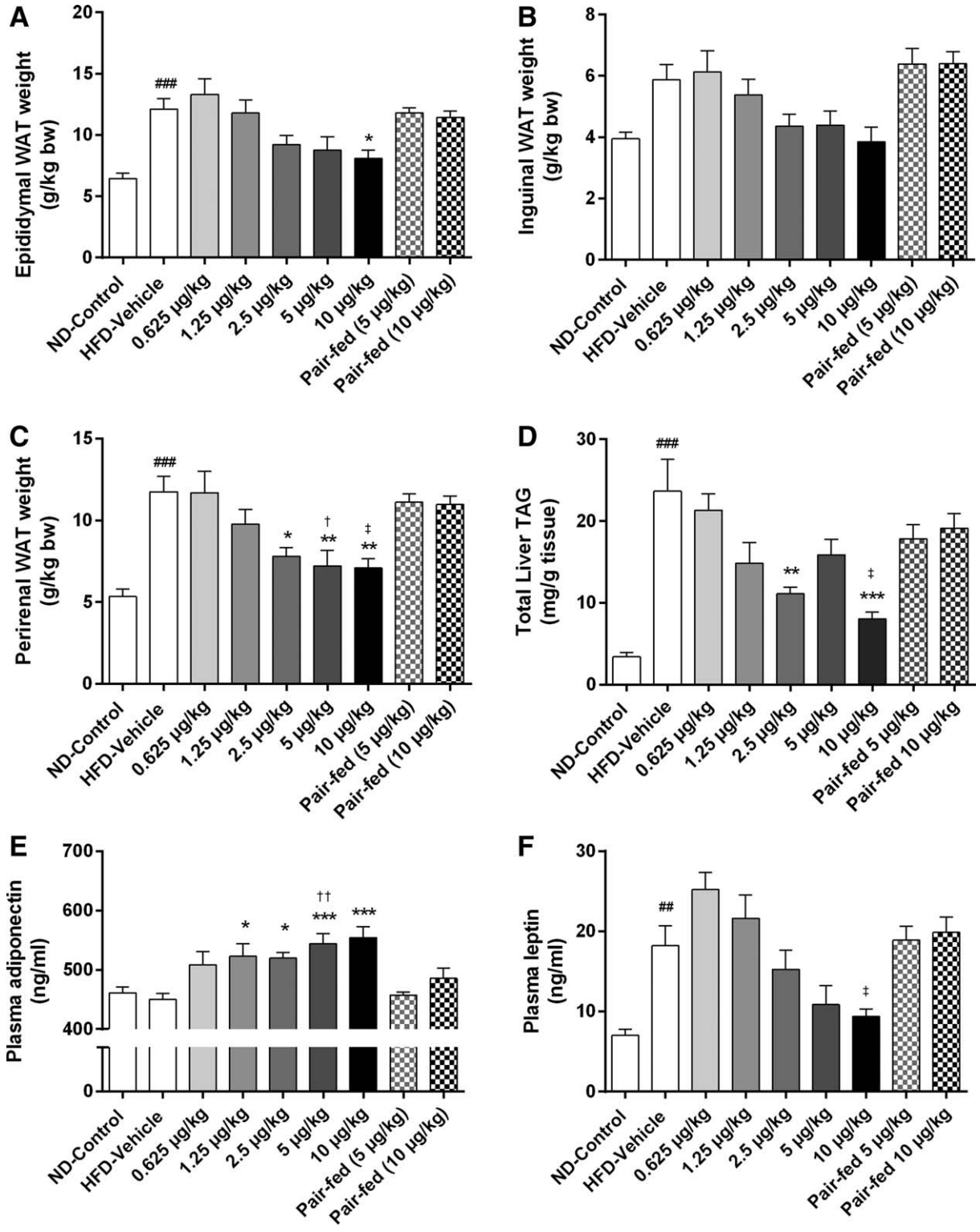


Figure 2 (**A–C**) Weight of isolated epididymal, inguinal, and perirenal white adipose tissue (WAT), respectively, after 56 days of treatment. (**D**) Total triacylglyceride content extracted from liver tissue after treatment with KBP-042 or saline for 56 days. (**E**,**F**) Plasma adiponectin and leptin levels, respectively, after 56 days of treatment. n = 10 for all groups except vehicle (n = 12). Statistical analysis between groups performed as a one-way ANOVA followed by Tukey's post hoc test with the following annotations: ##$P < 0.01$, ###$P < 0.001$ vs. normal diet control (ND). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. high-fat diet (HFD) vehicle. †$P < 0.05$, ††$P < 0.01$ vs. pair-fed 5 µg/kg. ‡$P < 0.05$ vs. pair-fed 10 µg/kg. Data are expressed as mean ± SEM.

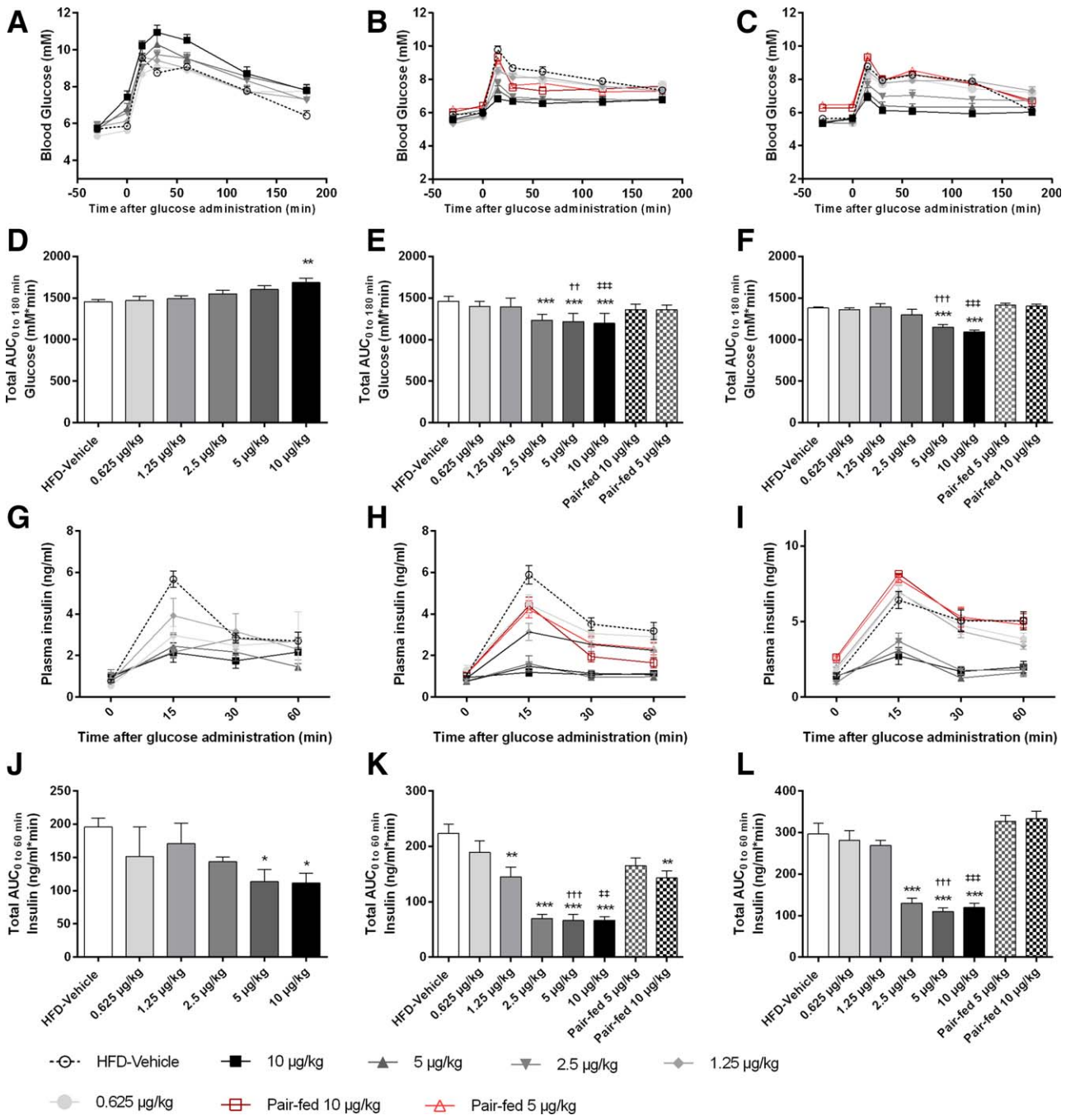


Figure 3 Blood glucose and insulin levels during oral glucose tolerance test (OGTT) performed in animals treated with KBP-042 or vehicle once (left), for 3 weeks (middle), or 7 weeks (right). Animals were challenged with an oral glucose bolus (2 g/kg) at time = 0 and dosed with either KBP-042 or saline at t = −30. (**A**–**C**) Blood glucose levels during acute OGTT, OGTT after 3 weeks, and OGTT after 7 weeks, respectively. (**D**–**F**) Area under the curve (AUC) for acute OGTT, OGTT after 3 weeks, and OGTT after 7 weeks, respectively. (**G**–**I**) Insulin levels during acute OGTT, OGTT after 3 weeks, and OGTT after 7 weeks, respectively. (**J**-**L**) Insulin levels during acute OGTT, OGTT after 3 weeks, and OGTT after 7 weeks, respectively, expressed as AUC. n = 10 for all groups except vehicle (n = 12). Statistical analysis between groups performed as a one-way ANOVA followed by Tukey's post hoc test with the following annotations: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. high-fat diet (HFD) vehicle. $\dagger\dagger P < 0.01$, $\dagger\dagger\dagger P < 0.001$ vs. pair-fed 5 µg/kg. $\ddagger\ddagger P < 0.01$, $\ddagger\ddagger\ddagger P < 0.001$ vs. pair-fed 10 µg/kg. Data are expressed as mean ± SEM. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

fed controls 5 and 10 min after glucose administration (Figure 5A, B). This manifested in a lowered $tAUC_{0–120\ min}$ for the 2.5 µg/kg KBP-042 group only in the first test after 3 weeks and not after 7 weeks of treatment. No effect was observed for pair-fed groups. Interestingly, when insulin levels were quantified, the tAUC for insulin was significantly reduced in KBP-042 1.25-10 µg/kg groups after 3 weeks of treatment (Figure 5G). After 7 weeks of treatment, groups treated with 2.5 µg/kg and 5 µg/kg KBP-042 had

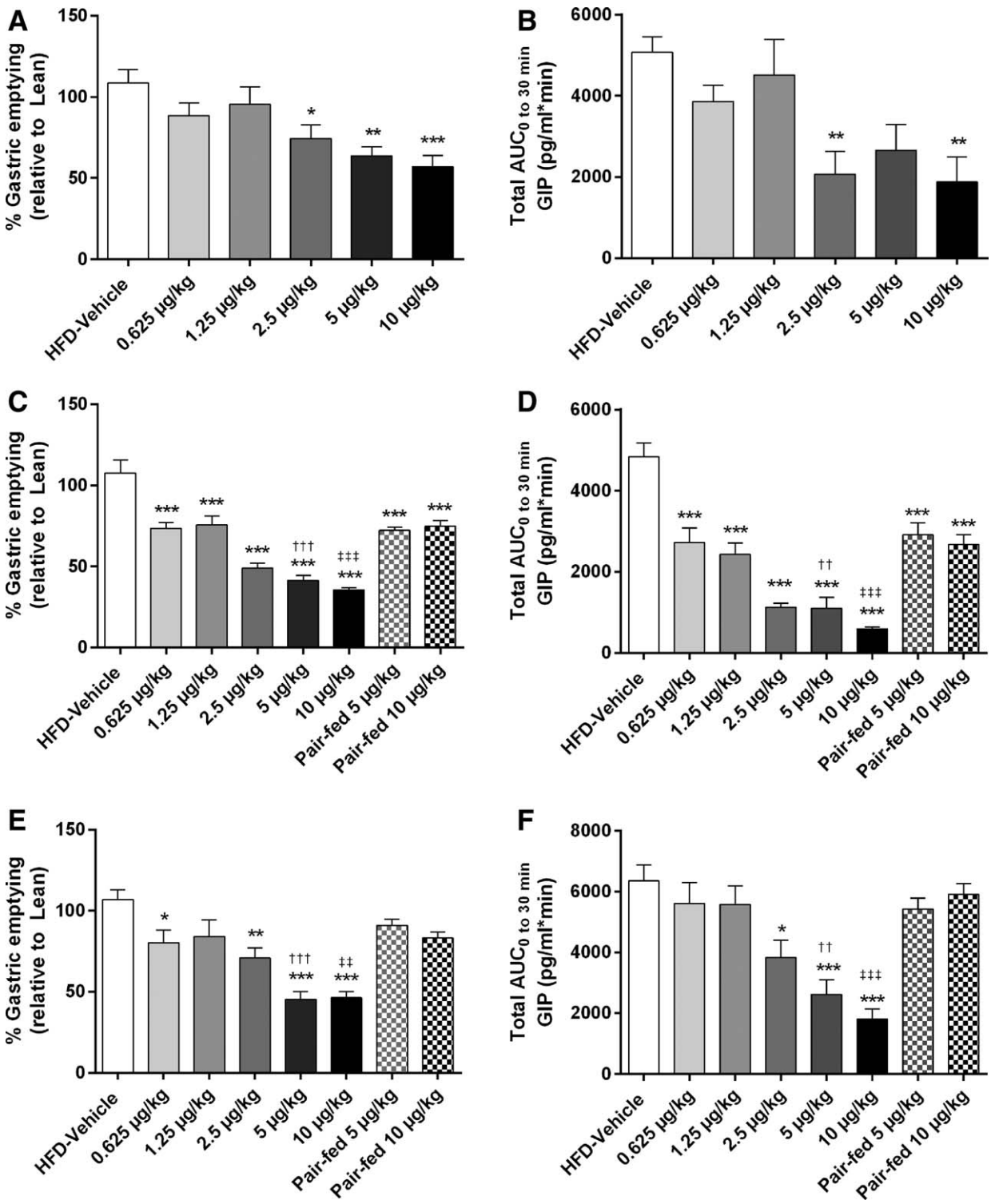
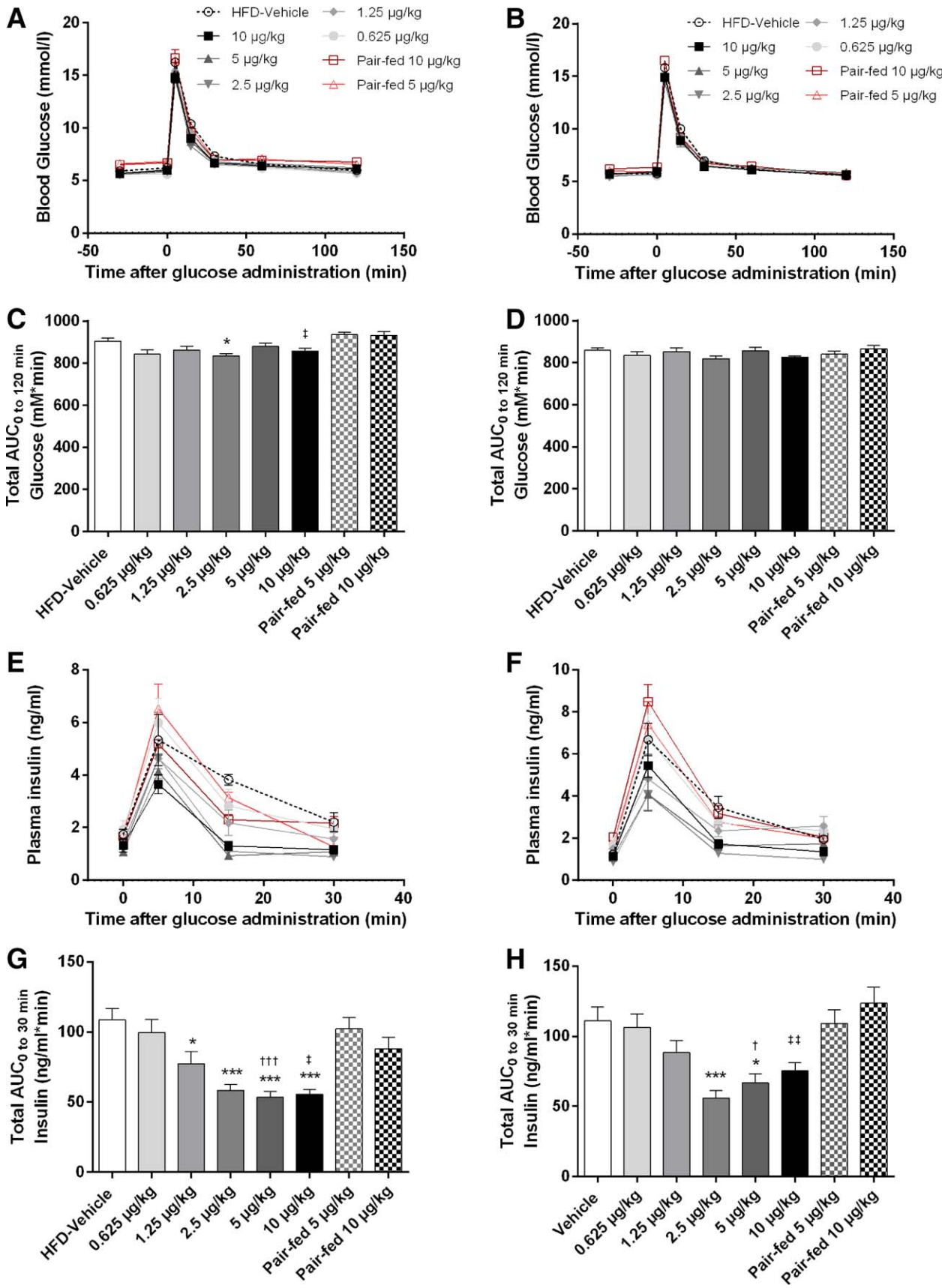


Figure 4 (**A**) Relative rates of gastric emptying measured 30 min after glucose challenge in the oral glucose tolerance test (OGTT) performed in treatment naive animals. (**B**) Area under the curve (AUC) of plasma levels of glucose-dependent insulinotropic peptide (GIP) during OGTT in treatment naive animals up to 30 min after glucose challenge. (**C**) Relative rates of gastric emptying measured 30 min after glucose challenge in the OGTT performed animals treated with KBP-042 for 3 weeks. (**D**) AUC of plasma levels of GIP during OGTT in animals treated for 3 weeks, up to 30 min after glucose challenge. (**E**) Relative rates of gastric emptying measured 30 min after glucose challenge in the OGTT performed animals treated with KBP-042 for 7 weeks. (**F**) AUC of plasma levels of GIP during OGTT in animals treated for 7 weeks, up to 30 min after glucose challenge. n = 10 for all groups except high-fat diet (HFD) vehicle (n = 12). Statistical analysis between groups performed as a one-way ANOVA followed by Tukey's post hoc test with the following annotations: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. HFD vehicle. $\dagger\dagger P < 0.01$, $\dagger\dagger\dagger P < 0.001$ vs. pair-fed 5 µg/kg. $\ddagger\ddagger P < 0.01$, $\ddagger\ddagger\ddagger P < 0.001$ vs. pair-fed 10 µg/kg. Data are expressed as mean ± SEM.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HFD-Vehicle
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1.25 µg/kg
0.625 µg/kg
Pair-fed 10 µg/kg
Pair-fed 5 µg/kg
Blood Glucose (mmol/l)
Time after glucose administration (min)
B
C
Total AUC0 to 120 min Glucose (mM*min)
D
E
Plasma insulin (ng/ml)
F
G
Total AUC0 to 30 min Insulin (ng/ml*min)
H
Vehicle

Figure 5.

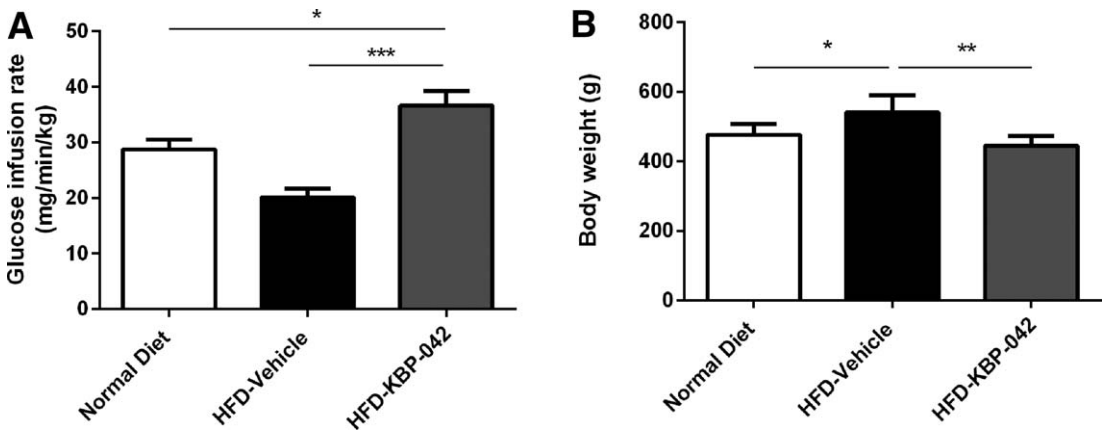

Figure 6 (**A**) Glucose infusion rate (GIR) at steady state during hyperinsulinemic–euglycemic clamp when blood glucose was clamped at basal levels after 21 days of treatment. (**B**) Body weight at hyperinsulinemic–euglycemic clamp experiment day after 21 days of treatment. Statistical analysis between groups performed as a one-way ANOVA followed by Tukey's post hoc test with the following annotations: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Data are expressed as mean ± SEM.

significantly reduced insulin levels while maintaining glucose tolerance (Figure 5H).

KBP-042 improved whole body insulin sensitivity in the hyperinsulinemic–euglycemic clamp

A hyperinsulinemic–euglycemic clamp study was performed to address the effect of KBP-042 on insulin sensitivity. For this study, ND rats were compared with insulin-resistant HFD rats and 5 μg/kg KBP-042 treated HFD rats. Figure 6A shows GIR reduced by ∼30% ($P = 0.057$) in the HFD group compared with ND. The treatment with KBP-042 led to a significant increase in GIR (82%, $P < 0.001$) compared with HFD vehicle. When KBP-042 treatment is compared with ND, GIR is increased with 27% ($P < 0.05$). As expected, body weight was increased after HFD for 10 weeks as compared with ND (Figure 6B), but treatment with KBP-042 for 21 days reduced weight with ∼18%, and the body weight was not significantly different from the ND rats at the end of the study.

Discussion

In this study, KBP-042 induced a significant weight loss over a period of 8 weeks, *albeit* with dramatic reductions in food intake initially. Kaolin consumption was, however, only stimulated in a higher dose than used in this study, thus indicating the reduction in food intake was not due to illness. However, minor nausea in the rats cannot be excluded. The highest KBP-042 groups sustain the weight loss (up to 20% compared with HFD vehicle) throughout the study, a phenomenon not seen in the pair-fed groups. The decreased food efficiency of the KBP-042-treated rats (2.5 μg/kg–10 μg/kg) and the large weight difference between treated and pair-fed rats, indicate increased energy expenditure. In general, amylin agonism blunts the reduction of energy expenditure that is normally caused by food restriction and weight loss, as well as changing RER (11,21), an indicator of fat utilization. Interestingly, amylin only increases energy expenditure when given as chronic infusion s.c. or i.c.v. (15,16,22), a finding likely related to short-lived activity of amylin (23). KBP-042 has a longer and more potent activation profile (17), despite a fast disappearance from plasma (<120 min) (18). However, energy expenditure, as well as potential fecal energy losses have to be formally assessed in future studies.

KBP-042 was able to significantly reduce TAG accumulation in the liver at both 2.5 μg/kg and 10 μg/kg. The reduction did not reach a significant level at 1.25 μg/kg and 5.0 μg/kg due to the relatively large individual variations in the hepatic TAG levels, but there is a tendency towards reduced hepatic TAG in these groups. Since ectopic deposition of lipids in the liver is related to increased insulin resistance, reducing the hepatic lipid-load could improve hepatic insulin sensitivity, hereby reducing gluconeogenesis in the fatty liver and increasing glucose tolerance (24). As of today, weight loss is the only remedy for ectopic lipid deposition, and KBP-042 serves as an excellent drug candidate to mediate this in an efficient manner. However, the extent to which a similar effect could be obtained by matching the weight loss remains to be explored. Importantly, the

Figure 5 (**A**,**B**) Intravenous glucose tolerance test (IVGTT) performed in animals treated for 3 weeks and 7 weeks, respectively, with either KBP-042 or saline. Animals were dosed s.c. at t = −30 and received i.v. glucose challenge at t = 0. (**C**) Area under the curve (AUC) 0 to 120 min for the IVGTT in panel A performed after treatment with KBP-042 for 3 weeks. (**D**) AUC 0 to 120 min for the IVGTT in panel B performed after treatment with KBP-042 for 7 weeks. (**E**) Plasma insulin levels during the IVGTT performed after 3 weeks of treatment. (**F**) Plasma insulin levels during the IVGTT performed after 7 weeks of treatment (legends as for panel A). (**G**) AUC for plasma insulin levels 0 to 30 min after glucose challenge in the IVGTT in panel A performed after treatment with KBP-042 for 3 weeks (legends as for panel B). (**H**) AUC for plasma insulin levels 0 to 30 min after glucose challenge in the IVGTT in panel B performed after treatment with KBP-042 for 7 weeks. n = 10 for all groups except high-fat diet (HFD) vehicle (n = 12). Statistical analysis between groups performed as a one-way ANOVA followed by Tukey's post hoc test with the following annotations: $^{*}P < 0.05$, $^{***}P < 0.001$ vs. HFD vehicle. $\dagger P < 0.05$, $\dagger\dagger\dagger P < 0.001$ vs. pair-fed 5 μg/kg. $\ddagger P < 0.05$, $\ddagger\ddagger P < 0.01$ vs. pair-fed 10 μg/kg. Data are expressed as mean ± SEM. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

analysis of the fatty acid composition of TAG further suggests that the fatty acid metabolism in the liver is unaltered, and the changes are an overall TAG reduction.

During acute OGTT, increases in plasma lactate and blood glucose were seen 30 min after administration of KBP-042, corresponding to previous studies showing acute hyperglycemia following acute administration of salmon calcitonin or rat amylin (25). This is likely explained by inhibition of insulin secretion, but also increased plasma lactate as seen in this study. This manifested as a tendency towards impaired glucose tolerance. Interestingly, the increase in plasma lactate was not present in animals treated chronically. In fact, chronic treatment led to improved oral glucose tolerance compared with both vehicle and pair-fed groups. Importantly, the improved glucose clearance was achieved with significantly lower plasma insulin levels, indicating improved insulin action. The improved glucose tolerance together with reduced liver TAG supports a general improved metabolism and insulin sensitivity. This is further supported by the reduction in adiposity, as plasma adiponectin is reduced in subjects who have obesity and related to for example, inflammation, insulin resistance, and energy metabolism (26,27), as well as type of phenotype in different fat depots (28). The observed increase in adiponectin is in alignment with the improvement in both glucose tolerance and insulin action as well as fatty acid removal from liver that KBP-042 induces (26,29-31). The reduced adiposity also manifested in lowering of plasma leptin, which corresponds well with previous demonstrations that KBP-042 increases the sensitivity towards leptin (18), a finding also seen with amylin (14,32).

IVGTT was performed to assess peripheral glucose homeostasis while circumventing the gastrointestinal system, which is obviously very affected by amylin agonism such as KBP-042 (33,34). Rats treated with KBP-042 maintained glucose tolerance with reduced insulin levels hence implying improved insulin sensitivity, *albeit* with an effect markedly lower than in the OGTT. This corroborates that KBP-042 has gastric emptying-independent effects on glucose tolerance. The reduced insulin levels both during IVGTT and OGTT could be explained by a direct KBP-042-mediated inhibition of both insulin and glucagon secretion directly in the islets of Langerhans (17), but maintaining or improving glycemia, glucose disposal rate, and insulin action after a significant weight loss is also well described in humans (5).

Plasma GIP levels and gastric emptying was assessed during the OGTT, and the rate of gastric emptying correlated to the GIP levels. In summary, KBP-042 reduces plasma incretin levels during OGTT, directly inhibits insulin and glucagon release from the islets of Langerhans (17), and reduces gastric emptying. These effects can also explain the reduced insulin levels in the OGTT, but not in the IVGTT. The reduced gastric emptying can mediate a beneficial effect on postprandial glucose levels, which along with fasting plasma glucose levels are very important factors in the reduction of risks related to hyperglycemia.

To formally assess the suggested increase in insulin action we performed a hyperinsulinemic–euglycemic clamp study. The reduced GIR seen in the HFD group compared with ND was expected since obesity is negatively correlated to insulin sensitivity and GIR (2). The large increase in GIR after treatment with KBP-042 illustrated the increase in insulin sensitivity. The KBP-042-induced weight loss could explain a large increase in GIR. However, here the rats treated with KBP-042 had similar body weight to the ND, but with a significantly increased GIR. This could suggest that insulin sensitivity is increased beyond what would be expected from weight loss, although this has to be further tested in weight-matched animals receiving the same diet.

In conclusion, KBP-042 induced a sustained weight loss over 8 weeks in obese prediabetic rats but not in pair-fed animals, leading to reduction in adipose tissues, ectopic TAG deposition, improved glucose tolerance, and improved insulin action. The combination of a weight-reducing and insulin-sensitizing agent is to our knowledge unique. KBP-042 thus shows great promise for the treatment of type 2 diabetes and obesity due to its multiple beneficial effects on several aspects of the metabolic syndrome. O

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Paper III:

KBP-088, a novel DACRA with prolonged receptor activation, is superior to davalintide in terms of efficacy on body weight

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KBP-088, a novel DACRA with prolonged receptor activation, is superior to davalintide in terms of efficacy on body weight

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Gydesen S, Andreassen KV, Hjuler ST, Christensen JM, Karsdal MA, Henriksen K. KBP-088, a novel DACRA with prolonged receptor activation, is superior to davalintide in terms of efficacy on body weight. *Am J Physiol Endocrinol Metab* 310: E821–E827, 2016. First published February 16, 2016; doi:10.1152/ajpendo.00514.2015.—This study aims to elucidate the mechanism behind the potent weight loss induced by dual amylin and calcitonin receptor agonists (DACRA) through comparison of the novel DACRA KBP-088 with the amylinomimetic davalintide with regard to in vitro receptor pharmacology and in vivo efficacy on food intake and body weight. KBP-088 and davalintide were tested for their ability to activate the amylin and calcitonin receptors as function of dose and time. Two doses of KBP-088 (1.67 and 5.0 μg/kg) were compared with similar davalintide doses in high-fat diet (HFD)-fed rats receiving subcutaneous dosing once daily for 62 days. Glucose tolerance was assessed after 3 and 7 wk of treatment. KBP-088 demonstrated activation of amylin and calcitonin receptors and prolonged receptor activation compared with davalintide as well as a potent reduction of acute food intake. KBP-088 transiently reduced food intake and induced and notably sustained a significant ~16% vehicle-corrected weight loss without significant weight loss in the calorie-restricted control groups. Additionally, KBP-088 reduced white adipose tissues and adipocyte hypertrophy. Finally, KBP-088 alleviated hyperinsulinemia and improved oral glucose tolerance even with significantly lower insulin levels after 3 and 7 wk of treatment. KBP-088 is a potent amylin and calcitonin receptor agonist with prolonged receptor activation compared with davalintide. Moreover, KBP-088 induced and sustained significant weight loss and reduced overall adiposity and adipocyte hypertrophy in HFD rats. Finally, KBP-088 improved oral glucose tolerance and alleviated hyperinsulinemia, underscoring the potential of KBP-088 as an antiobesity agent with benefits on glucose control.

obesity; amylin; DACRA; adiposity; treatment

OBESITY IS A CONSEQUENCE of the modern-day lifestyle, and the number of obese people is increasing. Associated with obesity is a number of comorbidities and reduced life expectancy. Of these, type 2 diabetes, nonalcoholic fatty liver disease, osteoarthritis, and cardiovascular disease are prominent, and the obesity-derived insulin resistance is considered a major detrimental event in terms of prognosis (21)(15)(6).

Treatments for obesity are few, and even with the recent approval of high-dose liraglutide as a treatment for obesity in the US, there is a very limited library of molecules leading to weight loss.

Amylin and amylin analogs such as davalintide are associated with control of appetite and thereby weight loss; however, they are significantly limited by a lack of efficacy especially in humans (10). The amylin analog pramlintide is the only molecule approved as therapy, and it is only as adjunct therapy to insulin for treatment of diabetes (12, 17), which underscores the challenge in translating potent receptor activation to in vivo efficacy. Dual amylin and calcitonin receptor agonists (DACRAs) are separated from amylin, as they elicit activation not only of the amylin receptor (AMY-R), but also of the calcitonin receptor (CTR) (1), and interestingly they also appear to activate the receptors for an extended period of time, leading to markedly superior effects on classical amylin-induced responses in vivo, such as food intake, weight reduction, and suppression of glucagon (1, 11, 16). However, the extent to which these effects can be translated into long-term efficacy on body weight and how they compare to previous amylin analogs is presently not known.

In this study, we studied a novel, highly potent DACRA, KBP-088, with a prolonged receptor activation profile in a long-term in vivo study head to head with davalintide, a potent AMY-R agonist, to clearly determine peptide properties predictive of in vivo efficacy on body weight.

RESEARCH DESIGN AND METHODS

Peptide therapy. Synthetic KBP-088 and davalintide (American Peptide) were dissolved in saline for subcutaneous delivery. The 5 μg/kg dose chosen for peptide administration in the current investigations was based on previous comparable DACRA studies in animal models of obesity and type 2 diabetes (1, 5) using sCT and the potent DACRA KBP-042.

In vitro receptor binding and activity. The receptor specificity and potency at the amylin and calcitonin receptor were determined by the ability of KBP-088 to induce β-arrestin and recruitment in cell lines overexpressing the human calcitonin amylin and calcitonin gene-related peptide receptors, respectively. U20S CALCR cells (DiscoverX cat. no. 93-0566C3), CHO K1 CALCR RAMP3 (DiscoverX cat. no. 93-0268C2) and CKO-K1 CALCRL RAMP1 (DiscoverX cat. no. 93-0269C2) cells were used to quantify β-arrestin by PathHunter Detection Kit (DiscoverX 93-0001) according to the manufacturer's instructions. The responses were analyzed and plotted as previously described (1, 2).

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 were obtained at 6 wk of age and housed at the Nordic Bioscience animal facility (21–23°C, 55–65% relative humidity, 12:12-h light-dark cycle) with ad libitum access to food and water.

Animals. Normal-diet age-matched lean rats (ND) were fed a standard pelleted chow, and high-fat diet-fed rats (HFD) a 60 kcal% fat diet (#58Y1; TestDiet, London, UK). At study start, HFD rats received 10% fructose (#F0127, Sigma-Aldrich, Brøndby, Denmark) in the drinking water, and, in order to avoid bacterial growth, citric acid was added (pH 3.6) in fructose drinking water; ND rats received tap water with an equal amount of citric acid.

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Table 1. *Amino acid sequences of the dual amylin and calcitonin receptor agonists*

	Sequences
KBP-088	AC- **C S** N **L S** T C **M** L G R L S Q E L H R L Q T **F** P K T **D V** G **A** N **A P** -NH_2
Davalintide	**K C** N **T A** T C **V** L G R L S Q E L H R L Q T **Y** P R T **N T** G **S** N **T Y** -NH_2

Amino acid sequence comparison of KBP-088 and davalintide. Differences in sequences are highlighted in boldface. KBP-088's NH_2-terminal cysteine has an acetyl modification.

Acute food intake. Acute food intake was tested in overnight-fasted HFD rats. The animals received vehicle, KBP-088 (5, 1.67 μg/kg sc), or davalintide (5, 1.67 μg/kg sc), and food intake was monitored 4, 24, 48, and 72 h postinjection.

Chronic in vivo study. We compared two doses of KBP-088 (1.67 and 5.0 μg/kg) with similar davalintide doses in HFD rats. Following 10 wk of HFD feeding, the rats were randomly (body weight) assigned to treatment groups ($n = 8$) receiving either vehicle (saline sc), KBP-088 (5, 1.67 μg/kg sc), or davalintide (5, 1.67 μg/kg sc) once daily. Pair-fed rats (saline sc) were food restricted to KBP-088 5 μg/kg and davalintide 5 μg/kg. Food intake and body weight were monitored on *days 1–20, 28, 35, 42, 49, 56*, and *62*. After 3 and 7 wk of treatment, OGTT was performed in overnight-fasted (12 h) rats, with blood glucose measured and EDTA-plasma obtained for hormonal analysis. Rats received glucose gavage (2 g/kg po). Blood samples were collected from the tail vein before drug administration (−30 min) and glucose challenge (0 min) and 15, 30, 60, and 120 min post-glucose challenge.

At study end, the animals were euthanized, anesthetized by inhalation (isoflurane) followed by exsanguation and dissection. Epididymal fat pads were fixed in 4% formaldehyde and then stained with hematoxylin. Sections were randomly and blindly selected and viewed under a microscope (12 sections per group; 6 sections for ND control, ×20 magnification). Pictures were taken and adipocytes counted using Olympus cell imaging software, and the average size of the adipocytes was calculated.

Blood samples were collected in EDTA tubes and centrifuged at 5,000 rpm for 10 min at 4°C. Blood glucose was monitored by Accu-Check Avia monitoring system (Roche Diagnostics, Rotkreuz, Switzerland). Plasma levels of insulin (Mercodia Rat Insulin ELISA; Mercodia, Uppsala, Sweden) was analyzed according to manufacturer's instruction.

Statistical analysis. All data are presented as means ± SE. The statistical analyses of various drug effects were conducted using one-way ANOVA, followed by Tukey's posttest for multiple comparison. ND controls and HFD rats as well as EC_{50} values weare compared 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.

RESULTS

KBP-088 induces prolonged receptor activation in vitro, whereas davalintide does not. Table 1 shows the sequence of KBP-088 and davalintide for comparison. In vitro analyses of the potency of KBP-088 and davalintide on the calcitonin, amylin and CGRP receptors showed that both KBP-088 and davalintide are highly potent ligands for the calcitonin and amylin receptors (Fig. 1). EC_{50} values for the CTR were calculated to 4.5 ± (1.4) × 10^{-9} M and 5.2 (± 1.2) × 10^{-9} M for KBP-088 and davalintide ($P = 0.68$), respectively, whereas the corresponding EC_{50} values for the AMY-R were 4.0 (± 1.7) × 10^{-10} M and 1.3 (± 1.7) × 10^{-9} M for KBP-088 and davalintide ($P = 0.38$), respectively. In contrast, on the CGRP-R we only observed a response with davalintide, whereas KBP-088 even at very high doses (10^{-7} M) did not induce β-arrestin recruitment (Fig. 1*C*). However, the mag-

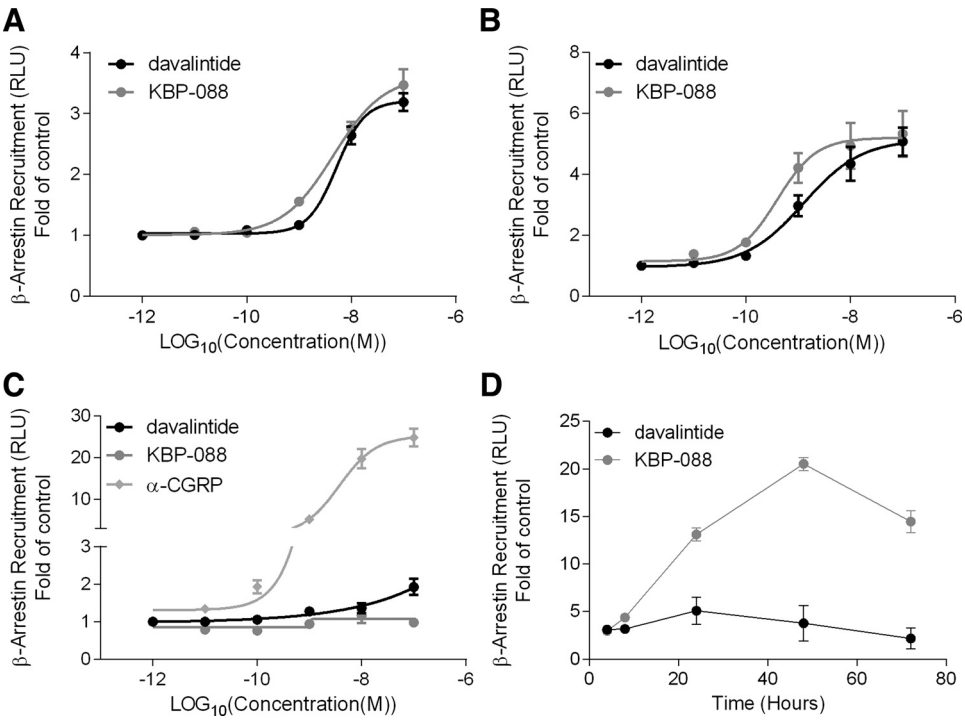

Fig. 1. Dose-range curves of KBP-088 and davalintide on induction of β-arrestin in calcitonin receptor (CTR; A), amylin receptor (AMY-R; B), and calcitonin gene-related peptide receptor (CGRP-R; C) -expressing cell lines. *D*: prolonged CT-R-specific β-arrestin response mediated by 100 nM KBP-088 or 100 nM davalintide in CTR-expressing cells for 4–72 h. *A–D* are pooled data from 3–4 independent experiments.

nitude of the CGRP-R induction elicited by davalintide was low compared with the endogenous CGRP-R agonist, α-CGRP. An important aspect of the DACRAs is their prolonged interaction with the CTR (2) and while davalintide has been reported to bind irreversibly to the AMY-R (9) it is not clear to what extent this translates into functional receptor activation over time. To address this, we compared davalintide to KBP-088 with receptor to prolonged receptor activation, and as seen in Fig. 1*D*, KBP-088 induces a potent prolonged receptor activation with activation still observed at 72 h, in line with other DACRAs (2). On the other hand, despite the potent short term activation of the receptors (Fig. 1, *A–C*), davalintide did not lead to prolonged receptor activation (Fig. 1*D*).

Both davalintide and KBP-088 attenuate short-term food intake, albeit only KBP-088 shows a prolonged reduction. A single dose of KBP-088 and davalintide resulted in significantly ($P < 0.01$) reduced food intake 4 h postinjection; however, only KBP-088 significantly reduced food intake 24 (~95%) and 48 (~32%) hours postinjection (Fig. 2*A*).

KBP-088 potently reduces appetite, body weight, and fat depots. Ten weeks of high-fat feeding resulted in a phenotype with significantly ($P < 0.001$, ~30%) increased body weight (HFD), hyperinsulinemia, impaired glucose control without hyperglycemia, but impaired insulin sensitivity (HOMA-IR) compared with the lean age-matched controls (ND) (Table 2) resembling an obese and prediabetic phenotype.

Table 2. *Model characterization*

	ND Control	HFD
Body weight (g)	466 ± 33	607 ± 23***
Fasting plasma glucose (ng/ml)	6.2 ± 0.1	5.6 ± 0.1**
Fasting plasma insulin (ng/ml)	1.0 ± 0.1	2.2 ± 0.2***
HOMA-IR (mM x μU/ml)	6.3 ± 0.6	14.8 ± 1.1***
Glucose tAUC in OGTT after 7 wk of treatment (mmol/l·min)	1,253 ± 20	1,422 ± 31***
Insulin tAUC in OGTT after 7 wk of treatment (ng/ml·min)	260 ± 17	358 ± 33*

Values are means ± SE; $n = 8$ rats per group. Model characterization of normal-diet lean (ND Control) and high-fat diet-fed (HFD) rats. HOMA-IR, homeostasis model assessment of insulin resistance; tAUC, total area under the curve; OGTT, oral glucose tolerance test. Statistical tests performed with Student's *t*-test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. ND Control.

To investigate the anti-obesity potential of KBP-088 in vivo we treated HFD rats for 8 wk, and compared the metabolic effects with equivalent davalintide dosing. Previously, DACRAs have shown a hypophagic effect (5); therefore, we included a pair-fed group to both KBP-088 and davalintide treated rats exploring impact of food restriction regarding body weight. KBP-088 and davalintide were subcutaneously administered (1.67 and 5 μg/kg sid) throughout 62 days. During the study period food intake was transiently attenuated by KBP-088 (Fig. 2, *B* and *C*) treatment, although cumulative food intake after the initial 2 wk of treatment was not significantly different

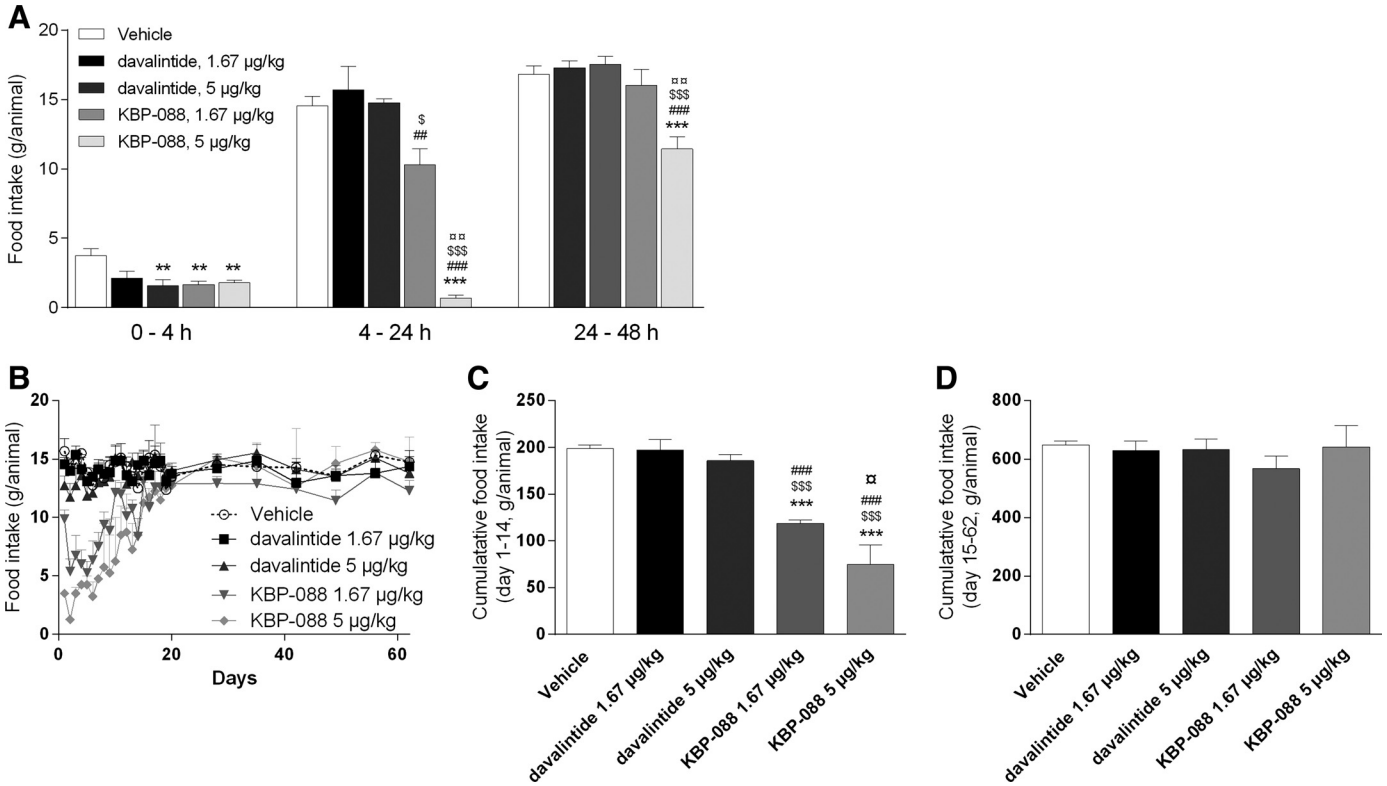

Fig. 2. A: short-term treatment effect on food intake (4–48 h) by 2 different concentrations of KBP-088 and davalintide in high-fat diet-fed (HFD) rats ($n = 8$ rats per group). Weekly food intake (*B*), cumulative food intake *days 1–14* (*C*), and *days 15–62* (*D*) in HFD rats dosed with davalintide and KBP-088 (1.67 and 5 μg/kg) for 62 days ($n = 8$ rats per group; KBP-088 5 μg/kg $n = 4$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. KBP-088 1.67 μg/kg; # vs. 1.67 μg/kg davalintide, $ vs. 5 μg/k davalintide g, * vs. vehicle. Statistical analysis between groups was evaluated by one-way ANOVA post hoc analyses. All data are means ± SE.

compared with davalintide and vehicle treated rats (Fig. 2*D*). At study end, body weight of KBP-088-treated animals was significantly lowered compared with vehicle- and davalintide-treated rats and associated pair-fed rats (Fig. 3*B*). The reduced food intake in the initial phase of the study (Fig. 2*A*) corresponds well with the significant weight loss observed in both KBP-088-treated groups and the KBP-088-associated pair-fed group compared with vehicle- and davalintide-treated rats (Fig. 3*A*).

Based on food intake and body weight change, food efficacy was calculated. We used the total food intake from *days 15–62*, as there were no significant difference in food consumption between the groups in this period. Expectedly, KBP-088 treatment (1.67 and 5 μg/kg) resulted in a marked attenuation of food efficiency (Fig. 3*C*), which was significantly different from vehicle- and davalintide-treated rats and the pair-fed group associated with the KBP-088 5 μg/kg group.

At termination, we isolated epididymal, inguinal, and perirenal fat depots. Interestingly and in conjunction with the significant body weight reduction, the weight of epididymal white adipose tissue was significantly reduced after treatment with 1.67 and 5 μg/kg KBP-088 (Fig. 4*A*). This reduction was not observed in the pair-fed control or in davalintide-treated rats. There was a trend toward reducing inguinal and perirenal adipose tissue (Fig. 4, *B* and *C*). Furthermore, the size of the adipocytes (Fig. 4*D*, *1–6*, *E*) in KBP-088-treated HFD rats was markedly reduced compared with vehicle- and davalintide-treated rats and corresponding pair-fed controls.

KBP-088 enhances glucose tolerance and potentially insulin sensitivity. As expected, the basal insulin levels were markedly increased in HFD rats compared with ND rats; however, hyperinsulinemia was significantly reduced in KBP-088 groups compared with vehicle (data not shown). To investigate the effect of KBP-088 on glucose tolerance, we performed an OGTT in *weeks 3* and *7*. Glucose tolerance was significantly improved by KBP-088 (1.67 and 5 μg/kg) and davalintide (5 μg/kg) to a similar extent (Fig. 5, *A* and *B*), evidenced by the ~12% decreases in the blood glucose AUC values for both treatment doses of 5 μg/kg (Fig. 5, *C* and *D*). Pair feeding did not improve glucose tolerance in either test. The glucose-induced insulin hypersecretion observed in vehicle- and pair-fed groups was markedly suppressed during OGTT by the two concentrations of KBP-088 and by davalintide at 5 μg/kg, which resulted in significantly reduced insulin AUC values in KBP-088- (1.67 and 5 μg/kg) and davalintide- (5 μg/kg) treated rats (Fig. 5, *E* and *F*).

Overall, these findings suggest that KBP-088 exerts a pronounced anorectic effect in HFD rats, a reduction of body weight, and an improvement in energy homeostasis in conjunction with alleviation of hyperinsulinemia, which is in line with previous findings for injectable DACRAs (5), and illustrates the need for prolonged receptor activation to induce these effects.

DISCUSSION

Amylin receptor agonists are highly interesting as candidates for the treatment of type 2 diabetes and obesity (4). However, despite the approval of the amylin receptor agonist pramlintide for the treatment of diabetes as adjunct to mealtime insulin, these ligands are notoriously limited in terms of efficacy both on glucose homeostasis and on weight control. Recent studies have indicated that DACRAs, dual amylin and calcitonin receptor agonists, have potency extending far beyond classical amylin agonists such as pramlintide, although the explanation for this remained to be elucidated.

In this study, we compared a novel DACRA, called KBP-088, to the amylin mimetic davalintide, an amylin, calcitonin, and calcitonin gene-related peptide receptor agonist, using a series of in vitro and in vivo tests to elucidate the mechanism underlying the superior activity of the DACRAs. By use of short-term in vitro assays, davalintide was roughly equipotent to KBP-088; however, when their ability to elicit long-term receptor activation was tested, davalintide did not induce this. On the other hand, KBP-088 activated the receptor for up to 72 h, demonstrating a superior receptor activation profile. Furthermore, these effects manifested directly in a prolonged ability to control appetite by KBP-088, which was not seen for davalintide. This was somewhat surprising, as davalintide previously had been shown to bind irreversibly to the AMY-R (9); however, due to some yet to be identified mechanism, this does not translate into prolonged receptor activation or prolonged suppression of appetite.

In this study, KBP-088 induced a marked weight loss. The drastic reduction in body weight observed at study start could be explained by the initial anorectic effect of KBP-088, as the food-restricted pair-fed controls lowered their body weight simi-

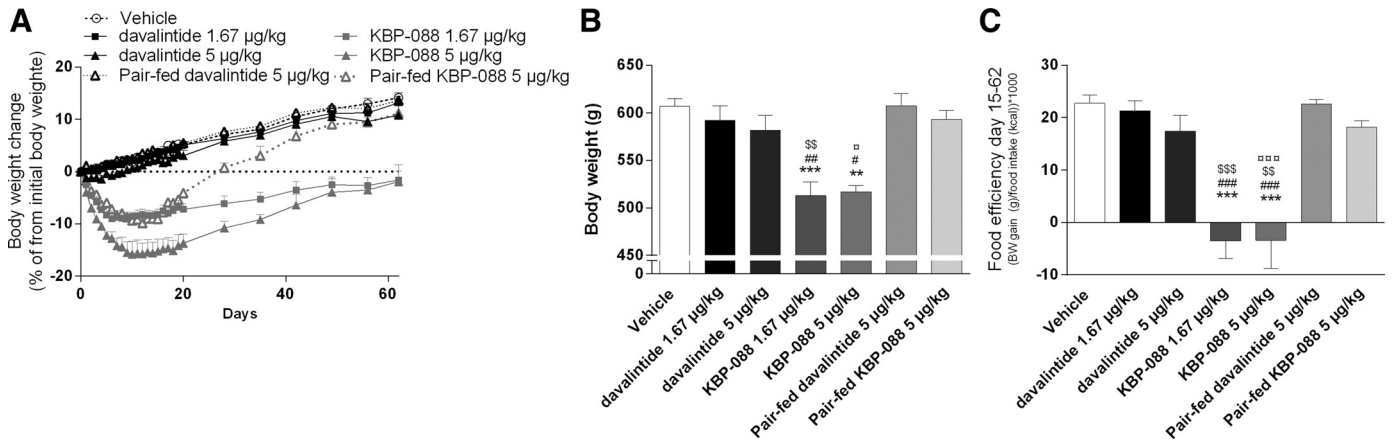

Fig. 3. Weekly (*A*), study end body weight (*B*), and food efficacy (*days 15*-62; *C*) in HFD rats dosed with davalintide and KBP-088 (1.67 and 5 μg/kg) for 62 days and pair-fed ($n = 8$ rats per group; KBP-088 5 μg/kg $n = 4$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; * vs. vehicle, # vs. 1.67 μg/kg davalintide, $ vs. 5 μg/kg davalintide, ¤ vs. pair-fed 5 μg/kg KBP-088.

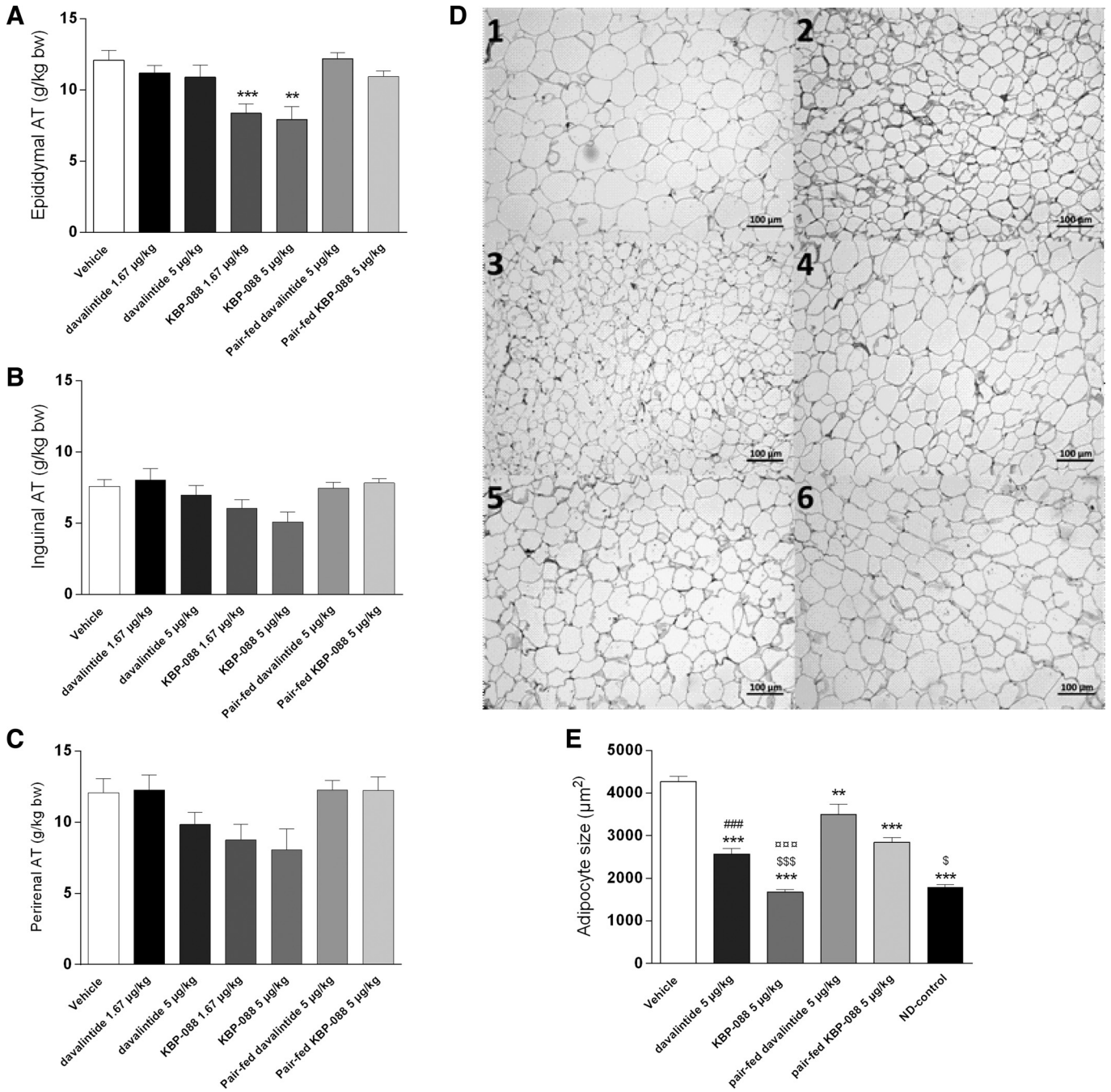

Fig. 4. Relative weight of epididymal (*A*), inguinal (*B*), and retroperitoneal (*C*) adipose tissue at study end. *D 1–6*: Hematoxylin-stained epididymal adipose tissue at ×10 magnification in *1*) vehicle, *2*) lean, *3*) KBP-088, *4*) davalintide, *5*) pair-fed KBP-088, and *6*) pair-fed davalintide, respectively (n = 8 rats per group; KBP-088 5 μg/kg n = 4). *E*: quantified adipocyte size (12 sections per group; 6 sections for ND-control, ×20 magnification, dimension: 2040 × 1536). *$P <$ 0.05, **$P < 0.01$, ***$P < 0.001$: * vs. vehicle, $ vs. 5 μg/kg davalintide, # vs. pair-fed 5 μg/kg davalintide, ¤ vs. pair-fed 5 μg/kg KBP-088. Statistical analysis between groups was evaluated by one-way ANOVA post hoc analyses. All data are means ± SE.

larly. Importantly, five animals were subtracted from the 5 μg/kg group due to a too-large weight loss. The maximal dose of KBP-088 was selected based on previous findings using salmon calcitonin, an amylin and calcitonin receptor agonist, and DACRAs (5); however, this peptide exerts a very potent anorectic effect, and in future studies the maximal doses will be of lower concentration. Interestingly, the food intake returned to normal within 3 wk, and the pair-fed group regained lost body weight, whereas the KBP-088-treated groups maintained the weight loss achieved, 16% throughout the study in the highest-concentration KBP-088 group. We speculate that the nonexisting prolonged response of davalintide underlay the lack of ability to suppress body weight at the doses chosen, although it transiently suppressed food intake. These are consistent with the need for infusion pumps and thereby continuous exposure to davalintide in order for it to exert a weight-reducing effect (9).

Considering the fact that KBP-088 significantly suppresses body weight compared with the pair-fed controls emphasizes

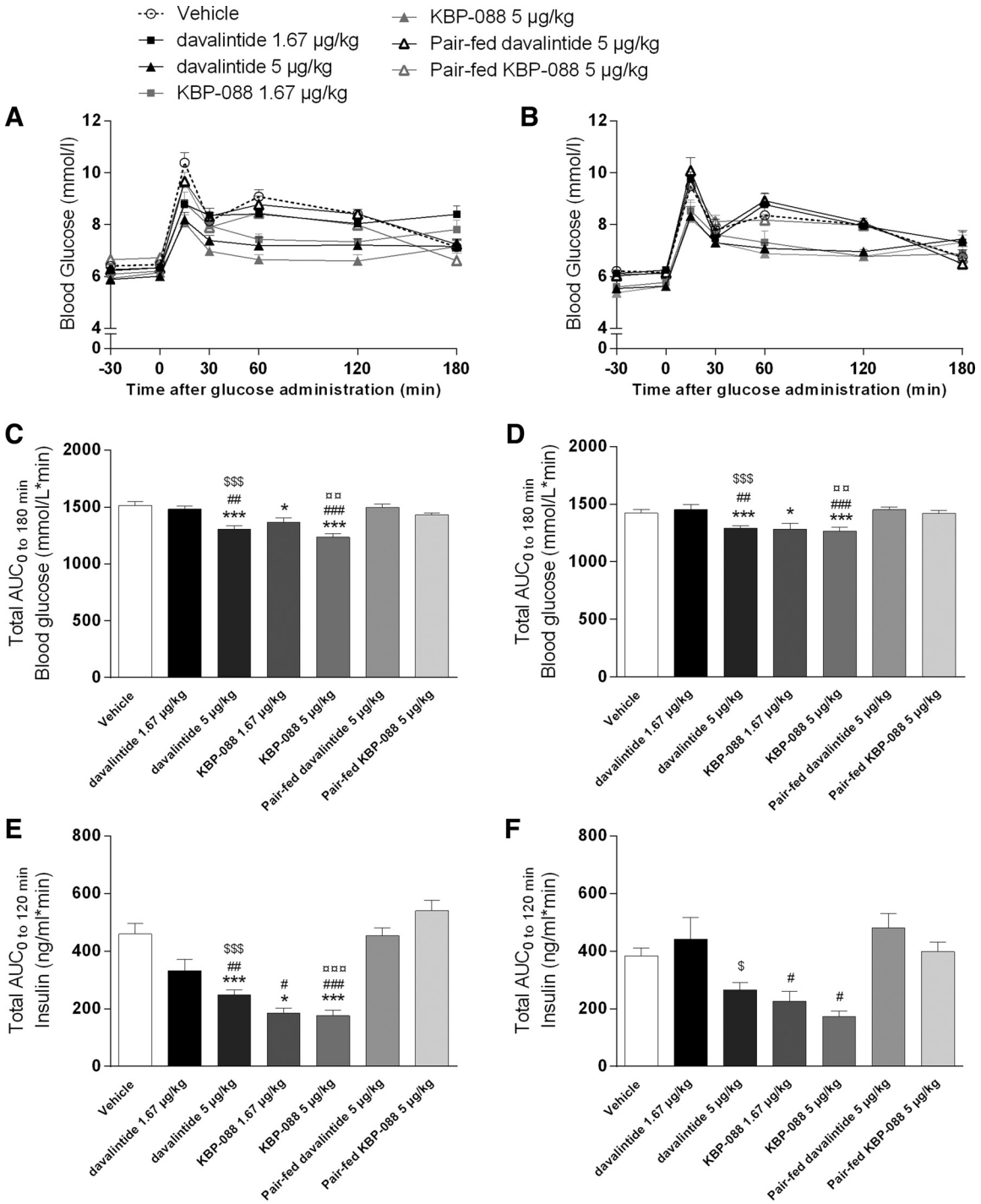

Fig. 5. *A* and *B*: plasma glucose during oral glucose tolerance test (OGTT) in HFD rats dosed with davalintide and KBP-088 (1.67 and 5 μg/kg) after 3 and 7 wk, respectively. Total AUC for glucose (*C* and *D*) and insulin (*E* and *F*) during OGTT after 3 and 7 wk, respectively ($n = 8$ rats per group; KBP-088 5 μg/kg $n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P <$ 0.vs. pair-fed 5 μg/kg davalintide, ¤ vs. pair-fed 5 μg/kg KBP-088. Statistical analysis between groups was evaluated by one-way ANOVA post hoc analyses. All data are means ± SE.

that KBP-088 has some beneficial effect on the weight reduction besides suppressed food intake. Furthermore, the decreased food efficiency of the KBP-088-treated rats, and the difference between treated and pair-fed rats, suggest an increased energy expenditure. Davalintide has enhanced pharmacological properties over rat amylin (8), albeit a short-lasting effect compared with KBP-088. Under normal conditions the rats will lower energy expenditure during weight loss; however, continuous infusion of amylin prevents this reduction (11, 18), and similar effects were observed with davalintide (9).

Interestingly, amylin increases energy expenditure only when given as a continuous infusion or icv (3, 7, 18), a finding likely related to the short-lived activation of the AMY-R. However, the energy expenditure needs to be formally assessed in the future.

Additionally, KBP-088 reduced overall adiposity as well as decreasing the size of adipocytes in the epididymal white adipose tissue over the study period. Weight loss has on multiple occasions been associated with beneficial effects on adipocytokines (14) and leptin metabolism – leptin sensitivity, which has previously been shown to be improved by DACRAs (5). Furthermore, weight loss and reduction in adipose cell size are involved in restoring plasma insulin concentration toward normal, concomitant with the return of normal tissue insulin sensitivity (13), and whether KBP-088 has a direct effect on adipocyte hypertrophy needs further investigations.

Short- and long-term treatment with KBP-088 improved glucose tolerance compared with both vehicle and pair-fed groups in accord with previous studies performed with DACRAs (5) and davalintide. The previously described effect of davalintide on glucose tolerance was performed in rats receiving a continuous infusion of davalintide (8). Notably, glucose tolerance was also improved in davalintide-treated rats even though there was a lack of prolonged receptor activation. This was probably due to the predosing of the rats with the peptides 30 min prior to an OGTT, which confirms the ability of davalintide to improve glucose tolerance short-term, as previously described. However, to evaluate the overall treatment effect of KBP-088 and davalintide on glucose tolerance, the OGTT must be performed without predosing. As KBP-088 has a prolonged response and reduces body weight, the glucoregulatory and insulinostatic effects would expectedly be present; however, as davalintide did not elicit long-term receptor activation, the glucose-lowering effect might have been lost.

In line with previous DACRA findings, albeit in contrast to other glucose-lowering agents such as sulfonylureas and GLP-1 analogs, the enhanced glucose disposal was achieved with an attenuated insulin secretion. This could imply an enhanced insulin sensitivity; however, this needs further investigations addressing insulin sensitivity and circumventing gastric emptying, as amylin agonism lowers the gastric emptying rate (19, 20).

In conclusion, the novel DACRA KBP-088 has prolonged receptor activation, and furthermore, KBP-088 induces and sustains a marked weight loss over 62 days in obese rats, which concomitantly leads to a reduced amount of adipose tissue. In addition, KBP-088 improves glucose tolerance and implies improved insulin action, underscoring the potential of KBP-088 as an antiobesity agent with additional benefits on glucose control.

GRANTS

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DISCLOSURES

All authors are employed by Nordic Bioscience. M. A. Karsdal and K. Henrikson own stock in Nordic Bioscience.

AUTHOR CONTRIBUTIONS

Author contributions: S.G., K.V.A., M.A.K., and K.H. conception and design of research; S.G. and K.V.A. performed experiments; S.G., K.V.A., J.M.C., and K.H. analyzed data; S.G., K.V.A., and K.H. interpreted results of experiments; S.G. and K.V.A. prepared figures; S.G., K.V.A., S.T.H., and K.H. drafted manuscript; S.G., K.V.A., S.T.H., M.A.K., and K.H. edited and revised manuscript; S.G., K.V.A., S.T.H., J.M.C., M.A.K., and K.H. approved final version of manuscript.

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Paper IV:

Optimization of tolerability and efficacy of the novel Dual Amylin and Calcitonin Receptor Agonist, KBP-089, through dose-escalation and combination with a GLP-1 analogue

Am J Physiol Endocrinol Metab 312: E000–E000, 2017.
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RESEARCH ARTICLE

Optimization of tolerability and efficacy of the novel dual amylin and calcitonin receptor agonist KBP-089 through dose escalation and combination with a GLP-1 analog

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Gydesen S, Andreassen KV, Hjuler ST, Hellgren LI, Karsdal MA, Henriksen K. Optimization of tolerability and efficacy of the novel dual amylin and calcitonin receptor agonist KBP-089 through dose escalation and combination with a GLP-1 analog. *Am J Physiol Endocrinol Metab* 312: E000–E000, 2017. First published March 14, 2017; doi:10.1152/ajpendo.00419.2016.—Amylin and GLP-1 agonism induce a well-known anorexic effect at dose initiation, which is managed by dose escalation. In this study we investigated how to optimize tolerability while maintaining efficacy of a novel, highly potent dual amylin and calcitonin receptor agonist (DACRA), KBP-089. Furthermore, we tested the GLP-1 add-on potential of KBP-089 in high-fat diet (HFD)-fed rats. KBP-089 potently activated both the amylin and calcitonin receptors in vitro and demonstrated a prolonged receptor activation as well as a potent reduction of acute food intake. HFD rats dosed every day or every second day obtained equal weight loss at study end, albeit with an uneven reduction in both food intake and body weight in rats dosed every second day. In a 4-fold dose escalation, KBP-089 induced a transient reduction in food intake at every escalation step, with reducing magnitude over time, and the following treatment with 2.5, 10, and 40 μg/kg resulted in an ~15% vehicle-corrected weight loss, a corresponding reduction in adipose tissue (AT), and, in all treatment groups, improved oral glucose tolerance ($P < 0.01$). Twofold and linear escalations suppressed body weight evenly with no significant reduction in food intake at either escalation step. KBP-089 (1.25 μg/kg) and liraglutide (50 μg/kg) reduced 24-h food intake by 29% and 37% compared with vehicle, respectively; however, when they were combined, 24-h food intake was reduced by 87%. Chronically, KBP-089 (1.25 μg/kg) and liraglutide (50 μg/kg) lowered body weight 8% and 2% in HFD rats, respectively, whereas the combination resulted in a 12% body weight reduction. Moreover, the combination improved glucose tolerance ($P < 0.05$). In conclusion, DACRAs act complementarily with GLP-1 on food intake and body weight. Furthermore, on escalation, KBP-089 was well tolerated and induced and sustained a significant weight loss and a reduction in AT in lean and HFD rats, underscoring the potential of KBP-089 as an anti-obesity agent.

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obesity; amylin, DACRA; adiposity; treatment; tolerance; GLP-1; insulin sensitivity

OBESITY IS THE RESULT of excessive caloric intake and a sedentary lifestyle. Complications such as insulin resistance, type 2 diabetes mellitus (T2DM), and nonalcoholic fatty liver disease, among others, often coexist with obesity. Achieving weight loss through low-energy diets, increased physical activity, and behavioral therapy represent the core components of lifestyle intervention in obesity management (31). Complementary therapies such as surgery and pharmacotherapy are used in persons not achieving sufficient weight loss with lifestyle interventions (8).

Amylin receptor agonists (pramlintide and davalintide) have shown promise for weight reduction in preclinical models (19) and clinical settings (3, 26), and in combination with leptin (22) and small-molecule weight loss agents (4). Due to its appetite-regulating capability, pramlintide has been shown to reduce insulin-induced weight gain, in combination with regulation of postprandial glucose levels, and therefore has been approved as adjunct therapy to mealtime insulin for the treatment of T2DM (25, 32). In the context of T2DM, pramlintide has been shown to cause small placebo-subtracted absolute reductions in hemoglobin A1c (Hb A1c) of 0.2–0.4% and weight loss of 2.1–2.3 kg in obese individuals (20). Side effects include temporary nausea in T2DM (20, 34).

The most recent therapy for obesity is high-dose liraglutide, which induces a reduction in body weight and sustains the achieved weight loss partially due to a lowered appetite (7). Furthermore, liraglutide also addresses elevated blood glucose levels, albeit still with limitations in terms of efficacy and challenges with tolerability (14, 17). Treatment with amylin and GLP-1 analogs induces potent reductions in food intake. Changes in satiety induced by activation of receptors in the brain (e.g., by amylin and GLP-1) can lead to nausea (5, 10), and to circumvent these tolerability issues, the peptides are escalated in dose over time (3, 15, 17).

Dual amylin and calcitonin receptor agonists (DACRAs) elicit activation not only of the amylin receptor (AMY3-R) but also of the calcitonin receptor (CTR) (1). Notably, DACRAs activate the receptors for an extended time period, leading to superior effects on classical amylin-induced responses in vivo, such as food intake, weight reduction, and suppression of glucagon (1, 9, 24, 30).

In this study, we characterized a novel, highly potent DACRA, KBP-089, in vitro as well as in vivo. We determined the potency and receptor activation profile using cell lines expressing the CTR and the AMY3-R. In vivo, we evaluated the tolerability of KBP-089 using dose-escalation regimes, assessed dosing frequency by comparing daily dosing with dosing every second day, and examined KBP-089 as mono-

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therapy and in combination with the GLP-1 analog liraglutide as treatment for obesity in high-fat diet-fed rats (HFD).

METHODS

Peptide Therapy

Synthetic KBP-089 (American Peptide Company, Vista, CA) and liraglutide (Bachem, Bubendorf, Switzerland) were dissolved in saline for subcutaneous delivery. The suboptimal doses chosen for KBP-089 administration in the current in vivo investigations were based on previous comparable DACRA studies in animal models of obesity using potent DACRA, KBP-042 (11), and KBP-088 (9) and on previous studies using liraglutide (16).

In Vitro Receptor Binding and Activity

KBP-089 receptor specificity and potency were determined by cAMP production and β-arrestin recruitment in cell lines with heterologous overexpression of the human calcitonin (CTa), amylin (CTa + RAMP3), and calcitonin gene-related peptide (CGRP) receptors (U2OS CALCR cells, DiscoverX catalog no. 93-0566C3; CHO-K1 CALCR-RAMP3, DiscoverX catalog no. 93-0268C2; and CHO-K1 CALCRL-RAMP1, DiscoverX catalog no. 93-0269C2, respectively). All in vitro cell experiments were conducted with 2,500 cells/well, incubated with ligands for 3 h at 37°C in a humidified incubator with atmospheric air supplemented with 5% CO_2 unless otherwise specified. Quantification of the intracellular cAMP was assayed using the cAMP femto Tb kit (no. 62AM7PEB; Cisbio Bioassays) according to the manufacturer's instructions. β-Arrestin recruitment was quantified by using the PathHunter detection kit (DiscoverX catalog no. 93-0001) according to the manufacturer's instructions. Data analysis was conducted as previously described (1, 2, 9).

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 (SD) rats (Envigo, Horst, The Netherlands) were obtained at 6 wk of age and housed at the Nordic Bioscience animal facility (21–23°C, 55–65% relative humidity, 12:12-h light-dark cycle) with ad libitum access to food and water.

Animals

From arrival and throughout the study periods, normal-diet age-matched lean rats (ND) were fed a standard pelleted chow (no. 5002; LabDiet, St. Louis, MO) and high-fat diet-fed rats (HFD) a 60 kcal% fat diet (no. 58Y1; TestDiet, London, UK) from arrival and a minimum of 10 wk before study initiation.

Acute Food Intake

Acute food intake tests were performed in overnight-fasted HFD rats (16 wk of age). The animals received either vehicle (saline) or KBP-089 and/or liraglutide subcutaneously in multiple concentrations and combinations, and food intake was monitored 4, 24, 48, and 72 h postinjection.

Chronic In Vivo Studies

Dosing once daily vs. every other day. To assess daily dosing vs. dosing every other day, male HFD rats (18 wk of age) were randomly assigned into three treatment groups (n = 5–6) and received either vehicle (saline) or KBP-089 (5 μg/kg) once daily (s.i.d.) or every other day (q.a.d.) for 15 days. Food intake and body weight was daily monitored. On *days 0* and *16*, a pharmacokinetic (PK) profile was conducted. The rats received a single injection of KBP-089 (5 μg/kg), and the amount of KBP was measured in plasma samples obtained 0, 10, 20, 40, 60, 120, and 240 min after dosing in an in-house ELISA as previously described (12).

KBP-089 tolerability in HFD rats. One hundred age-matched male SD rats (50 HFD and 50 ND, 18 wk of age) were assigned into treatment groups and normalized according to body weight (n = 10), and a four-step dose escalation of KBP-089 (0.625, 2.5, 10, and 40 μg/kg) was applied, followed by 6 wk of treatment with each group's final dose, as follows. All four treatment groups received 0.625 μg/kg KBP-089 from study start; one group continued 0.625 μg/kg treatment throughout the study. Three of the treatment groups receiving 0.625 μg/kg KBP-089 were escalated to 2.5 μg/kg at *day 7*; one group continued 2.5 μg/kg treatment throughout the study. Two of the treatment groups receiving 2.5 μg/kg KBP-089 were escalated to 10 μg/kg at *day 21*; one group continued 10 μg/kg treatment throughout the study. One of the treatment groups receiving 10 μg/kg KBP-089 was escalated to 40 μg/kg at *day 35* and continued 40 μg/kg treatment throughout the study. Food intake and body weight were monitored daily through the escalation period and once weekly during the treatment period. An oral glucose tolerance test (OGTT) was performed 3 wk into the treatment period.

In addition, we did a similar dose-escalation study with smaller escalations of KBP-089 in male ND rats (n = 10 rats/treatment group, 16 wk of age). These rats were escalated either once weekly (0.625, 1.25, 2.5, 5, and 10 μg/kg; E1) at *days 0*, *7*, *14*, *21*, and *28* or twice weekly (0.625, 0.94, 1.25, 1.88, 2.5, 3.75, 5, 7.5, and 10 μg/kg; E2) at *days 0*, *4*, *7*, *11*, *14*, *18*, *21*, *25*, and *28* with daily monitoring of food and body weight.

KBP-089 and liraglutide in HFD rats. Seventy HFD rats (18 wk of age) were assigned into treatment groups according to body weight (n = 9–10 rats/treatment group). The rats received suboptimal doses of KBP-089 (0.625 and 1.25 μg/kg sc), liraglutide (25 and 50 μg/kg), and their combinations (0.625 + 25 μg/kg and 1.25 + 50 μg/kg) and vehicle for 10 wk. Body weight was monitored daily, and after 3 and 8 wk of treatment, OGTTs were performed. At study end, animals were euthanized (anesthetized by inhalation of isoflurane), followed by exsanguination and dissection. Epididymal, retroperitoneal, and subcutaneous inguinal fat were surgically removed and weighed.

Glucose Tolerance Tests

The rats received glucose by oral gavage (2 g/kg). EDTA plasma samples were collected from the tail vein before glucose challenge (0

Table 1. *Amino acid sequence of hCT, sCT, KBP-089, davalintide, and rat amylin*

Name	NH_2-Term. Mod.	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	COOH-Term. Mod.
hCT		C	G	N	L	S	T	C	M	L	G	T	Y	T	Q	D	F	N	K	F	H	T	F	P	Q	T	A	I	G	V	G	A	P						-NH_2
sCT		C	S	N	L	S	T	C	V	L	G	K	L	S	Q	E	L	H	K	L	Q	T	Y	P	R	T	N	T	G	S	G	T	P						-NH_2
KBP-089	AC-	C	S	N	L	S	T	C	M	L	G	R	L	S	Q	D	L	H	R	L	Q	T	Y	P	K	T	D	V	G	A	N	A	P						-NH_2
Davalintide		K	C	N	T	A	T	C	V	L	G	R	L	S	Q	E	L	H	R	L	Q	T	Y	P	R	T	N	T	G	S	N	T	Y						-NH_2
rAMY		K	C	N	T	A	T	C	A	T	Q	R	L	A	N	F	L	V	R	S	S	N	N	L	G	P	V	L	P	P	T	N	V	G	S	N	T	Y	-NH_2

Amino acid sequences of human calcitonin (hCT), salmon calcitonin (sCT), KBP-089, davalintide, and rat amylin (rAMY) are shown. Modifications in KBP-089 consist of an NH_2-terminal acetyl group and a COOH-terminal amide group.

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min) in both tests and at 15, 30, 60, and 120 min after glucose challenge.

Biochemical Analysis

Blood samples were collected in EDTA tubes and centrifuged at 5,000 rpm for 10 min at 4°C. Blood glucose was monitored using the Accu-Check Avia monitoring system (Roche Diagnostics, Rotkreuz, Switzerland). Plasma levels of insulin (Mercodia rat insulin ELISA; Mercodia, Uppsala, Sweden) were analyzed according to the manufacturer's instructions.

Statistical Analysis

All figure data are means ± SE. Group differences were assessed using analysis of variance followed by post hoc Dunnet's (compared with vehicle) or Tukey's multiple comparison test. Lean age-matched controls were compared with HFD vehicle using Student's *t*-test. All analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA). Concentration-response curves were fitted in GraphPad Prism using the variable slope (four parameter) setting with least-squares fit. EC_{50} values were determined in individual experiments, and the average of

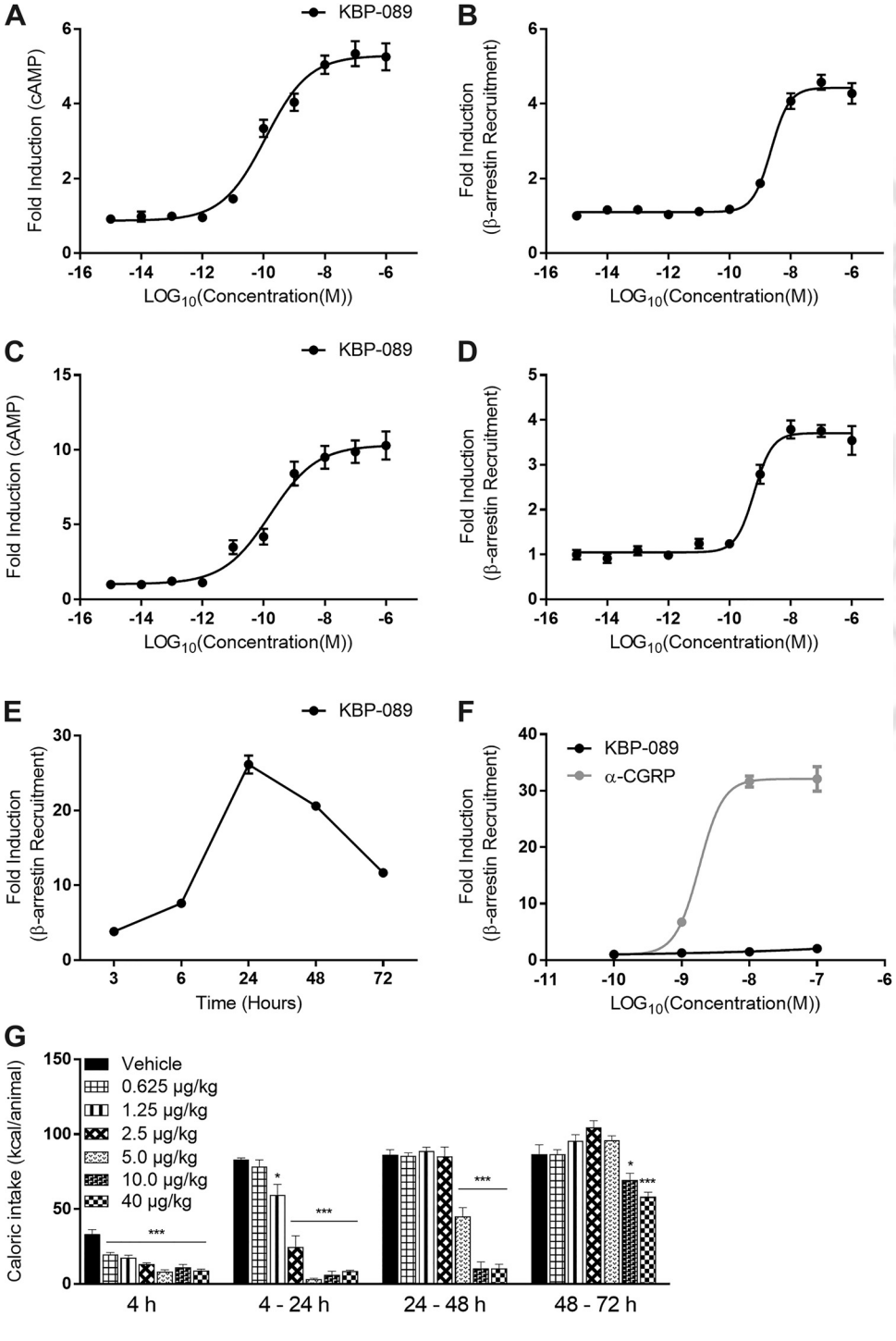

Fig. 1. *A–D*: Concentration-response curves of KBP-089 for cAMP (*A*) and β-arrestin induction (*B*) in calcitonin receptor (CTR)-expressing cell line or cAMP (*C*) and β-arrestin induction (*D*) in amylin receptor (AMY3-R)-expressing cell line. *E*: prolonged CTR-specific β-arrestin response mediated by 100 nM KBP-089 in CTR-expressing cells for 4–72 h. *F*: β-arrestin induction in CGRP receptor-expressing cell line. For *A–F*, pooled data are from 3–4 independent experiments. *G*: effect of a single subcutaneous KBP-089 injection on caloric intake ($n = 6–8$ rats per group). $^{*}P < 0.05$; $^{***}P < 0.001$ compared with vehicle.

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Table 2. *Amylin and calcitonin receptor pEC_{50} values for KBP-089 and sCT*

	CTR		AMY3-R	
	cAMP	β-Arrestin	cAMP	β-Arrestin
Ligand	pEC_{50}	pEC_{50}	pEC_{50}	pEC_{50}
Salmon calcitonin‡	9.8 ± 0.1 (3)	8.2 ± 0.0 (3)	8.8 ± 0.3 (3)	8.7 ± 0.1 (3)
KBP-089	9.9 ± 0.6 (3)^{ns}	8.7 ± 0.1 (3)†	9.9 ± 0.4 (3)*	9.2 ± 0.1 (3)†
Fold difference (EC_{50} KBP-089/EC_{50} sCT)	1.3	3.0	10.4	2.9

pEC_{50} values for cAMP production and β-arrestin recruitment were determined for each individual experiment, and data are means ± SD of individual experiments. Values in parentheses are the number of individual experiments performed in this study. All parameters were measured in cells expressing human CTR or human AMY3-R. *$P < 0.05$; †$P < 0.001$, KBP-089 compared with sCT (ns, not significantly different). ‡Previously published (Andreassen et al. 2014).

means ± SD was used to calculate the pEC_{50}. A value of $P < 0.05$ was considered statistically significant.

RESULTS

KBP-089 Induces Prolonged Receptor Activation In Vitro

KBP-089 is a novel DACRA, and the sequence can be found in Table 1 with the sequences of related peptides. The receptor activation profile corresponds to that in previous publications, with salmon calcitonin (sCT) being the most potent ligand on both receptors and human calcitonin and amylin being selective for their corresponding receptors (2, 9, 28). The ability of KBP-089 to activate the calcitonin- and amylin receptors was assessed by cAMP production and β-arrestin recruitment (Fig. 1, *A–D*), and corresponding pEC_{50} values were determined (see Table 2). pEC_{50} values for KBP-089 for β-arrestin on both CTa and AMY3a were significantly higher compared with the sCT pEC_{50} value, and in addition, the KBP-089 pEC_{50} value for cAMP on AMY3a was markedly higher than the sCT pEC_{50} value. Furthermore, an important aspect of the DACRAs is their prolonged interaction with the CTa (2). To address this, we tested prolonged receptor activation with KBP-089, and as shown in Fig. 1*E*, KBP-089 induced a potent prolonged receptor activation with activation still observed after 72 h, in line with other DACRAs (1, 9). Finally, we tested the response on the CGRP receptor, and even at high concentrations, KBP-089 did not induce β-arrestin recruitment (Fig. 1*F*).

KBP-089 Potently Attenuates Acute Food Intake

A single dose of KBP-089 dose-dependently reduced caloric intake (Fig. 1*G*). Four hours postinjection, all concentrations of KBP-089 significantly suppressed caloric intake, and at 24 h a similar suppression, except from the KBP-089 0.625 μg/kg dose, was observed. At 48 h postinjection, KBP-089 still suppressed food intake with 5, 10, and 40 μg/kg doses (40 μg/kg ~28% of vehicle intake, $P < 0.001$). Interestingly, a single injection of 10 and 40 μg/kg KBP-089 markedly suppressed caloric consumption in rats. Furthermore, in terms of exposure, no difference between first and last dose was observed.

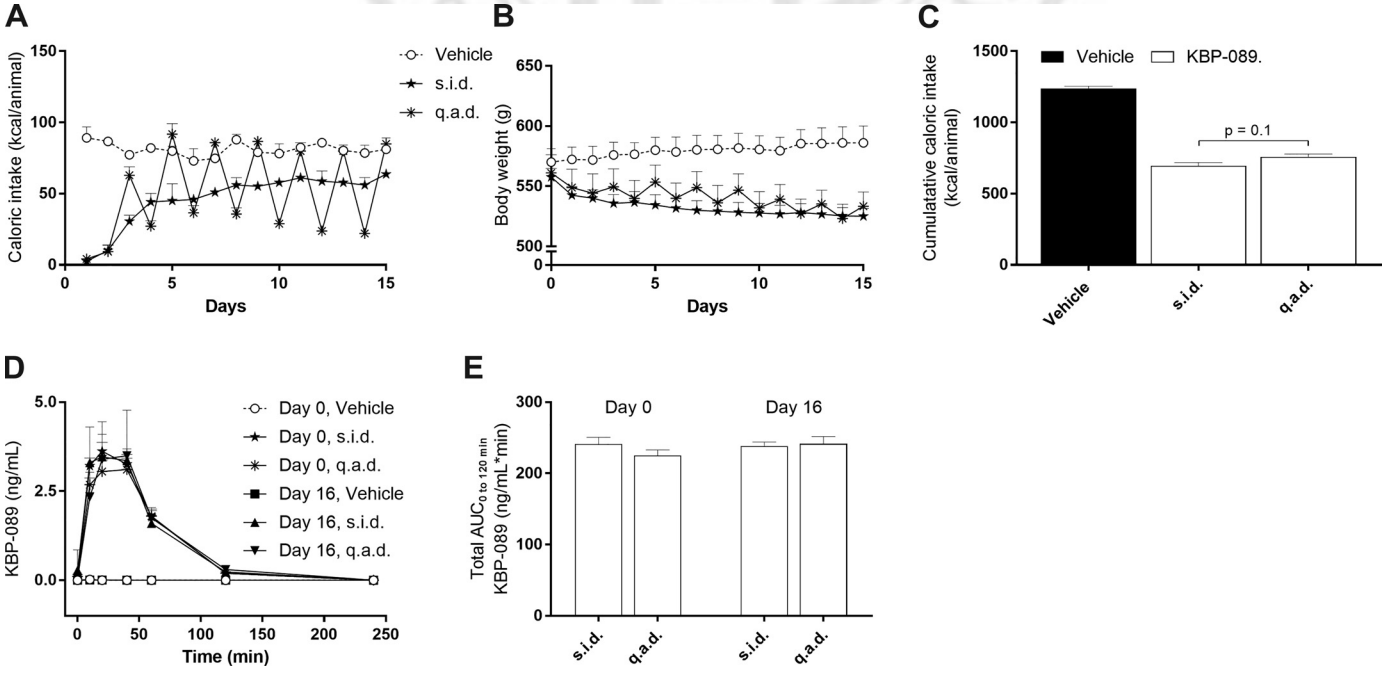

Fig. 2. *A–C*: daily caloric intake (*A*), body weight (*B*), and cumulative caloric intake (*C*) in high-fat diet-fed rats dosed with KBP-089 (5 μg/kg) s.i.d. or q.a.d. for 15 days (n = 5–6 rats per group). *D* and *E*: corresponding PK profiles of KBP-089 (s.i.d. and q.a.d) at baseline (*D*) and at study end after 16 days (*E*). All data are means ± SE.

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KBP-089 Efficacy is Independent of Pharmacokinetic Profile

To assess KBP-089 tolerance, HFD rats were treated for 15 days with KBP-089 (5 μg/kg) either s.i.d. or q.a.d. Expectedly, KBP-089 transiently suppressed food intake in the group dosed once daily. The animals dosed every other day had a markedly suppressed food intake on dosing days; however, the food intake was increased on nondosing days (Fig. 2*A*). Concomitantly, body weight was evenly reduced by KBP-089 s.i.d, whereas KBP-089 q.a.d. had a more uneven weight loss, albeit resulting in equal body weight reduction (Fig. 2*B*). Cumulative food consumption was slightly reduced in s.i.d. dosed rats compared with q.a.d. dosed rats (Fig. 2*C*).

To determine pharmacokinetics of KBP-089, we performed a PK study before study start and after 15 days of KBP-089 (5 μg/kg) treatment (Fig. 2*D*). The plasma concentration of KBP-089 increased immediately after dosing (T_{max} was between 20 and 50 min), and KBP-089 was observed in plasma for 80 min. $T_{½}$ was ~1 h, and after 120 min, KBP-089 was cleared from

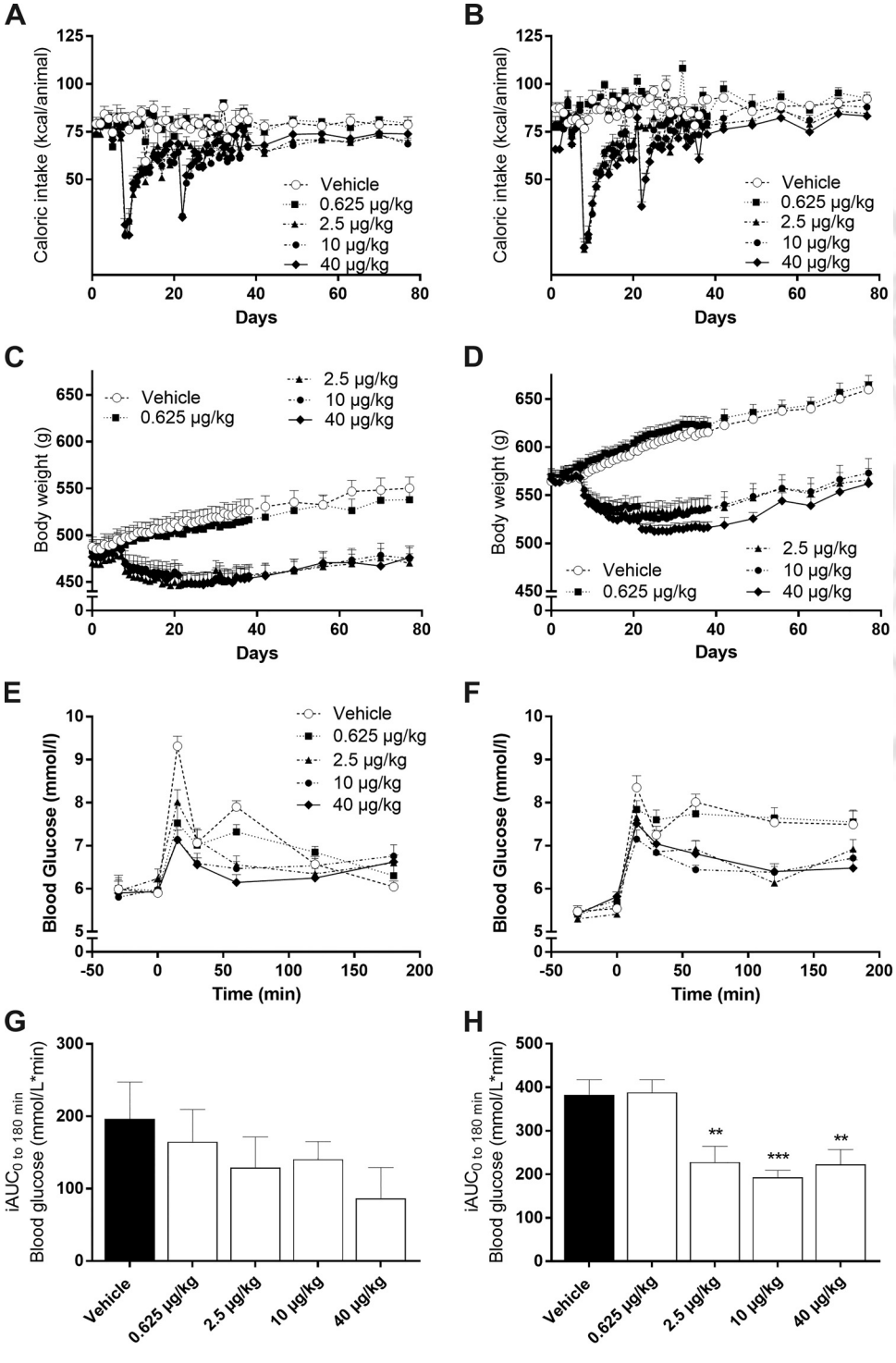

Fig. 3. *A–D*: daily caloric intake (*A* and *B*) and body weight (*C* and *D*) during the escalation period and the treatment period in four-step dose-escalated lean and high-fat diet-fed rats, respectively. *E–H*: corresponding plasma glucose levels during oral glucose tolerance test (*E* and *F*) and incremental area under the curve (iAUC) values for glucose (*G* and *H*) ($n = 10$ rats per group). $^{**}P < 0.01$; $^{***}P < 0.001$, compared with vehicle. All data are means ± SE.

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plasma. There was no difference between values for area under the curve (AUC; Fig. 2*E*) on *day 1* and *day 15*, indicating that KBP-089 does not accumulate even with repeated exposure.

KBP-089 is Tolerable in High Concentrations After Dose Escalation

Amylin receptor and GLP-1 receptor agonists are known to evoke nausea and vomiting (5, 10), a phenomenon handled clinically using dose escalation (15, 17). Furthermore, high doses of DACRA therapy were shown to induce kaolin ingestion, an indication of an adverse gastrointestinal (GI) response to therapy (11). In the current study, we determined whether dose-escalating KBP-089 therapy led to weight loss with a more "modest" suppression of food intake, i.e., a more tolerable profile.

A 4-fold dose escalation followed by 6 wk of treatment (0.625, 2.5, 10, and 40 μg/kg) in HFD and ND rats (565 ± 7 vs. 488 ± 11 g, $P < 0.001$) was applied. Dose escalation induced a transient reduction in food intake at every escalation step in ND (Fig. 3*A*) and HFD rats (Fig. 3*B*), but with attenuated duration and magnitude over time compared with those not escalated. The following 6 wk of treatment with 2.5, 10, and 40 μg/kg resulted in an ~15% vehicle-corrected weight loss in ND (Fig. 3*C*) and HFD rats (Fig. 3*D*) and a corresponding reduction in overall adipose tissue (data not shown). Food efficiency was calculated on the basis of food intake and body weight change, and as previously observed, food efficiency was decreased by DACRA treatment (9, 11) (data not shown). Moreover, all treatment groups showed improved oral glucose tolerance (ND rats, Fig. 3*E*; HFD rats, Fig. 3*F*), resulting in significantly lowered ($P < 0.01$) incremental AUC values (ND rats, Fig. 3*G*; HFD rats, Fig. 3*H*).

As was evident from the previous study, fourfold concentration increments still led to substantial suppression of food intake. Accordingly, we switched to twofold escalations (0.625, 1.25, 2.5, 5, and 10 μg/kg; E1) once weekly and linear escalations (0.625, 0.94, 1.25, 1.88, 2.5, 3.75, 5, 7.5, and 10 μg/kg; E2) twice weekly, and as shown in Fig. 4*A*, smaller dose escalations were associated with a lower reduction in food intake at the indicated increases in dose level. Furthermore, the cumulative reduction in food intake was larger in the group without escalation than in the two escalated groups (Fig. 4*B*). Importantly, weight loss following dose escalation reached the same magnitude as that without escalation, albeit the time to get to maximum weight loss was prolonged (Fig. 4*C*). Expectedly, the food efficiency was lowered in all therapy groups (Fig. 4*D*).

KBP-089 Acts Complementarily with GLP-1 on Food Intake, Body Weight, and Glucose Tolerance

To assess whether KBP-089 acts in combination with GLP-1 on food intake and body weight, we did acute food intake studies with KBP-089 and GLP-1 analog, liraglutide, and a long-term treatment study in HFD rats. The effect of a single subcutaneous injection of KBP-089, liraglutide, and their combinations were tested on the cumulative 72-h caloric intake using multiple concentrations (Fig. 5*A*). A single injection of liraglutide (100, 200, and 400 μg/kg) significantly suppressed the caloric consumption compared with vehicle, whereas the lower concentrations of liraglutide (25 and 50 μg/kg) were not able to change the intake. Concomitantly, the high concentrations of KBP-089 (2.5, 5, and 10 μg/kg) were able to induce a significant reduction in the 72-h caloric intake compared with vehicle, whereas the low concentrations of KBP-089 (0.625 and 1.25 μg/kg) were unable to attenuate 72-h caloric intake. Interestingly, all the combinations used were able to induce a marked suppression in the amount of calories consumed over 72 h compared with intake by vehicle rats.

To assess whether KBP-089 acts complementarily with liraglutide, low doses of liraglutide and KBP-089 were used alone or in combination for 10 wk. On the basis of effects

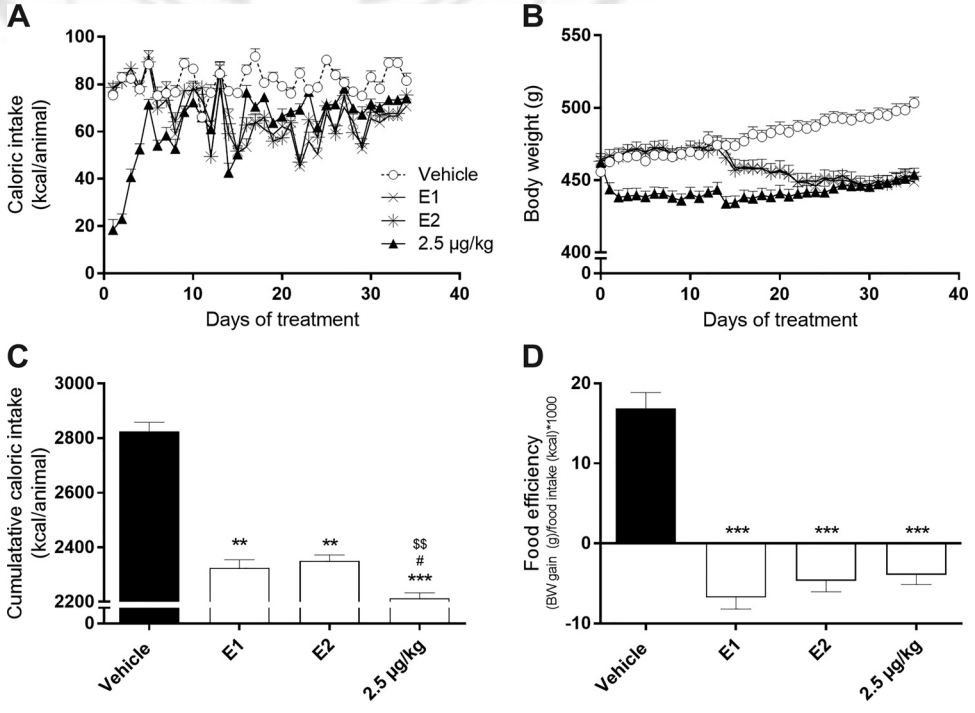

Fig. 4. *A* and *B*: daily caloric intake (*A*) and body weight (*B*) during the escalation period in 2-fold escalations (0.625, 1.25, 2.5, 5, and 10 μg/kg; E1) once weekly at *days 0*, *7*, *14*, *21*, and *28* and linear escalations (0.625, 0.94, 1.25, 1.88, 2.5, 3.75, 5, 7.5, and 10 μg/kg; E2) twice weekly at *days 0*, *4*, *7*, *11*, *14*, *18*, *21*, *25*, and *28* in lean rats fed a normal diet. *C* and *D*: cumulative caloric intake (*C*) and food efficiency (*D*) during the escalation period ($n = 10$ rats per group). $^{**}P < 0.01$; $^{***}P < 0.001$ compared with vehicle. #$P < 0.05$ compared with E1. \$\$$P < 0.01$ compared with E2. All data are means ± SE.

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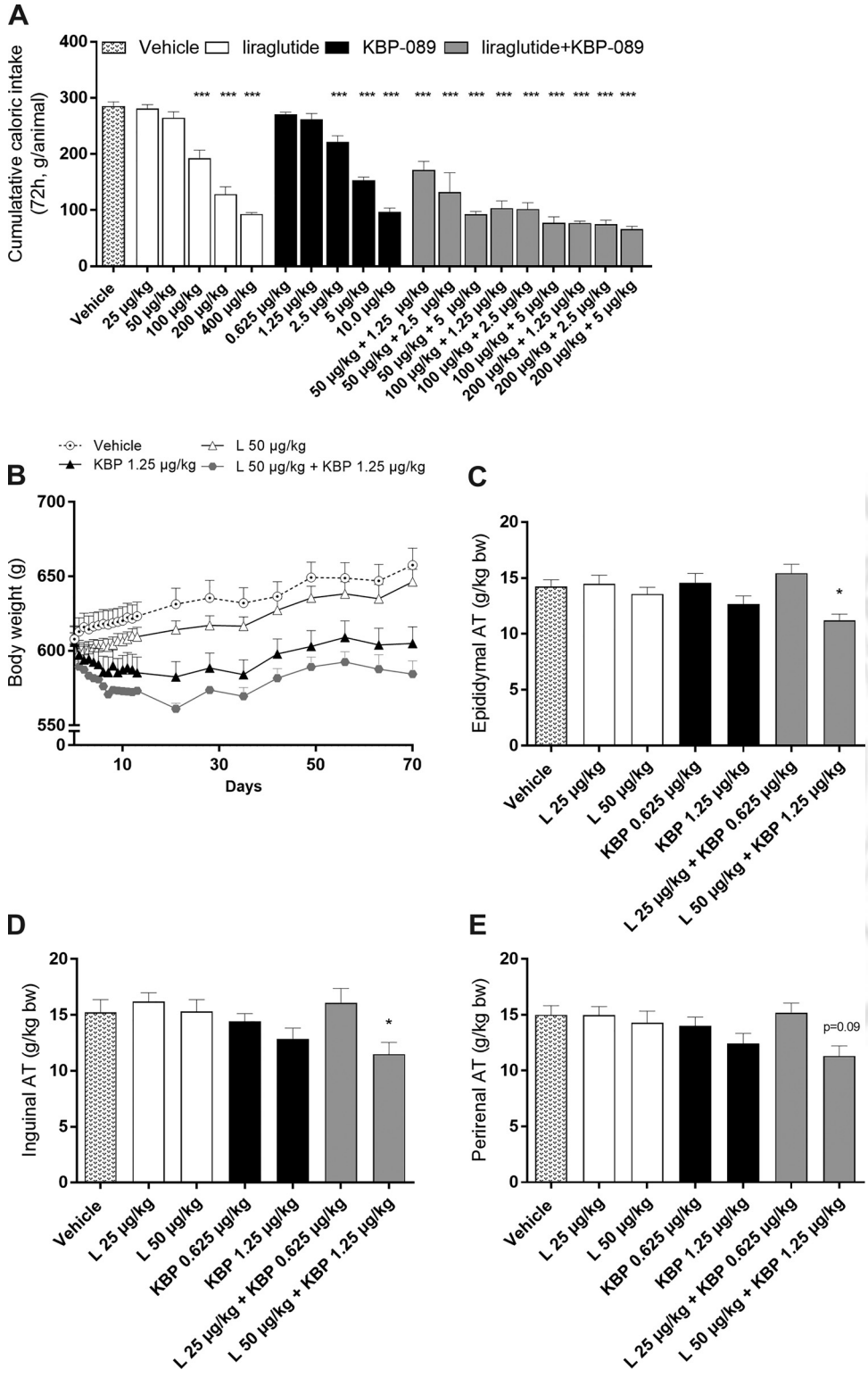

Fig. 5. *A*: cumulative caloric intake (72 h) after a single dose of liraglutide, KBP-089, or combinations of liraglutide and KBP-089. *B*: body weight during treatment period. *C–E*: weight of epididymal (*C*), inguinal (*D*), and perirenal adipose tissue (AT; *E*) at study end ($n = 8–10$ rats per group). $^{*}P < 0.05$; $^{***}P < 0.001$ compared with vehicle. All data are means ± SE.

observed in Fig. 5*A*, we chose the suboptimal doses for the chronic study. Ten weeks of treatment with low-dose liraglutide (50 μg/kg) and KBP-089 (1.25 μg/kg) resulted in a 2% and 8% vehicle-corrected weight loss, respectively, whereas the combination resulted in a 12% vehicle-corrected body weight reduction (Fig. 5*B*). Concomitantly, only a combination of liraglutide and KBP-089 (50 μg/kg liraglutide and 1.25 μg/kg KBP-089) was able to lower overall adiposity, resulting in a significant reduction in epididymal, inguinal, and perirenal adipose tissue (Fig. 5, *C–E*). Moreover, this combination improved oral glucose tolerance after 3 (Fig. 6, *A* and *C*) and 8 wk (Fig. 6, *B* and *D*) of treatment. Insulin levels were lowered in rats treated with low concentrations of KBP-089 and slightly potentiated in rats treated with low concentrations of liraglutide

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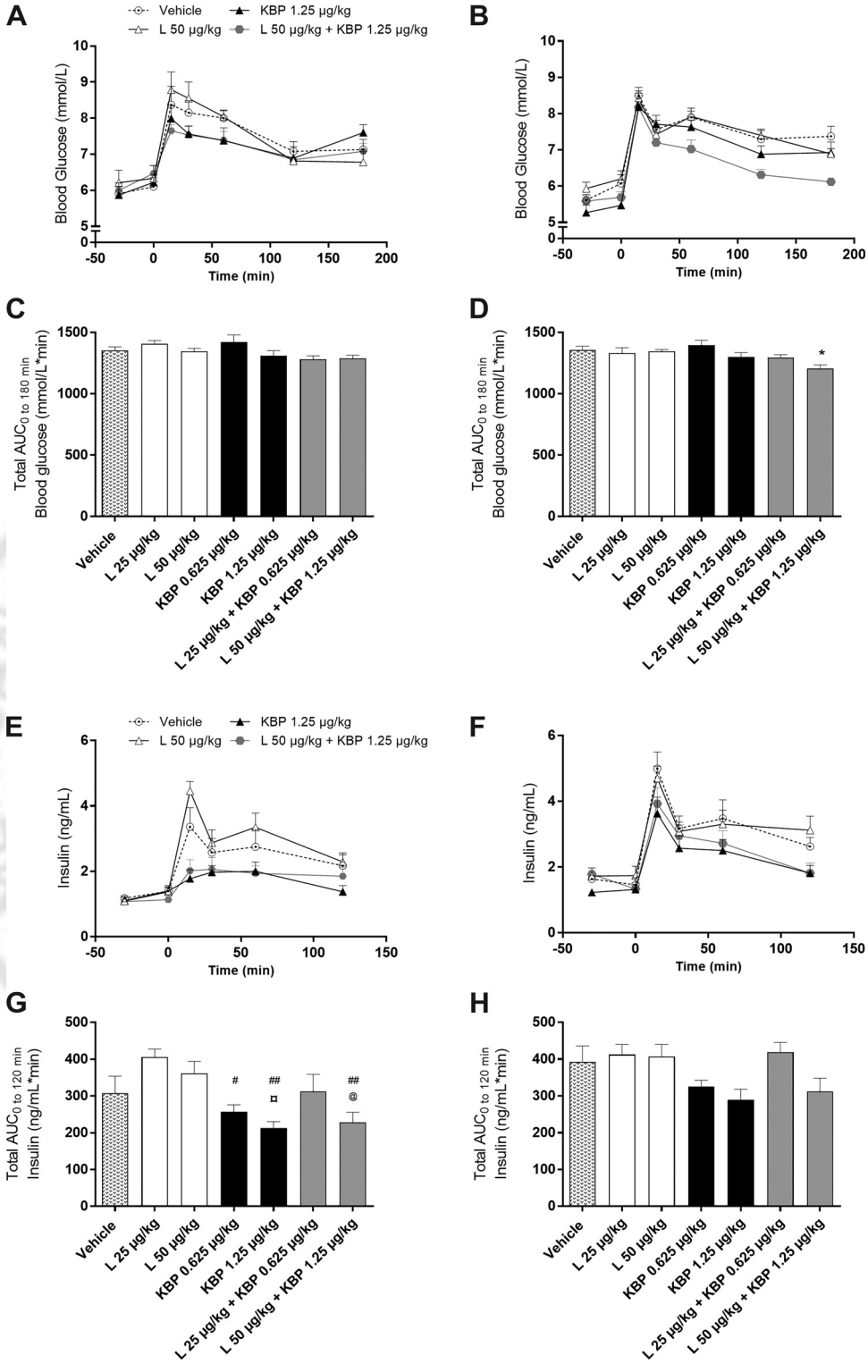

Fig. 6. Plasma glucose (*A* and *B*) and insulin levels (*E* and *F*) during oral glucose tolerance test (OGTT) after 3 and 8 wk, respectively, in high-fat diet-fed rats dosed with liraglutide (50 μg/kg), KBP-089 (1.25 μg/kg), or a combination of liraglutide and KBP-089 (50 and 1.25 μg/kg, respectively) for 10 wk. Total area under the curve (AUC) is shown for glucose (*C* and *D*) and insulin levels (*G* and *H*) during OGTT after 3 and 8 wk, respectively, in all treatment groups (liraglutide, 25 and 50 μg/kg; KBP-089, 0.625 and 1.25 μg/kg; or combinations of liraglutide and KBP-089, 25 + 0.625 μg/kg and 50 + 1.25 μg/kg, respectively; $n = 8–10$ rats per group). $^*P < 0.05$ compared with vehicle. $\#P < 0.05$; $\#\#P < 0.01$ compared with 25 μg/kg liraglutide. $¤P < 0.05$; $@P = 0.07$ compared with 50 μg/kg liraglutide. All data are means ± SE.

after 3 wk (Fig. 6*E*) of treatment, resulting in significantly different AUC values in KBP-089- and liraglutide-treated rats (Fig. 6*G*). The combination of the two insulin-opposing treatments (50 μg/kg liraglutide and 1.25 μg/kg KBP-089) resulted in an intermediate insulin level that was not significantly different from that in vehicle-treated rats; however, it was significantly lower compared with that in liraglutide-treated rats (Fig. 6*G*). Insulin levels during the OGTT performed after 8 wk of treatment with KBP-089 showed a reducing trend (Fig. 6*F*), albeit AUC was not significantly different from vehicle (Fig. 6*H*). A similar trend was observed in the combination (50 μg/kg liraglutide and 1.25 μg/kg KBP-089), whereas insulin levels in obese rats treated with low-dose liraglutide were not different from those in rats treated with vehicle.

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DISCUSSION

In this article we present data on the novel dual amylin and calcitonin receptor agonist (DACRA) KBP-089. Our data show that KBP-089 is a highly potent DACRA, based on activation of the AMY3-R and the calcitonin receptor, with no activation of the CGRP receptor, and importantly, KBP-089 possesses the ability to induced prolonged receptor activation, a trait known to be crucial for in vivo activity of other members of the DACRA family (9).

The in vitro investigation was limited to AMY3-R, and investigations into AMY1-R and AMY2-R activation are warranted to provide important pharmacological information due to differences in receptor subtype off-target activation by CGRP (21). However, the in vivo data presented in this article suggests that KBP-089 does not have an AMY1-R activation profile that is significantly different from that for AMY3-R or that such a difference is not important for the observed pharmacological effect. A recent study of rodent area postrema neurons suggests that the different subtypes are coexpressed in the same individual neurons (18); hence, no AMY-R subtype appears be to predominant, or tissue specific, and could be indicative of amylin receptor expression in other AMY-R relevant tissues.

The in vitro efficacy indeed translated to in vivo efficacy, as shown by the massive suppression of food intake following a single injection of KBP-089. Interestingly, on this parameter KBP-089 was more potent than previously reported DACRAs (9, 11), as well as other previously described amylin-receptor agonists such as pramlintide and davalintide (19, 33), and the suppression clearly exceeded a 24-h time frame, potentially indicating that less frequent dosing could be feasible. To address this question, we compared chronic dosing using 5 μg/kg s.i.d. or 5 μg/kg q.a.d. KBP-089, and importantly, both dose regimens led to similar weight loss during the 15-day study; however, in the q.a.d. arm, notable fluctuations in food intake and body weight were observed, likely resulting in a challenge in terms of tolerability.

Peptides with known anorectic effects, such as amylin and GLP-1 analogs, are associated with GI tolerability problems (3, 13), and we speculate that the massive suppression of food intake following initial dosing is indicative of adverse effects on the GI tract, as also supported by kaolin intake at high doses of KBP-042 (11). To investigate whether a similar weight loss, albeit with a less pronounced suppression of appetite, could be obtained, we mimicked the clinical situation for pramlintide and GLP-1 analogs and used dose escalation (3, 17). In these studies, we found that dose escalation resulted in a similar magnitude of weight loss, with lower suppression of appetite; however, the time before maximum weight loss was prolonged. Importantly, the increments of the dose escalation are very important, because over-large increments led to substantial suppression of food intake. These data correlate well with what was expected from the literature and the dose escalation of anorectic peptides (3, 17). These data indicate that dose-escalation strategies can be tested in rat models using appetite suppression and weight regulation as the output, and clearly indicate that dose escalation is a good option for increasing tolerability of this type of molecule.

Finally, because GLP-1 analogs and amylin receptor agonists are thought to work, at least on the appetite regulation, through a similar mechanism of action (13), we studied the potential combination of KBP-089 with liraglutide. Both classes of molecules exhibit weight-lowering effects, and previously, combinations of GLP-1 and amylin agonism have shown synergistic reductions in food intake in nonhuman primates (6), and peptide hybrids composed of an exenatide analog and davalintide have shown beneficial effects body weight and glucose control in obese rats (27, 29). Hence, the combination of GLP-1 and amylin therapies for the treatment of metabolic disease has been intensively discussed (23), because combination-based therapies are increasing in this area of disorders. We found a good effect on appetite suppression when combining the two peptides, resulting in efficacy observed at doses considered virtually ineffective when given as stand-alone doses. Importantly, this manifested in efficacy on body weight, as well.

In conclusion, KBP-089 is a novel, promising DACRA with a substantial potency for control of metabolic parameters, and dose-escalation strategies are implemented, it is highly likely that the expected GI events can be overcome. Finally, KBP-089 acts complementarily with GLP-1, indicating the potential for an add-on therapy causing additional weight loss.

GRANTS

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DISCLOSURES

M. A. Karsdal and K. Henriksen own stock in Nordic Bioscience. All other authors disclose no conflict of interest.

AUTHOR CONTRIBUTIONS

S.G. and K.H. conceived and designed research; S.G. and K.V.A. performed experiments; S.G. and K.V.A. analyzed data; S.G. and K.V.A. interpreted results of experiments; S.G. prepared figures; S.G. and K.H. drafted manuscript; S.G., S.T.H., L.I.H., M.A.K., and K.H. edited and revised manuscript; S.G., K.V.A., S.T.H., L.I.H., M.A.K., and K.H. approved final version of manuscript.

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Paper V:

The Dual Amylin and Calcitonin Receptor Agonist (DACRA), KBP-089, Improves Metabolic and Hepatic Features of Nonalcoholic Steatohepatitis in High Fat, High Cholesterol Fed Rats

The Dual Amylin and Calcitonin Receptor Agonist (DACRA), KBP-089, Improves Metabolic and Hepatic Features of Nonalcoholic Steatohepatitis in High Fat, High Cholesterol Fed Rats.

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Abstract

Obesity and non-alcoholic fatty liver disease (NAFLD) are the most common causes of nonalcoholic steatohepatitis (NASH) and subsequently chronic liver disease, such as fibrosis. While no treatments are approved, weight loss and insulin sensitizers have shown promise on metabolic and hepatic parameters; hence, drugs causing weight loss and alleviating insulin resistance are highly interesting as candidates for treatment of NAFLD and NASH.

We started rats on high fat diet (HFD) for 8 weeks to induce obesity followed by a high fat, high cholesterol and cholate diet (HFCC) for 56 days to induce NASH. After HFD, the rats were assigned into treatment groups receiving either vehicle (saline) or escalated to 0.625, 1.25, 2.5 and 5.0 μg/kg KBP-089.

KBP-089 induced and sustained a significant 16.5% vehicle-corrected weight loss, reduced overall adiposity and enhanced insulin action during and oral glucose tolerance test. Furthermore, KBP-089 dose-dependently reduced the HFCC diet induced hepatomegaly and reduced circulating levels of triglycerides and AST. At the histological level, KBP-089 impressively reduced both the combined NAFLD activity score and HFCC induced fibrosis stage.

In conclusion, KBP-089 is a weight reducing agent that is well tolerated when introduced by dose escalation. Importantly, KBP-089 improves metabolic and hepatic features of NASH in a human like NASH model system, hence revealing the potential of KBP-089 as a therapeutic target in the treatment of NASH.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is considered the hepatic manifestation of the metabolic syndrome and is increasingly becoming common in parallel with the increasing prevalence of obesity[83,152]. NAFLD is characterized by excessive fat accumulation in the liver in the absence of excessive alcohol consumption or any other specific causes of hepatic steatosis[82] – a lipid accumulation above 5% of the liver weight is classified as NAFLD[83]. When the balance between lipid uptake and utilization is abrogated, lipids accumulate in the liver. NAFLD encompasses a variety of liver pathologies with different clinical manifestations, extending from simple lipid accumulation in the hepatocytes to nonalcoholic steatohepatitis (NASH) with intralobular inflammation, hepatocellular ballooning and fibrosis[100,288,289], and is projected to be the leading cause of liver transplants in the future[153]. Presently, there are no approved pharmacological treatments for NASH.

Lifestyle changes focusing on healthy eating, weight loss and regular exercise is a cornerstone in NAFLD therapy in adults[154–156] and children[157], and bariatric surgery has been shown to reverse NASH and even substantial fibrosis[158,159]. Surgery however is only performed in a minority of the patients and is associated with peri- and postoperative hazards, hence there is clearly a need for pharmacological therapies to treat NASH[160,161]. Consequently, insulin sensitizers such as the PPAR-γ agonists pioglitazone[109,110] and rosiglitazone[111] have been introduced as "off-label" treatments for NASH[112,290,291], and while these agents have proven efficacious in patients with NASH – with regards to hepatic histology and glucose control – their use is limited by the conservative beneficial effects and the undesired side effects associated with their use, namely weight gain. Insulin sensitizers, which improve glucose control but also reduces body weight could potentially be a more efficacious treatment, as weight loss is the most important parameter for NASH and a significantly weight loss per se results in resolutions of disease[156].

The therapeutics currently under evaluation in clinical trials are focused on relatively downstream events of liver injury such as inflammation and fibrogenesis – it might be useful to target upstream events such as weight loss, insulin sensitivity, control of satiety and energy efficiency[177,178], hence possibly preventing the prevalence of progression into fibrosis and cirrhosis and targeting the core of MS.

KBP-089 is a dual amylin and calcitonin receptor agonist (DACRA) that activate both the amylin receptor and the calcitonin receptor, and possess superior activity in terms of receptor activation and duration of activation compared to classical amylin receptor agonists[268,269]. Notably, the extended activation of the receptors appears to increase the *in vivo* efficacy[269], thus addressing a major limitation of amylin agonists.

KBP-089 has along with another DACRA called KBP-042 shown anti-obesity potential as well as the ability to reduce liver steatosis in obese rats[274,279]. KBP-089 is associated with transient hypophagia at dosing initiation and activation of receptors in the brain (eg. by

amylin and GLP-1) that induce satiety changes can lead to nausea[292,293]; however, recently it was demonstrated that KBP-089 was well tolerated even in high concentrations when introduced by dose-escalation.

In this study, we evaluate the effects of KBP-089 in a rat model with excessive hepatic lipid accumulations, inflammation and mild fibrosis including a characterization of the KBP-089 effects on a series of metabolic parameters, such as bodyweight and glucose metabolism and importantly liver steatosis, inflammation and fibrosis.

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[269,270,274]

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 (2016-15-0201-00910). Male Sprague Dawley rats (Harlan, Venray, The Netherlands) 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. From arrival to study start, the rats were fed a 60 kcal% fat diet (5.1 kcal/g) (#58Y1, TestDiet, London, UK). 8 weeks post arrival and throughout the study, the rats were fed a 65 kcal% fat (mostly cocoa butter) with 2% cholesterol and 0.5% cholate added (HFCC) (5.30 kcal/g) (#D09052204, Research Diet, New Brunswick, NJ, USA). The rats received food and tap water *ad libitum*.

In vivo study

KBP-089 in high fat, high cholesterol and cholate fed rats.

After 8 weeks of high fat feeding, the rats were randomly assigned into experimental groups according to body weight, ensuring an equal average value of body weight at study start. The rats were dose escalated in four steps (0.625, 1.25, 2.5 and 5.0 µg/kg KBP-089) once weekly at day 1, 7, 14 and 21 followed by 4 weeks of treatment with either dose. All four treatment groups received 0.625 µg/kg from study start – one group continued 0.625 µg/kg treatment throughout the study. Three of the treatment groups receiving 0.625 µg/kg were escalated to 1.25 µg/kg at day 7 – one group continued 1.25 µg/kg treatment. Two of the 1.25 µg/kg groups were escalated to 2.5 µg/kg at day 21 – one group continued 2.5 µg/kg treatment throughout the study and finally, one of the 2.5 µg/kg treatment groups was escalated to 5 µg/kg at day 35 and continued 5 µg/kg treatment throughout the study period. The vehicle group received saline (s.c.). Food intake and body weight were daily monitored through the escalation period and once weekly during the treatment period. We measured aspartate transaminase (AST), alanine transaminase (ALT), and triglyceride levels after the 8 weeks of high fat feeding and at study end, and performed an oral glucose tolerance test (OGTT) 2 weeks into the treatment period.

Glucose tolerance tests

The rats received glucose per oral gavage (p.o.) (2 g/kg) and blood samples were collected from the lateral tail vein prior to the glucose challenge (0 min) and 15, 30, 60, and 120 minutes post glucose challenge in the OGTT.

Liver histology

To address tissue fat accumulation, potential inflammation and fibrosis, livers were surgically removed, fixed in 4% formaldehyde and paraffin embedded. 5µm sections from liver tissue were stained with Sirius Red and Masson's Trichrome, slides were examined under a light microscope, and magnification for each picture is stated. Quantitatively image analysis of steatosis (% of area) was performed using Image J software. Further, the combined NAFLD activity score (NAS) (0-8) composed of a steatosis (0-3), ballooning (0-2) and inflammation (0-3) scores[31] was performed. Two independent individuals scored steatosis, ballooning, inflammation and fibrosis blindly (magnification x20, 8 images per animal; 4 pictures per depth; 5 animals per group).

Biochemical analysis

Blood samples were collected in Hep/Li 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). Plasma levels of insulin (Mercodia Rat Insulin ELISA, Mercodia AB, Uppsala, Sweden) was analysed according to manufacturer's instruction. Alanine transaminase (ALT), aspartate transaminase (AST), and triglycerides (TG) levels were measured in-house (ADVIA® 1800, Siemens, Germany).

Statistical analysis

All data are presented as means ± SEM. The statistical analysis of various drug effects were conducted using one-way ANOVA followed by Dunnets's post test for multiple comparison for parametric data and Kruskal-Wallis test with Dunn's post test for non-parametric data. Comparison between baseline and study end in vehicle rats as well as the difference between vehicle and KBP-089 (5 μg/kg) treated rats with regards to the parameters included in the NALFD activity score (NAS), and fibrosis score were evaluated by 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.

Results

KBP-089 potently reduced body weight and fat depot size

After 8 weeks of high fat feeding, the rats were significantly obese compared to age-matched lean controls (data not shown). Post induction of experimental obesity, the diet was changed to HFCC and the rats assigned to the different treatment groups. KBP-089 treatment was subcutaneously administered and introduced by a four-step dose escalation once weekly followed by treatment with either dose (E0.625, E1.25, E2.5 and E5 μg/kg). Food intake was transiently attenuated by non-escalated 2.5 μg/kg KBP-089 (Figure 1A) as previously observed with DACRAs[269,274,279]. In the escalated groups, a transient reduction in food intake was observed when the rats were escalated to 2.5 μg/kg (Figure 1A), albeit cumulative caloric intake at study end was not significantly different in either of the escalation groups compered to vehicle rats (Figure 1B).

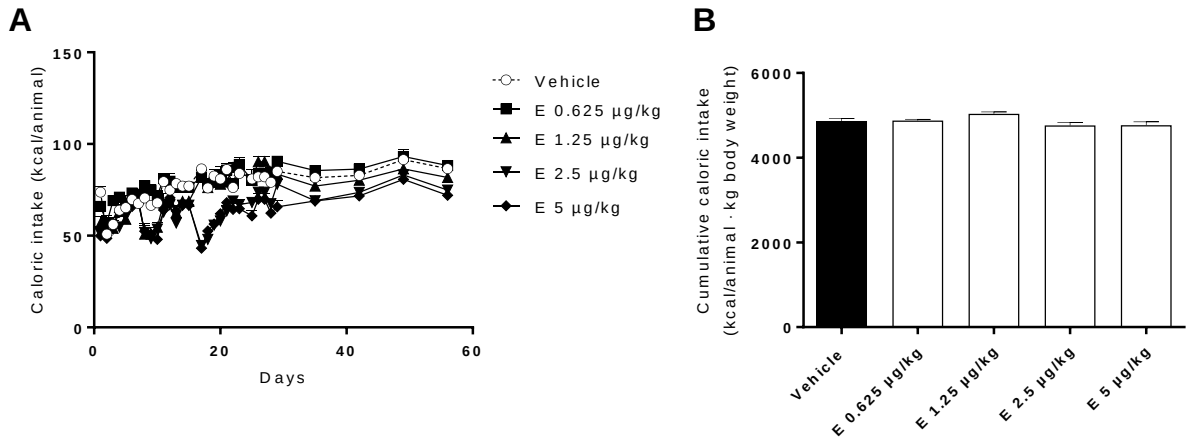

Figure 1: KBP-089 was well tolerated when introduced by dose escalation. KBP-089 treatment was introduced by a four-step dose escalation once weekly followed by treatment with either dose (E0.625, E1.25, E2.5 and E5 µg/kg). (A) Caloric intake monitored daily initially, and then weekly in high fat, high cholesterol and cholate fed rats. Expressed as daily intake per animal. (B) Cumulative caloric intake in rats. (n = 10 rats per group). Statistical analysis between groups was evaluated by an ordinary one-way ANOVA with Dunnet's multiple comparisons test (not significant).

8 weeks of KBP-089 treatment – dose escalation from 0.625 µg/kg and the following 4 weeks of treatment with 1.25, 2.5 and 5 µg/kg KBP-089 (E1.25, E2.5 and E5 µg/kg) resulted in a ~8%, 16% and 17% vehicle-corrected weight loss, respectively, hence significantly attenuating food efficiency (data not shown). At study end, epididymal, inguinal and perirenal fat pads were weighed and in line with the massive reduction in body weight, a corresponding reduction in epididymal (Figure 2B), inguinal (Figure 2C), and perirenal (Figure 2D) adipose tissue compared to vehicle was observed.

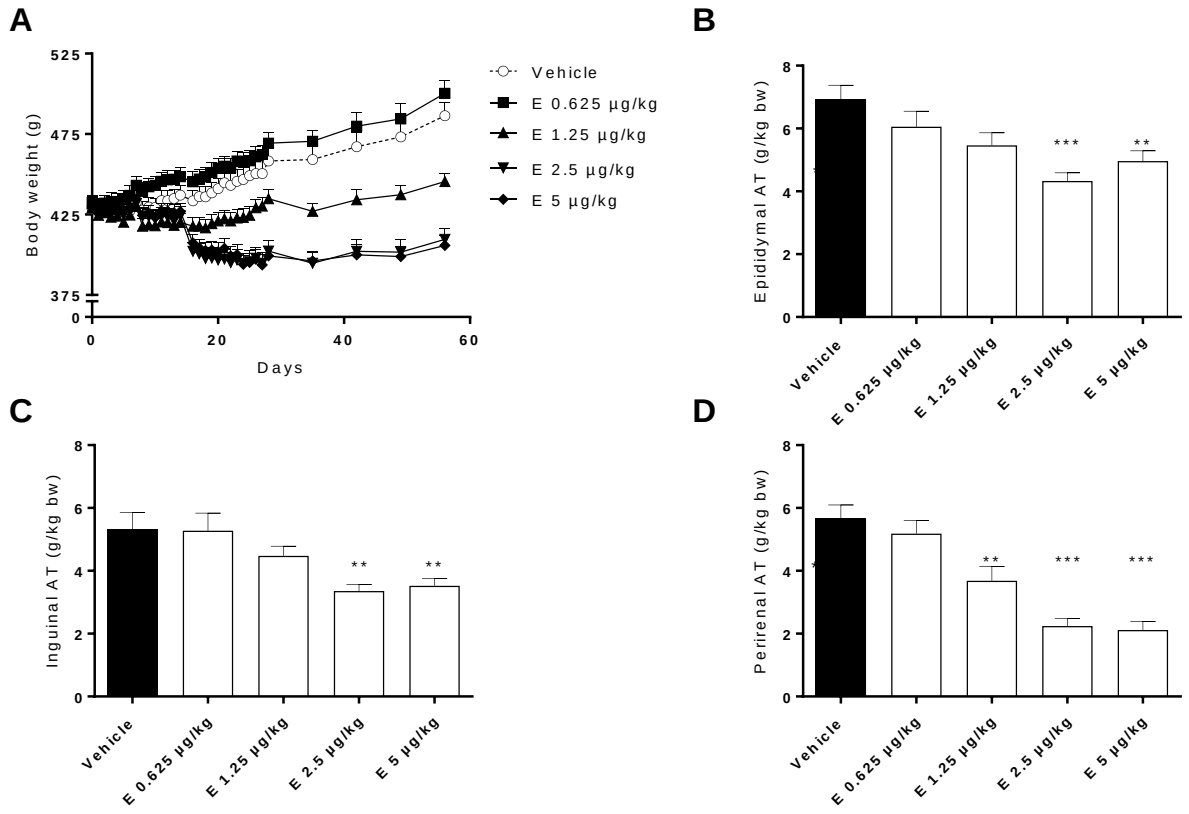

Figure 2: KBP-089 potently reduced body weight and fat depots in HFD rats. KBP-089 treatment was introduced by a four-step dose escalation once weekly followed by treatment with either dose (E0.625, E1.25, E2.5 and E5 µg/kg). (A) Body weight monitored daily initially, and then weekly in high fat, high cholesterol and cholate fed rats. Relative weight of (B) epididymal, (C) inguinal and (D) peritoneal adipose tissue (AT) at study end. (n = 10 rats per group). Statistical analysis between groups was evaluated by an ordinary one-way ANOVA with Dunnet's multiple comparisons test, $p<0.05$. *compared to vehicle.

KBP-089 enhances insulin action

An OGTT was performed after 2 weeks of treatment (Figure 3). All treatment groups showed a trend towards lower blood glucose levels compared to vehicle 15 minutes post glucose challenge (Figure 3A); however, incremental area under the curve (iAUC) was only significantly reduced the highest treatment group when compared to vehicle (Figure 3C). Interestingly, the glucose stimulated insulin secretion observed in vehicle rats was dose-dependently suppressed by KBP-089 during the OGTT (Figure 3B), hence resulting in significantly reduced insulin iAUC values in KBP-089 treated rats (Figure 3D).

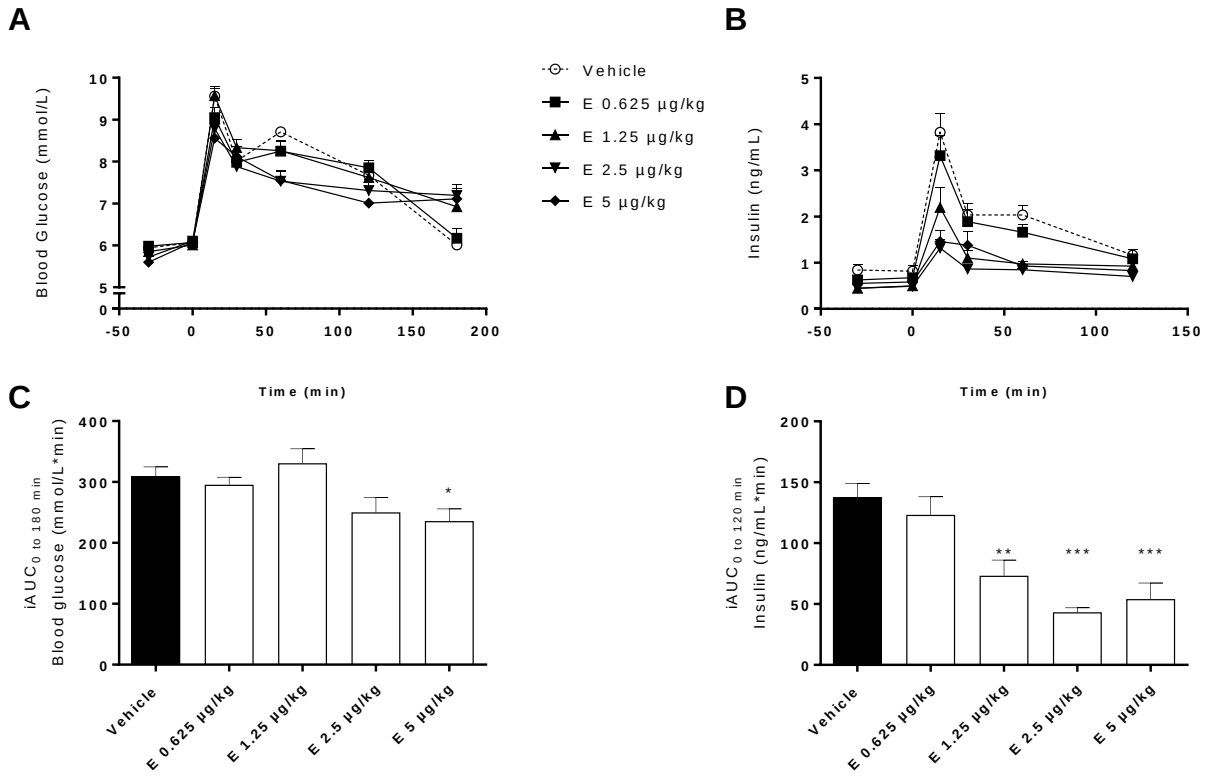

Figure 3: KBP-089 enhances insulin action in HFCC rats. (A) Plasma glucose and (B) insulin during oral glucose tolerance test (OGTT) in high fat, high cholesterol and cholate fed (HFCC) rats treated with four-step dose escalated KBP-089. The rats were escalated once weekly followed by treatment with either dose (E0.625, E1.25, E2.5 and E5 µg/kg). Incremental area under the curve (iAUC) values for (C) glucose and (D) plasma insulin during OGTT after 2 weeks of treatment. (n = 10 rats per group). Statistical analysis between groups was evaluated by an ordinary one-way ANOVA with Dunnet's multiple comparisons test, $p < 0.05$, * compared to vehicle.

KBP-089 lowers circulating levels of aspartate transaminase and triglyceride

Plasma for determination of ALT, AST, and TG levels was collected after 8 weeks of HFD (baseline) and at study end. 8 weeks of HFCC diet increased the circulating levels of AST, ALT, and TG levels in vehicle rats compared to baseline (Table 1).

Biomarker	Baseline	Study end	P value
AST	87.7 ± 2.1 U/L	141.8 ± 12.2 U/L	p<0.001
ALT	47.9 ± 1.5 U/L	146.3 ± 14.6 U/L	p<0.001
TG	0.84 ± 0.04 mmol/L	1.14 ± 0.07 mmol/L	p<0.01

Table 1: Baseline and study end values of alanine transaminase (ALT), aspartate transaminase (AST), triglycerides (TG), total cholesterol, high-density lipoproteins (HDL), and low-density lipoproteins (LDL) in untreated rats fed a high fat, high cholesterol and cholate diet. (n = 10 rats per group). The results are presented as mean ± SEM (n=10). Statistical analysis between baseline and study end was evaluated by Student's t-test.

At study end, the level of aspartate transaminase (AST) in plasma was significantly reduced by KBP-089 treatment (E2.5 µg/kg, $p<0.05$; E5 µg/kg, $p<0.01$ and 2.5 µg/kg, $p<0.05$) (Figure 4A). Similarly, ALT levels showed a lowering trend (Figure 4B), however, the values were not significantly reduced compared to vehicle at study end. The HFCC induced increase in circulation TG was lowered by KBP-089 – significantly in the E2.5 µg/kg group (Figure 4C).

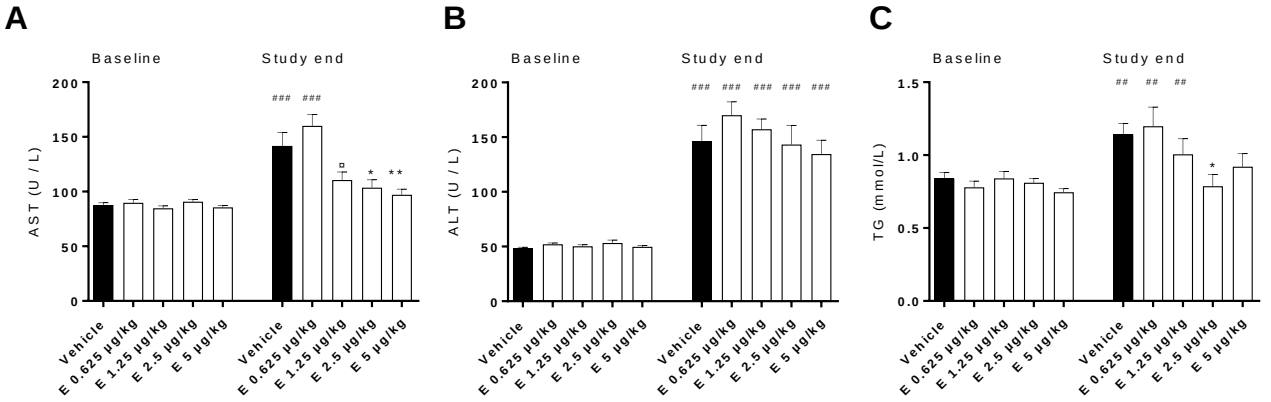

Figure 4: The effect of KBP-089 on aspartate transaminases and triglyceride. Baseline and study end values of (A) aspartate transaminase (AST), (B) alanine transaminase (ALT), and (C) triglycerides (TG). (n = 10 rats per group). Statistical analysis between groups was evaluated by an ordinary one-way ANOVA with Dunnet's multiple comparisons test and difference between baseline and study end by Student's t-test, $p<0.05$. * compared to vehicle, #compared the groups baseline value, ¤$p = 0.06$.

KBP-089 reduces accumulation of lipids in the liver

At study end, hepatic steatosis and ballooning were assessed in liver (Figure 5). As seen in figure 5I, the HFCC diet led to a genuinely dramatic increase in lipid accumulation and ballooning. We calculated the fat content, which was ~23% in the vehicle rats.

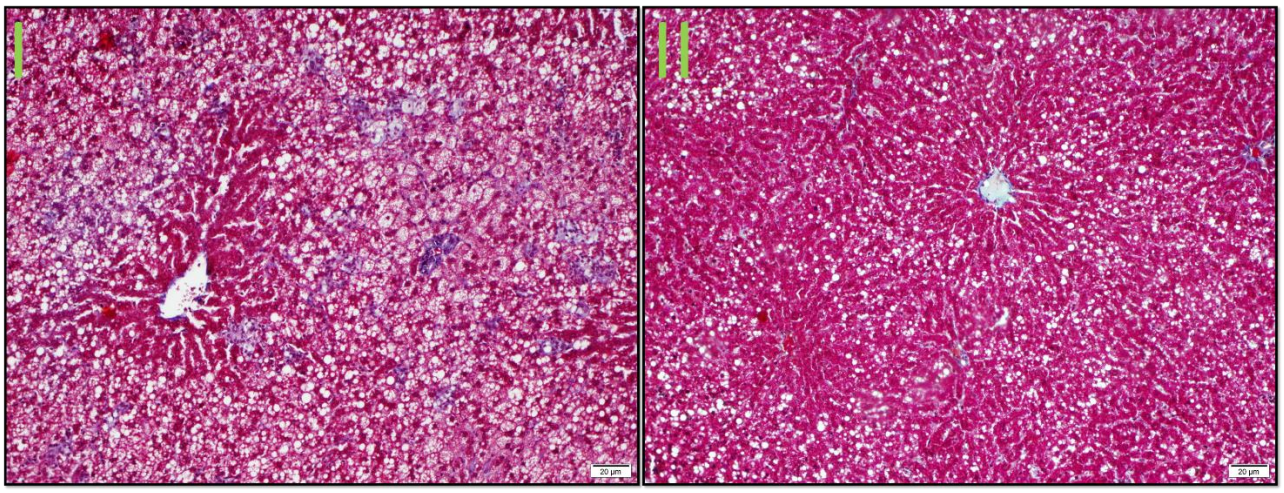

Figure 5: Masson's Trichrome stained liver sections from HFCC rats. Representative pictures of Masson's Trichrome stained livers from (I) vehicle and (II) rats escalated to 5 µg/kg KBP-089 (magnification of x10).

After 8 weeks of treatment, the HFCC diet induced hepatomegaly was dose-dependently reduced by KBP-089 (Figure 6A); however, this reduction in liver weights was equalized when normalized to the individual body weight (data not shown). We evaluated the livers using the NAFLD activity score (NAS). The blinded histological assessment of NAS was performed on Masson's Trichrome and Sirius Red stained terminal hepatic tissue from vehicle and KBP-089 E5 µg/kg treated HFCC rats. HFCC feeding induced massive lipid accumulation, ballooning and mild inflammation in the vehicle livers. Notably, after treatment with KBP-089 for 8 weeks, this inappropriate storage of lipids was reduced by treatment with KBP-089 (Figure 6B) and concomitantly, NAS was significantly reduced (Figure 6C).

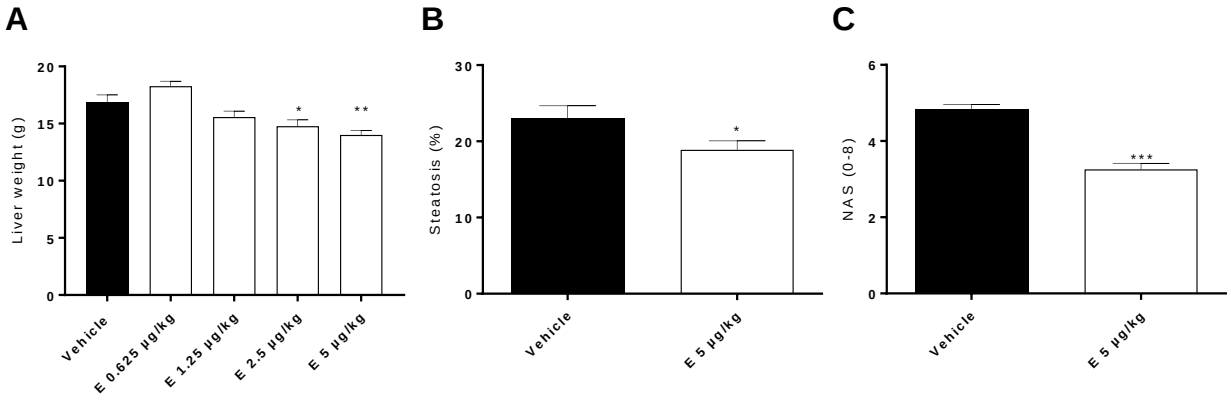

Figure 6: KBP-089 reduces diet induced hepatomegaly, liver steatosis and NAFLD activity score in HFCC rats. (A) Liver weights at study end, (B) quantitatively image analysis of steatosis (% of area) using Image J software and (C) combined NAS score (0-8) composed of a steatosis (0-3), ballooning (0-2) and inflammation (0-3) scores[31]. Steatosis, ballooning and inflammation were scored blinded by two independent persons (magnification x20, 8 images per animal; 4 pictures per depth; 5 animals per group). Statistical analysis between groups was evaluated by (A) an ordinary one-way ANOVA with Dunnet's multiple comparisons test, and (B, C) Student's t-test, $p < 0.05$, * compared to vehicle.

KBP-089 reduces inflammation and fibrosis stage in HFCC rats

We evaluated the terminal hepatic tissue from vehicle and KBP-089 E5 µg/kg treated HFCC rats. As seen in figure 7, 8 weeks of HFCC induced both marked hepatic steatosis, inflammation and mild fibrosis. The obese HFCC model had zone 3 perisinusoidal and periportal fibrosis – alone or with both traits present – and some of the livers even had bridging fibrosis.

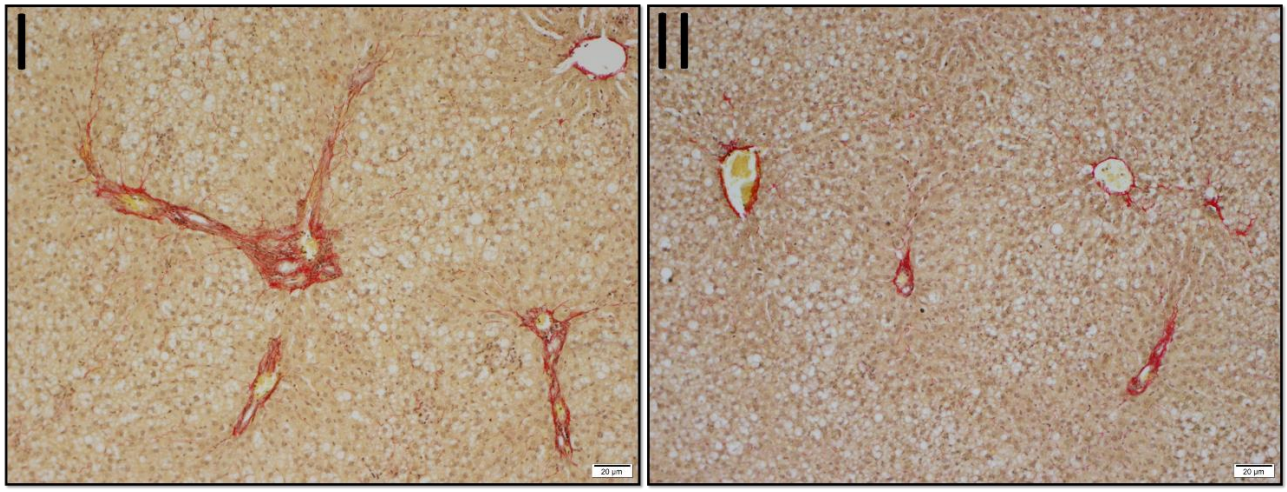

Figure 7: Sirius red stained liver sections from HFCC rats. Representative pictures of Sirius Red stained livers from (I) vehicle, and (II) HFCC rats escalated to 5 µg/kg KBP-089 treatment (magnification x10).

Furthermore, HFCC dieting profusely induced inflammation resulting in an inflammatory score of ~2, which was spectacularly reduced by approximately 60% by KBP-089 treatment (Figure 8A). Finally, the HFCC induced fibrotic traits were reduced in the hepatic sections from the KBP treated rats, resulting in an impressive ~45% reduction in fibrosis score compared to the vehicle HFCC rats (Figure 8B).

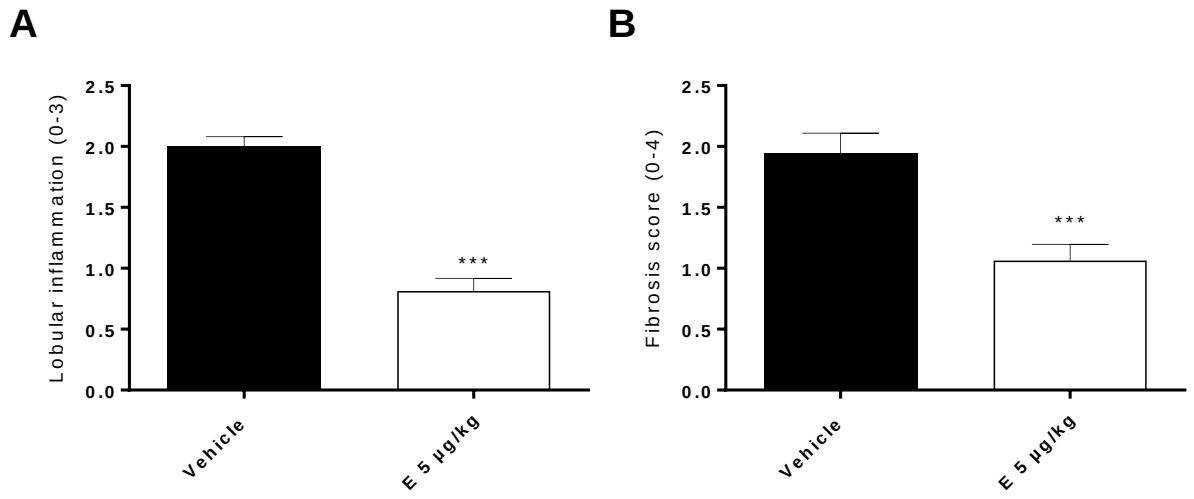

Figure 8: KBP-089 reduces inflammation and fibrosis scores in HFCC rats. (A) Lobular inflammation and (B) fibrosis score in vehicle and HFCC rats escalated to 5 µg/kg KBP-089 treatment. Two blinded independent persons (magnification x20 for inflammation, x10 for fibrosis, 8 images per animal; 4 pictures per depth 5 animals per group) scored inflammation and fibrosis[31].

Discussion

The present study describes the effects of the dual amylin and calcitonin agonist, KBP-089, in an experimental rat model of NASH. KBP-089 was able to induce and sustain significant weight loss, enhance insulin action and improve general liver health. In this study, we focused on the pathophysiology between diet induced obesity and insulin resistance, and the development of NASH. As previously reported, in rats, a diet high in fat and cholesterol leads to a phenotype resembling human NASH[294–296], albeit without eliciting obesity. Therefore, we included fattening period so the rats were significantly obese when they started the HFCC diet. Notably, this model has metabolic and hepatic features of NASH including steatosis, ballooning and inflammation. Interestingly, this model also revealed features as zone 3 perisinusoidal and periportal fibrosis – alone or with both traits present – and bridging fibrosis, which is in line with fibrotic findings in female rats fed a high fat, high cholesterol diet for 16 weeks[295].

KBP-089 was in this study introduced by dose escalation, which has been shown effective when introducing high concentrations of KBP-089[270], and was able to induce and sustain a significant weight loss equalling the weight loss previously described with standard dosing[274]. Importantly, KBP-089 possessed the ability to improve insulin action, consistent with other DACRAs[269,274,279].

An interesting aspect of the findings in the HFCC model regarding the reduction of fatty acid accumulation in the liver and enhanced insulin action is the known relation between liver fat, insulin resistance and NASH [297,298]. Insulin sensitizers as rosiglitazone[111] and pioglitazone[109,110] have beneficial effects on the histologic features of livers in patients with NASH. Likewise, it has recently been found that the weight reducing agent, liraglutide, led to histological resolution in patients with NASH[169]. Hence, our findings suggest that KBP at least due to its weight reducing capacity and improvement of insulin action could be a potential treatment candidate for NASH. This is corroborated by the KBP-089 induced reduction in AST levels, hepatic inflammation and fibrosis in this severe model – inflammation and the fibrotic traits were significantly reduced. The protective effect of KBP-089 on the HFCC dietary model of NASH supports the hypothesis that therapies directed toward alleviation of insulin resistance and obesity could enhance the care of patients with NASH.

In conclusion, the obese HFCC rat model reflects the human pathophysiology of NASH. KBP-089 is well tolerated when introduced by dose escalation and it induces a substantial weight loss. Importantly, KBP-089 improves metabolic and hepatic features of NASH, hence revealing the potential of KBP-089 as a therapeutic target that alleviates obesity and insulin resistance.

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Author contribution

S.G. and K.H designed the study. S.G. performed the study. S.G., S.J.D., A.T.L. and N.S. analysed data. S.G. drafted the manuscript. S.G. and K.H revised the manuscript. S.G., S.J.D., A.T.L., N.S., M.A.K., and K.H approved the final version of the manuscript.

CHAPTER IV

IV. Discussion

This series of studies describes the metabolic effects of treatment with dual amylin- and calcitonin receptor agonists (DACRAs), called KBP-042, KBP-088 and KBP-089. These peptides activate both the amylin and the calcitonin receptor, and importantly, they possess the ability to induce prolonged receptor activation – a trait found to be crucial for potent *in vivo* activity. KBPs induce and sustain a pronounced weight loss *in vivo*, leading to markedly improved metabolic parameters including food preference, and these are beyond those observed simply by diet-induced weight loss. Furthermore, KBPs improve inflammatory status and fibrosis stage in a rat model of NASH. Finally, these peptides were well tolerated when introduced by dose escalation.

Amylin receptor agonists such as pramlintide[299] and davalintide[300] have shown promise as pharmacological intervention in obesity and type 2 diabetes. Despite the approval of the amylin receptor agonist pramlintide for the treatment of diabetes as adjunct to mealtime insulin, these ligands are notoriously limited in terms of *in vivo* efficacy – both on glucose homeostasis and weight control.

KBP-042, KBP-088 and KBP-089 are based on the peptide backbone of salmon calcitonin and selected on prolonged receptor activation, acute food intake and body weight reductions. The knowledge obtained in previous studies with salmon calcitonin encouraged a development of novel dual agonists targeting both the amylin- and calcitonin receptors. KBP-042 was the first peptide discovered and it was first presented in 2014[268] followed by an injectable form showing that the use of injections reduced the variations on bioavailability[273]. Later other similar peptides like KBP-088 and KBP-089, also included in this thesis, were developed. KBP-042, KBP-088 and KBP-089 target the same receptors, albeit with slightly different potencies. This appears *in vivo* where KBP-088 induced a more pronounced hypophagic response in obese rats than KBP-042, which was tolerable at 10 µg/kg. KBP-089 and KBP-088 have comparable receptor potencies and were therefore only used in 2.5 µg/kg when introduced without dose escalation. Nevertheless, these peptides have similar sequences and *in vitro* and *in vivo* properties, and have been used interchangeably in this thesis to address various parts of our metabolic questions as a part of the overall development plan for these.

Dual Amylin and Calcitonin Receptor Agonists Mediated Receptor Activation

Our data shows that KBPs are highly potent in terms of activation of the amylin and the calcitonin receptor – with no activation of the CGRP receptor. Importantly, KBPs elicit prolonged receptor activation; a trait we prove to be crucial for potent *in vivo* activity as seen by the massive suppression of food intake following a single injection of KBP-089.

When KBP-088 was compared to davalintide – an amylin, calcitonin and calcitonin gene-related peptide receptor agonist – *in vitro*, davalintide was roughly equipotent to KBP-088 concerning short-term activation of the amylin and the calcitonin receptor. Notably, when their ability to elicit long-term receptor activation was tested, davalintide did not match KBP-088, which activated the receptor for up to 72 hours demonstrating a superior receptor activation profile. Furthermore, these effects manifested directly into a prolonged ability to control appetite by KBP-088, which was not seen for davalintide. This was somewhat surprising, as davalintide previously has been shown to bind irreversibly to the amylin receptor[300], albeit due to some yet to be identified mechanism this does not translate into prolonged receptor activation or prolonged suppression of appetite – a lack effect underlined *in vivo* when not using continuous infusion.

Despite the potent *in vivo* efficacy, peptides are not detectable in plasma for long nor do they accumulate – not even with repeated exposure. Plasma concentrations of KBP increase immediately after dosing while after 120 minutes, the plasma is cleared. This could be due to the internalization of the activated receptor. Normally, an internalized receptor stops signaling; however, it has been shown that the CTR elicits continuous signaling from the early endosome, which could be part of the explanation for the prolonged *in vivo* efficacy observed[220,301]. Furthermore, sCT binds virtually irreversible to the CTR and has a slower receptor dissociation rate than amylin, which also prolongs the signaling[220,301]. This has not yet been investigated for KBP, albeit as KBP has a similar receptor activation profile as sCT it is reasonable to assume such similar properties in terms of lower dissociation rate compared to amylin as well as irreversible binding, which is supported by the short plasma half life yet long pharmacodynamic effects *in vivo*.

The Anti-Obesity Effects of Dual Amylin and Calcitonin Receptor Agonists

In all chronic studies, a potent weight reduction was observed initially. This drastic reduction in body weight could be explained by the initial anorexic effect of KBPs as the food restricted pair-fed controls lowered their body weight similarly. Interestingly, the caloric intake returned normalized within the first two weeks depending on the ligand, and when the food consumption normalized the pair-fed group regained lost body weight whereas the KBP treated rats maintained the weight loss achieved. This large difference in body weight and the decreased food efficiency of the KBP treated rats indicate an increased EE in the KBP treated rats. Previously, data showing a food independent weight reduction

when administering amylin or amylinomimetics have been published[280,302], supporting our data. Considering the fact that KBPs significantly suppressed body weight compared to the pair-fed controls emphasizes that these peptides have some beneficial effects on body weight and composition besides suppressed food intake, likely increasing EE. Rats normally suppress EE during weight loss; however, continuous infusion of amylin prevents this[253,258] and there has been similar observations with davalintide[275]. Notably, amylin only increases EE when administered as continuous infusion or icv[258,259,267], a finding likely related to the low amylin receptor potency. The KBP mediated effect on EE is to be formally assessed in the future.

Throughout the studies, KBP induced a 15-20% vehicle-corrected weight loss with the highest concentrations. This body weight reduction extends far beyond what has previously been found with amylin agonism in rats. This is corroborated in the davalintide comparison study where KBP-088 treated rats lose significantly more weight than davalintide treated rats. We speculate that the absence of prolonged receptor activation by davalintide underlies the lack of ability suppress body weight at the doses chosen, although it transiently suppressed food intake, and these findings are supported by the need for infusion pump mediated delivery to ensure weight reduction[275].

In line with the reduction in body weight, KBP treatment reduced overall adiposity and decreased the adipocyte hypertrophy in the epididymal white adipose tissue, which is involved in restoring circulating insulin concentrations towards normal, concomitant with the return of normal tissue insulin sensitivity[303]. Whether these peptides have a direct effect on adipocyte hypertrophy needs further investigations; however, amylin has been demonstrated to directly stimulate adipocytes *in vitro* potentiating the effect of insulin and thereby may influence IR[304]. MR scans revealed a slight increase in lean mass in KBP treated rats compared to untreated rats. Previous studies have associated amylin receptor activation with a specific reduction in fat mass rather than lean mass[253,305], while inhibiting amylin signaling centrally increases fat mass[266], hence potentially explaining the difference in body composition.

Another important aspect of body weight loss could be to manipulate volunteer food consumption and composition of food chosen. This hypothesis was thought to be relevant for KBPs as amylin agonism has effect on the release of dopamine in the hypothalamus[306] and induce alterations in the melanocortigenic system[307] both of which are mediators of the reward/pleasure circuits that are known to affect feeding patterns[308]. Interestingly, patients treated with amylin analog pramlintide also experience a voluntary change in eating patterns and reduce 'binge eating'[309]. From the food preference study, it could be speculated that dosing with KBP offers some of the beneficial effects that the patients experience after surgical intervention. Patients who have had weight reducing surgical intervention experience a change of food consumption towards less energy dense and sweet diet[310], hence making KBPs a relevant option for treating obese patients and thereby aiding a body weight reduction and in combination with a healthier lifestyle, this might improve the results even

further. The mechanisms of action behind these changes are not completely clear, albeit alterations in food reward or taste functions have been suggested as possible explanations[311]. Whether this is the case for KBP remains to be elucidated.

The Glucoregulatory Effects of Dual Amylin and Calcitonin Receptor Agonists

In terms of hyperglycemia and IR, amylin analogues have shown promise[146,300]; however, they do not possess the intrinsic ability to improve fasting plasma glucose levels and insulin tolerance. In contrast, these data reveals the ability of KBP to improve fasting plasma glucose levels and HbA1c in ZDF rats and improve glucose tolerance in both ZDF and HFD rats independent of the gastric emptying effect. The glucose tolerance was improved compared to both vehicle and pair-fed rats in line with previous findings for salmon calcitonin[195] and KBP[273] and also compared to davalintide. Notably, davalintide did improve glucose tolerance even though there was a lack of prolonged receptor activation. This is due to the pre-dosing of the rats with the peptides 30 minutes prior to OGTT, which confirms the ability of davalintide to improve glucose tolerance short-term as previously described[300]. Previous findings on davalintide also showed improved glucose tolerance during OGTT, however, the experiments were performed with continuous infusion of the peptide[300]. In contrast to other glucose lowering agents such as sulfonylureas and GLP-1 analogues, the enhanced glucose disposal was achieved with an attenuated insulin secretion. Of interest, it was previously found that KBP directly lowered glucose stimulated insulin secretion in isolated islets[268]. Thus, we speculate that KBPs directly relieve the ß-cell stress in an insulin resistant environment by enhancing insulin action. An insulinostatic concept was also demonstrated for the insulin sensitizer, pioglitazone, which has a direct insulinostatic effect on the ß-cell that may contribute to its capacity to lower insulinemia and anti-diabetic action[312,313].

The KBP-improved glucose tolerance is partly mediated through lowering of gastric emptying rate, which has previously been attributed an amylin agonism mechanism[237,314], hence affected by KBP. To assess peripheral glucose homeostasis while circumventing this influence of gastric emptying, intravenous glucose tolerance tests were performed. Rats treated with KBP maintained glucose tolerance with reduced insulinemia implying an improved insulin sensitivity, hence corroborating that KBP has effects on glucose tolerance, which are independent of gastric emptying.
The suggested improvement in insulin action was formally tested in a hyperinsulinemic–euglycemic clamp set-up in obese hyperinsulinemic HFD rats. The glucose infusion rate (GIR) was expectedly lowered in the HFD vehicle rats compared the normal diet age-matched rats since obesity is negatively correlated to insulin sensitivity and GIR[27]. KBP treatment induced a marked increase in GIR, hence supporting enhanced insulin sensitivity. The KBP-induced weight loss would explain a large increase in GIR; however, as mentioned above KBPs have effects beyond what weight loss provides and further, in this particular study, the rats treated with KBP had similar body weight to the ND rats,

albeit a significantly higher GIR. This supports the finding in the weight-matched study – namely that insulin sensitivity is increased beyond what would be expected from weight loss.

To further investigate whether KBP had beneficial metabolic effects beyond what was achieved with weight loss, a weight-matched control group was implemented. The study supported this hypothesis as substantially lower glucose and insulin levels were observed during an oral glucose tolerance test in the KBP treated group when compared to weight-matched controls. The improvement in insulin levels manifested in an improvement in glucose control too. It cannot be ruled out that the weight-matched animals had an 'artificial' increase in glucose intolerance due to significant food restriction or prolonged fasting, although the rats did not show any signs of malnutrition or abnormal behavior. This mild lowering of insulin could be an explanation for the limited improvement in glucose tolerance when compared to the improvement observed in KBP treated rats. This would have been likely after a marked weight loss[315–317].

Adiponectin and leptin levels were measured after chronic treatment with KBP. Adiponectin was significantly increased by KBP treatment. In contrast, plasma leptin was reduced. Weight loss has on multiple occasions been associated with beneficial effects on adipocytokines[318]. The increased level of adiponectin supports an improved metabolic status as plasma adiponectin is reduced in obese people and related to inflammation, IR, and energy metabolism[319,320], as well as the type of phenotype in the different fat depots[321]. The decreased overall adiposity is likewise reflected in lowering of plasma leptin that supports previous findings where KBP improves leptin sensitivity[273] – leptin sensitivity which is also improved by amylin[280,322]. Furthermore, KBP treated rats drastically reduced GIP and GLP-1 that were elevated in untreated rats following a glucose challenge. This pronounced suppression of these glucose dependent hormones could cause the reduced glucose stimulated insulin secretion in treated rats; however, since glucose homeostasis is maintained or even improved, we speculate that the reduction in incretin response is acceptable. Interestingly, it has been suggested that inhibition of GIP signaling actually prevents obesity in knockout mice on a calorie dense diet[323]; however, this is not the case in hyperphagic leptin deficient mice, where GIP knockout does not prevent excess weight gain[324]. The mechanism behind the suppression of GIP and GLP-1 is unclear, albeit a lowering of gastric emptying rate will naturally reduce the secretion of GIP and GLP-1. This is however not all there is to it. We measured GIP at different time points during the OGTT and the suppression of GIP was evident at all time points even when the glucose should have passed. This could indicate that KBP might have a direct effect on GIP; however, this needs further investigation.

Optimization of Tolerability

KBPs induce hypophagia at dose initiation and peptides with known anorectic effects, such as amylin and GLP-1 analogues, are associated with gastrointestinal tolerability problems in humans[325,326]. The massive suppression of food consumption following initial dosing might be indicative of adverse effects on the GI tract, which is also supported by kaolin intake at high doses of KBP in the pica test. The kaolin consumption was only stimulated at a high concentration – a higher concentration than used in the chronic studies, thus indicating the reduction in food intake was not due to illness, albeit nausea cannot be excluded.

To explore whether a similar weight loss was obtainable with a less pronounced induction of hypophagia, clinical setups for pramlintide and GLP-1 analogues using dose-escalation were mimicked[325,327]. Dose-escalation induced in a similar magnitude of weight loss with lower suppression of appetite; however, the time to reach maximum weight loss was prolonged. Notably, the increments of the dose-escalation steps are very important. Too large increments lead to substantial suppression of intake, possibly indicating an adverse response in the GI tract. These findings correlate well with what was expected from literature and dose-escalation of anorectic peptides[325,327]. These data support that dose-escalation is an option for increasing tolerability of this type of molecule, and that dose-escalation strategies can be tested in rat models using appetite suppression and weight regulation as the output. Moreover, in the acute settings, the suppression of food consumption clearly exceeded a 24-hour period; hence, potentially these peptides could be administered less frequently. To address this, different dosing regimens were investigated. Chronic dosing using 5 μg/kg once daily (s.i.d.) or 5 μg/kg every other day (q.a.d.) was compared, and importantly both dose regimens led to similar weight loss during the 15 day study. In the q.a.d. dosed rats, notable fluctuations in caloric intake and body weight were observed, likely resulting in a challenge in terms of tolerability. Surely, it is of interest to challenge these dosing regimens, and to test less frequent dosing as the peptides have the ability to suppress caloric intake up to 72 hours – perhaps escalation to an even higher dose that would allow less dosing.

Dual Amylin and Calcitonin Receptor Agonist and GLP-1 Synergy

As GLP-1 analogues and amylin receptor agonists are thought to work, at least on the appetite regulation, through a similar mechanism of action[326], we studied the potential combination of KBP with liraglutide. Various combinations were tested and it was found that a combination of the two peptides had superior efficacy in terms of suppressing acute caloric intake than either of the peptides alone. Chronically, two suboptimal doses of KBP and liraglutide were investigated and we found a good effect when combining the two peptides, resulting in body weight efficacy observed at doses considered virtually ineffective when given as stand-alone doses. Both amylin and GLP-1 agonism exhibit weight-lowering effects and previously, the combination of GLP-1 and amylin have shown synergistic

reductions in food intake in non-human primates[328]. Furthermore, also peptide hybrids composed of an exenatide analogue and davalintide have shown reduction of body weight and improved glucose control in obese rats[329,330]. Hence, the combination of GLP-1 and amylin therapies could be of interest for the treatment of metabolic disease as combination-based therapies are increasing in this area of disorders.

The Effects of Dual Amylin and Calcitonin Receptor Agonists on Nonalcoholic Steatohepatitis

The metabolic effects of KBPs were mostly tested in the hyperinsulinemic obese model. This high fat fed rat model is useful for highlighting the beginning IR along with obesity as they do mimic the status of the majority of insulin resistant/pre-diabetic patients[331]. Even though this model is heavily obese, insulin resistant, and has ectopic steatosis, there is no inflammation nor elevated liver markers in the plasma. Thus, to further explore the anti-steatotic effect of KBP observed in HFD, the high fat, high cholesterol and cholate model (HFCC) was used.

As previously reported, a diet high in fat and cholesterol develop a phenotype resembling human NASH[294–296], albeit without eliciting obesity. As the focus in this model was the pathophysiology between diet-induced obesity, IR and the development of NASH, we included fattening period so the rats were significantly obese when they started the HFCC diet and KBP treatment. This obese HFCC model has metabolic and hepatic features of human NASH including steatosis, ballooning and inflammation. Interestingly, this model also revealed features such as zone 3 perisinusoidal and periportal fibrosis – alone or with both traits present – and bridging fibrosis, which is in line with fibrotic findings in female rats fed a high fat, high cholesterol diet for 16 weeks[295].

An interesting aspect of the findings in the HFD and the HFCC model regarding the reduction of fatty acid accumulation in the liver and enhanced insulin action is the known relation between liver fat, IR and NASH[297,298]. Insulin sensitizers as rosiglitazone[111] and pioglitazone[109,110] have beneficial effects on the histologic features of livers in patients with NASH. Likewise, it has recently been found that the weight reducing agent, liraglutide, led to histological resolution in patients with NASH[169]. Hence, our findings suggest that KBP at least due to its weight reducing capacity and improvement of insulin action could be a potential treatment candidate for NASH. This is corroborated by the KBP induced reduction in AST levels, hepatic inflammation and fibrosis in this severe model – inflammation and the fibrotic traits were not eliminated, albeit significantly reduced. The protective effect of KBP on the HFCC dietary model of NASH supports the hypothesis that therapies directed toward obesity and IR could enhance the care of patients with NASH.

In summary, these studies have indicated that KBPs have potency extending far beyond classical amylin agonists. The peptides were well tolerated when introduced by dose escalation and improved metabolic and hepatic features markedly. These data clearly

reveals that KBPs at least due to the weight reducing capacity and beneficial effects on various metabolic features could be a novel treatment candidate for obesity and related morbidities as type 2 diabetes and NASH.

CHAPTER V

V. Conclusion

The overall conclusion to the hypothesis is that a prolonged receptor activation enhanced the *in vivo* efficacy and that KBPs induce metabolic improvement beyond what is observed with caloric restriction and simple diet induced weight loss. This underlines that KBPs have potential as novel treatments for obesity and related co-morbidities.

More specifically the conclusions are as followed:

1. KBPs are highly potent in terms of activation of the AMY-R and the CTR – without activating the CGRP-R, and have a superior ability to elicit prolonged receptor activation compared to davalintide
2. KBPs decrease food intake and alter food preference in rats.
3. KBPs induce and sustain substantial weight loss in obese rats independent of caloric consumption.
4. KBPs improve glucose tolerance with lowered hyperinsulinemia independent of caloric consumption and weight loss in obese rats.
5. KBPs improve insulin and leptin sensitivity in obese rats.
6. KBPs attenuate fasting plasma glucose and HbA1c and enhance glucose tolerance and insulin action in ZDF rats.
7. KBPs are well tolerated when introduced by dose escalation; however, with later onset of maximal weight loss.
8. KBPs can be dosed every other day while still inducing similar weight loss.
9. KBPs are superior to davalintide in terms of efficacy on body weight and work complementary with GLP-1 agonist, liraglutide.
10. KBPs reduce lipid accumulation in liver and muscle in obese rats as well as liver steatosis, inflammation and fibrosis scores in NASH rats.

CHAPTER VI

VI. Perspectives

The prospect of the synthetic dual amylin and calcitonin receptor agonists as potential therapeutic agents in obesity and related co-morbidities is quite intriguing. Several aspects in terms of mode of action, and how the anti-obesity effects and improved metabolic health observed in rats translate into humans remain to be fully evaluated.

Do DACRAs increase energy expenditure?

The discrepancy between the KBP induced weight loss and the caloric restricted pair-fed group groups clearly suggests an increase in EE of the treated animals. Amylin administration has previously been demonstrated to maintain metabolic rate during energy restriction and enhance fat utilization[253,332] perhaps by activation of brown adipose tissue[267]. To formally elucidate whether DACRAs modulates metabolic activity measures of e.g. EE, respiratory exchange ratio and physical activity are of great importance, and could be conducted using metabolic cages and activity wheels.

The body composition (e.g. lean versus fat mass) was determined using a magnetic resonance scanner. KBP treated rats had significantly lower fat mass compared to untreated and interestingly, treated rats had a slightly increased lean mass. Lean mass is determined as muscle tissue mass equivalent of all the body parts containing water, excluding fat, bone minerals, and such substances, which do not contribute to the NMR signal (hair, claws, etc.). Nevertheless, it would be of great interest to image the body composition and thus determine the efficacy of muscle mass exclusively.

Are DACRAs insulin sensitizers? And where does the glucose go?

Another interesting finding is the improved glucose tolerance and enhanced insulin action, which is independent of body weight. KBP improved glucose tolerance and insulin action beyond what was observed with a regular diet induced weight loss. We used the hyperinsulinemic-euglycemic in the HFD rats; however, to formally assess insulin sensitivity in KBP treated rats compared to weight-matched rats the gold standard hyperinsulinemic-euglycemic clamp should be performed[333] in combined with a radio-active labeled tracer (e.g. ^{14}C- or ^{3}H-deoxyglocuse) glucose uptake/clearance of peripheral tissues[334]. Additionally, it would be of great interest to combine the radiolabelled clamp study with *in vitro* studies investigating glucose uptake in cultured peripheral tissues (e.g. muscle fibers[335], adipocytes[304], hepatocytes[336] during normal or gluco- and lipotoxic

conditions to mimic diabetic conditions[337]. Furthermore, it would be interesting to further explore the direct effect DACRAs on pancreatic islets – also in a gluco- and lipotoxic environment.

Could DACRAs be dosed once weekly?

The peptides are dose once daily. In paper III, dosing every other day was tried and interestingly the rats reduced their body weight similarly. It would be interesting to explore more dosing regimens and frequencies, as dosing e.g. once weekly could be preferable for some patients.

Does the calcitonin receptor have any relevance for obesity and associated morbidities?

Another aspect in elucidating the mechanisms of action is to determine which of the two receptors targeted is responsible for the effects observed. The anti-obesity potential of these peptides has been established; however, it would be interesting to separate the contributions of the different receptors.
Furthermore, obesity is associated with hyperamylinemia and it has been hypothesized that amylin sensitivity is reduced in the obese state. It could be of interest to test KBP as well as calcitonin and amylin treatment animals with diet or drug induced hyperamylinemia, hence possibly lowered sensitivity to amylin.

Combination therapies
Additionally, the combinational therapy with GLP-1 will be of outmost importance. In paper IV, suboptimal dosing was used to enlighten a potential KBP-GLP-1 synergy. In the future, this synergy as well as combination with e.g. insulin and leptin should be further explored as this could hold the key to improve metabolic and glycemic control in preclinical and clinical settings.

Clinical perspectives

Finally, it will be exhilarating to monitor the anti-obesity, anti-diabetic and anti-NASH effects of the KBPs in clinical development – after all, treating people is the main goal.

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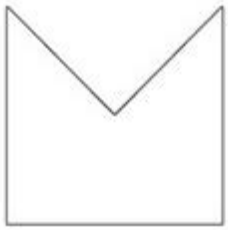

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