A Novel Oral Dual Amylin and Calcitonin Receptor Agonist (KBP-042) Exerts Anti-Obesity and Anti-Diabetic Effects in Rats

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ABSTRACT
The present study investigated a novel oral dual amylin and calcitonin receptor agonist (DACRA), KBP-042, in head-to-head comparison with salmon calcitonin (sCT) in regards to in vitro receptor pharmacology, ex vivo pancreatic islet studies and in vivo proof of concept studies in diet-induced obese (DIO) and Zucker diabetic fatty (ZDF) rats.

In vitro, KBP-042 demonstrated superior binding affinity and activation of amylin and calcitonin receptors, and ex vivo, KBP-042 exerted inhibitory action on stimulated insulin and glucagon release from isolated islets.

In vivo, KBP-042 induced a superior and pronounced reduction in food intake in conjunction with a sustained pair-fed corrected weight-loss in DIO rats. Concomitantly, KBP-042 improved glucose homeostasis and reduced hyperinsulinemia and hyperleptinemia in conjunction with enhanced insulin sensitivity.

In ZDF rats, KBP-042 induced a superior attenuation of diabetic hyperglycemia and alleviated impaired glucose and insulin tolerance. Concomitantly, KBP-042 preserved insulinotropic and induced glucagonostatic action, ultimately preserving pancreatic insulin and glucagon content.

In conclusion, oral KBP-042 is a novel DACRA, which exerts anti-obesity and anti-diabetic efficacy by dual modulation of insulin sensitivity and directly decelerating stress on the pancreatic alpha and beta-cells.

These results could provide the basis for oral KBP-042 as a novel therapeutic agent in type 2 diabetes.

INTRODUCTION
Targeting hyperglycemia in type 2 diabetes primarily focuses on improving insulin secretion and/or reducing insulin resistance (11; 37), although correction of hyperglucagonemia is equally important for optimal glycemic control (13). Furthermore, the majority of diabetic patients are overweight or obese, which contributes to insulin resistance and type 2 diabetes (20). Thus, optimally, novel anti-diabetic drugs should improve all these parameters.

Glucagon-like peptide (GLP)-1 analogues (17) have demonstrated glucoregulatory effects through stimulation of insulin secretion, decreased glucagon secretion and weight reduction (7). Another therapeutic approach is to enhance insulin action, to avoid extensive hyperinsulinemia and increased insulin resistance (18; 42). Presently, the insulin-sensitizing agents, such as the biguanides (e.g. metformin) and the thiazolidinediones (e.g. glitazones), primarily reduce blood glucose, but fail to reduce hyperglucagonemia and body weight. Additionally, the glitazones are associated with several adverse effects including weight gain (1), highlighting the urge for novel therapeutic insulin-sensitizing agents.

The amylin analogue pramlintide improves postprandial hyperglycemia by inducing glucagonostatic action, slowing gastric emptying, increasing satiation and facilitating weight loss, thereby targeting several of the defects commonly seen in diabetic patients, albeit, it lacks the ability to enhance insulin secretion and/or insulin action, and hence is used solely as an adjunct to prandial insulin (44),(12; 38). These findings have led to a search for more potent amylin analogues with enhanced pharmacological properties (32; 33).

Two peptide hormones, salmon and eel calcitonin, are unique in their ability to activate both the amylin receptor and the calcitonin receptor with potencies that are superior to other ligands (4; 10). Recently, oral delivery of salmon calcitonin (sCT) demonstrated glucoregulatory efficacy by improved fasting and postprandial glycemic control with a concomitant weight loss in diet-induced obese (DIO) rats (15; 31). Importantly, oral sCT attenuated diabetic hyperglycemia and preserved
pancreatic beta-cell function and mass in Zucker diabetic fatty (ZDF) rats (14). Hence, novel oral
peptides with these abilities could be promising interventions in type 2 diabetes
In this paper, we present KBP-042, a novel dual amylin and calcitonin receptor agonist (DACRA),
which was profiled in a series of *in vitro* receptor activation tests for the amylin, calcitonin and the
calcitonin gene-related peptide (CGRP) - receptors. Finally, to establish proof-of-concept for *in vivo*
efficacy of oral KBP-042, we performed a head-to-head study with oral sCT to explore the anti-
obesity and anti-diabetic efficacy in DIO and ZDF rats.
RESEARCH DESIGN AND METHODS

Peptide therapy.

For oral delivery of salmon calcitonin (sCT) or KBP-042, the carrier agent N-(5-chlorosalicyloyl)-8-aminocaprylic acid (5-CNAC) (21) (vehicle) was obtained from Biomics Biotechnologies Co. Ltd. (Nantong, China), and recombinant custom sCT or KBP-042 peptide (Unigene Laboratories, Boonton, NJ, USA) was mixed with 5-CNAC (150 mg/kg). The doses chosen for sCT/KBP-042 peptide administration were based on previous studies in animal models of obesity and type 2 diabetes (14; 16) and in vitro settings (5).

In vitro receptor binding and activity.

The relative receptor specificity and potency at the amylin and calcitonin receptor was determined by the ability of KBP-042 to induce cAMP, β-arrestin and receptor-binding in cell lines over expressing the human calcitonin, amylin and CGRP receptors, respectively.

Calcitonin and amylin receptor binding. U20S CALCR cells and CHO K1 CALCR RAMP3 cells (Path-Hunter® β-arrestin Cell Line, DiscoverX: 93-0566C3 and 93-0268C2) were incubated with 250 pM (125I)-(Tyr22)-sCT (NEX423, Perkin Elmer, Waltham, MA, USA) and unlabelled sCT (H-2260, Bachem AG, Bubendorf, Switzerland) or KBP-042 peptide (10^-6- 10^-12 M) for 30 min at ambient temperature. Cell lysates radio-active content was counted by scintillation using a 1470 Wallac Wizard™ Gamma Counter.

β-arrestin and cAMP quantification. U20S CALCR cells, CHO K1 CALCR RAMP3 cells (as described above), and CKO-K1 CALCRL RAMP1 cells (Path-Hunter® β-arrestin Cell Line, DiscoverX: 93-0269C2) were used to quantify β-arrestin by PathHunter® Detection Kit (93-0001, DiscoverX) according to the manufacturer’s instructions. Cyclic AMP (cAMP) assays were conducted with 3-Isobutyl-1-methylxanthine (IBMX) as a non-specific inhibitor of cAMP.
phosphodiesterase. cAMP FEMTO TB KIT (62AM7PEB, Cisbio Bioassays, Codolet, France) was used to quantify cAMP content according to the manufacturer’s instructions.

**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 selectively bred Sprague Dawley diet-induced obese (DIO) and male obese Zucker diabetic fatty (fa/fa) (ZDF) and lean (fa/+) rats (Charles River Laboratories, Sulzfeld, Germany) were obtained at 5 weeks of age and housed under controlled temperature on a normal 12-h light-dark cycle with unrestricted access to water and food. For DIO rats, chow consisted of regular rodent chow and a 60 kcal% high fat diet (#D12495) (Research Diets Inc., NJ, USA), respectively, and was administered for a total of 12 weeks. ZDF rats were fed Purina Laboratory Diet 5008 (Brogaarden, Lynge, Denmark) and allowed 1 week of acclimation prior to experiments.

For short-term treatment, eight 17-week old DIO rats were assigned by body weight and glucose levels to receive oral administration of oral vehicle (150 mg/kg 5-CNAC) or oral KBP-042 (150 mg/kg 5-CNAC mixed with KBP-042 at doses of 0.5, 1 and 2 mg/kg) for 3 days (b.i.d.) using plastic feeding tubes (FTP-18-75, Instech Solomon, Plymouth Meeting, PA USA). Cumulative food intake and body weight was measured at study end, and oral glucose tolerance test (OGTT) (2 g/kg) (16) performed after overnight fasting.

For longer-term treatment, ten 17-week old DIO rats were assigned by body weight and glucose levels to receive oral administration of vehicle (5-CNAC), sCT, or KBP-042 at 1 mg/kg dose for a total of 4 weeks (bidaily). Furthermore, a pair-feeding group to KBP-042 was included to assess the influence of food intake. Body weight and food intake were recorded weekly. To assess drug effect
on gastric emptying, overnight-fasted rats received 96 mg/kg acetaminophen by oral gavage (4 ml/kg) and the appearance of acetaminophen in plasma was monitored 30 min post-administration (32). Gastric emptying was calculated as % change relative to vehicle: % change = 100 X [(peptide-plasma acetaminophen/vehicle-plasma acetaminophen) – 1]. At study end, OGTT (2 g/kg) was performed in overnight-fasted rats with blood glucose measured and EDTA-plasma obtained for hormonal analysis. Homeostasis model assessment of insulin resistance (HOMA-IR) analysis was calculated and was used for the estimation of hepatic insulin resistance using the formula (35):

\[
\text{HOMA-IR} = \left( \text{FI (}\mu\text{U/ml)} \times \text{FBG (mM)} \right)/22.5,
\]

where FI and FBG represents fasting insulin and fasting blood glucose, respectively. While HOMA-IR was developed for humans, it can be used as a surrogate measurement for insulin resistance in rodents (8; 25; 34).

For chronic treatment, 10 six-week old ZDF rats were assigned by body weight, HbA1c and glucose levels to receive oral administration of vehicle (5-CNAC), sCT, or KBP-042 (0.25, 0.5, 1 or 2 mg/kg) for 7 weeks. Lean ZDF rats served as control. Fasting (12 h) and non-fasting blood glucose levels were measured and HbA1c analyzed at study end. OGTT (1 g/kg) (14) was performed after overnight fasting, and intraperitoneal (IPITT) (1 U/kg) and intravenous (IVITT) (0.4 U/kg) insulin tolerance testing was performed after 6 h fasting. For assessment of insulin sensitivity, calculation of the rate constant for blood glucose disappearance (\(K_{\text{ITT}}\)) (3) was determined from blood glucose levels at 5, 10 and 15 min post insulin injection during IVITT. Finally, animals were euthanized via an overdose of pentobarbital sodium (200 mg/kg, IV) and decapitated. Pancreases were excised, homogenized and extracted in acid-ethanol for subsequent determination of insulin and glucagon content (27). Protein content of these extracts was estimated by the bicinchoninic acid (BCA) method (41).

**Islet isolation and measurement of insulin and glucagon release.**
Male lean healthy Sprague Dawley (SD) rats (Taconic, Lille Skensved, Denmark) were obtained at 10-12 week old and islets isolated by retrograde collagenase solution injection via the bile-pancreatic duct (19). Isolated islets were hand-picked and transferred to incubation media. Islets (4 islets/0.2 ml incubation buffer per well) were preincubated for 60 min at 37°C in 0.2 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, supplemented with 10 mmol/l HEPES and 0.2 % bovine serum albumin as reported previously (2), modified to include 5.5 mmol/L glucose. After preincubation, identical buffer medium was exchanged including 5.5, 11 and 22 mmol/L glucose for glucose-stimulated insulin-secretion (GSIS) or with L-arginine (10 mmol/L, L-arginine hydrochloride 21%) (Sigma Aldrich Denmark, Copenhagen, Denmark) for arginine-stimulated glucagon-secretion (ASGS) with or without salmon calcitonin or KBP-042 at 10^{-8} M dose and incubated for 60 min at 37°C. Immediately after incubation, aliquots of incubations-media were removed for analysis of insulin and glucagon and results were normalized to islet numbers.

**Biochemical analyses**

Blood glucose was monitored by Accu-Check® Avia monitoring system (Roche Diagnostics, Rotkreuz, Switzerland) and HbA1c levels by DCA Vantage Analyzer (Siemens, Erlangen, Germany). Levels of insulin (Mercodia Rat Insulin ELISA, Mercodia AB, Uppsala, Sweden), glucagon (Glucagon Quantikine® ELISA, R&D Systems Europe, Abingdon, UK), and leptin (Rat Leptin ELISA, Millipore Corporation, Billerica, MA, USA), was analyzed according to manufacturer’s instruction.

**Statistical analyses**

All data are presented as mean ± standard error of the mean (SEM). The statistical analysis of drug effects versus vehicle effects was conducted using one-way ANOVA followed by the Dunnett’s post hoc test. Student’s t-test was performed to compare lean control group and vehicle. All
analyses were performed using GRAPHPAD PRISM software (GraphPad Prism, San Diego, CA. U.S.A). A value of $P < 0.05$ was considered to be significant.

RESULTS

**KBP-042 is a superior and specific dual amylin and calcitonin receptor agonist**

KBP-042 is an analogue of both salmon and eel calcitonin as the peptide sequence comparison listed in Table 1 illustrates. To assess activity on the amylin and the calcitonin receptor, we investigated ligand mediated cAMP production, $\beta$-arrestin recruitment and competitive ligand binding using salmon calcitonin as comparator. KBP-042 activated both the amylin receptor and the calcitonin receptor resulting in potent activation of both receptors (Fig. 1A-B and table 2). When compared to sCT, the potency of KBP-042 was found to be superior on these parameters (Table 2), apart from $\beta$-arrestin recruitment by the AMY-R; however, the calculated EC$_{50}$ in the $\beta$-arrestin assay was affected by a significantly higher E$_{\text{max}}$ for KBP-042 than for sCT (Figure 1B). In the competitive binding studies, KBP-042 bound to both receptors with higher affinity than sCT (Table 2). Finally, no induction of the CGRP-receptor was observed (Figure 1C)(33).

**Short-term oral KBP-042 administration improves energy balance and glucose tolerance in DIO rats.**

Initially, we assessed the short-term metabolic effect of increasing doses of KBP-042 on energy balance and glucose tolerance during OGTT in DIO rats. During the treatment, KBP-042 induced a significant dose-related reduction in body weight (Fig. 1D) and food intake (Fig. 1E) when compared to vehicle. Additionally, DIO vehicle rats demonstrated impaired glucose tolerance, which, in contrast, was markedly improved by KBP-042 treatment in a dose-related manner (Fig.
Longer-term oral KBP-042 treatment is metabolically superior to oral sCT in DIO rats.

To assess the anti-obesity efficacy of KBP-042 in vivo, we investigated the effect of longer-term KBP-042 administration in DIO rats head-to-head with an equivalent dose of sCT, and a pair-fed group for KBP-042 to explore the impact of food restriction on weight-and glucoregulatory action. KBP-042 and sCT (1 mg/kg, b.i.d.) were administered orally for 4 weeks. Importantly, bidaily oral dosing with KBP-042 and sCT at 1 mg/kg resembled equivalent plasma exposure (data not shown). KBP-042, sCT, and pair-fed groups showed significantly lowered body weight and food intake when compared to vehicle (Fig. 2A-B), and after 3 and 4 weeks of treatment KBP-042 was superior to sCT and its corresponding pair-fed group (Fig. 2A). Thus, throughout the intervention period, KBP-042 induced a significant hypophagic response superior to sCT (Fig. 2B). Additionally, at 3 weeks of treatment, the rate of gastric emptying was slightly increased in pair-fed rats (2.6 ± 0.2 %, NS), albeit significantly decreased in KBP-042 (-25.3 ± 1.8%, $P < 0.001$) and sCT (-22.2 ± 2.1%, $P < 0.001$) treated rats, when compared to vehicle. Importantly, fasting blood glucose was modestly, but significantly reduced only for the KBP-042 group, corresponding to improvement in indices of insulin resistance (HOMA-IR), when compared to vehicle and pair-fed rats at study end (Fig. 2C-D). Interestingly, hyperleptinemia was significantly reduced in sCT and KBP-042 treated rats, when compared to vehicle and pair-fed rats (Fig. 2E), while hyperinsulinemia was significantly reduced only in the KBP-042 rats when compared to vehicle group (Fig. 2F), no difference was observed between KBP-042, sCT and pair-fed groups.

Longer-term oral KBP-042 treatment improves glucose tolerance and pancreatic glucoregulatory hormones in DIO rats.
To investigate the treatment effect of KBP-042 on glucose tolerance we performed an OGTT at study end. KBP-042 and sCT treatment markedly reduced blood glucose excursions following oral glucose exposure (Fig. 3A) and, to a similar extent, significantly reduced glucose iAUC values during OGTT, when compared to vehicle and pair-fed groups (Fig. 3B).

To explore the glucoregulatory mode of action for KBP-042, we focused on pancreatic islet hormone response to glucose and demonstrated that (excessive) insulin secretion observed in DIO and pair-fed groups was markedly suppressed during OGTT (Fig. 3C) and, similarly, plasma insulin iAUC values were significantly reduced in KBP-042 and sCT treated rats (Fig. 3D). Furthermore, KBP-042 and sCT treatment induced a glucagonostatic action (Fig. 3E) and significantly reduced plasma glucagon iAUC values during the initial period of the OGTT, when compared to vehicle and pair-fed (Fig. 3F).

**Chronic oral KBP-042 treatment exerts superior anti-hyperglycemic efficacy to oral sCT in ZDF rats.**

To assess the anti-hyperglycemic efficacy of KBP-042 in vivo, we investigated the metabolic effect of chronic KBP-042 administration in ZDF rats with a head-to-head comparison to sCT. Multiple doses of KBP-042 and sCT (0.25, 0.5, 1 and 2 mg/kg, b.i.d.) were administered orally for 7 weeks. KBP-042 treatment at doses of 1 and 2 mg/kg and sCT at 2mg/kg induced a significant vehicle-corrected gain in body weight (approximately 8-10%) in line with previous findings (14). In ZDF rats, fasting and postprandial blood glucose levels decreased significantly over 7 weeks by KBP-042 treatment at 1 and 2 mg/kg doses (Fig. 4A and B), resulting in HbA1c reduction by 1.6% at study end (Fig. 4C), an effect only observed at the highest dose of sCT. During OGTT, KBP-042 and sCT dose-dependently improved glucose tolerance at doses of 0.5, 1 and 2 mg/kg (Fig. 4D and E), as evidenced by the significant decrease in iAUC during OGTT, which was significantly
reduced by approximately 50% at KBP-042 doses of 1 and 2 mg/kg (Fig. 4E). As observed above, KBP-042 treatment at 1 mg/kg dose exhibited a superior glycemic control when compared to sCT (Fig. 4E).

**Chronic oral KBP-042 treatment exerts superior pancreatic glucoregulatory effects to oral sCT in ZDF rats and directly modulates insulinotropic and glucagonostatic action in islets.**

To explore the glucoregulatory mode of action for KBP-042, we focused on pancreatic derived plasma hormones heavily involved in glycemic control. As expected, ZDF rats were severely hyperinsulinemic at baseline compared to the control rats (8.6 ± 0.4 vs 0.3 ± 0.0 ng/ml, \( P < 0.001 \)), reflecting an accelerating beta-cell hypersecretion to compensate for the impaired insulin action. As previously observed (14), plasma insulin levels in the ZDF vehicle rats were progressively reduced during the study period resulting in approximately 76% decrease at study end. In contrast, KBP-042 treatment significantly sustained hyperinsulinemia at doses of 1 and 2 mg/kg, which in contrast was only observed for sCT at 2 mg/kg dose (Fig. 5A). As previously observed (14), fasting hyperglucagonemia was likewise a phenotypic trait in the ZDF rats when compared to control rats (140.9 ± 8.9 vs 57.9 ± 7.4 pg/ml, \( P < 0.001 \)). In contrast, KBP-042 treatment at doses of 1 and 2 mg/kg significantly reduced plasma glucagon levels at study end (Fig. 5B), which, as for insulinemia, was only observed at the 2 mg/kg dose of sCT. Notably, these insulinotropic and glucagonostatic effects could likely not be ascribed the incretin hormone glucagon-like peptide-1 (GLP-1) (9), as KBP-042 treatment significantly decreased (normalized) plasma GLP-1 at doses of 1 and 2 mg/kg, which was observed only for 2 mg/kg dose of sCT (data not shown), which is in line with previous findings (14). As expected in ZDF rats, pancreatic insulin content was significantly reduced at study end when compared to control rats (0.57 ± 0.03 vs 1.38 ± 0.27 ng/μg protein, \( P < 0.01 \)). Importantly, KBP-042 at doses of 1 and 2 mg/kg significantly increased pancreatic insulin content compared to vehicle, which, in contrast, was only observed for sCT at 2 mg/kg treatment.
dose (Fig. 5C). Additionally, pancreatic glucagon content was significantly reduced in ZDF rats when compared to control rats (55.86 ± 10.81 vs 139.5 ± 16.27 pg/μg protein, $P < 0.001$), presumably as a result of pancreatic islet dysfunction and deterioration. Contrastingly, KBP-042 and sCT treatment at doses of 1 and 2 mg/kg preserved pancreatic glucagon content, although only at KBP-042 dose of 2 mg/kg was statistical significance reached when compared to vehicle (Fig. 5D). Finally, KBP-042, similar to sCT, significantly reduced glucose-stimulated insulin-secretion (GSIS) (Fig. 5E) and arginine-stimulated glucagon-secretion (ASGS) (Fig. 5F) in isolated islet from lean healthy rats.

**Chronic oral KBP-042 treatment enhances insulin sensitivity superior to oral sCT in ZDF rats.**

To clarify whether KBP-042 preserved pancreatic function by alleviating insulin resistance, we performed insulin tolerance testing. We found that chronic treatment with KBP-042 and sCT led to significant improved insulin tolerance for all doses tested during IPITT illustrated with the highest dose of 2 mg/kg sCT and KBP-042 (Fig. 6A). However, as glucagonostatic action could influence counter regulation, and thus bias the true effect on insulin action, we compared blood glucose iAUC values during IPITT and only KBP-042 doses at 1 and 2 mg/kg, and sCT at 2 mg/kg, were significantly reduced when compared to vehicle (Fig. 6B). In confirmation, during short-term IVITT, that KBP-042 at doses of 1 and 2 mg/kg significantly enhanced insulin action and glucose disposal (Fig. 6C), with superiority to sCT at the 1 mg/kg dose.

**DISCUSSION**

Peptides with amylin-like properties, such as pramlintide (44), and recently davalintide (32), have shown promise as pharmacological intervention in obesity and type 2 diabetes; however, they are somewhat limited by poor efficacy *in vivo*, and fail to reduce fasting blood glucose. The present
studies introduce a novel oral DACRA, namely KBP-042, which was identified through \textit{in vitro} receptor pharmacology screening and \textit{in vivo} testing in animal models of obesity and type 2 diabetes.

The \textit{in vitro} receptor screening showed that eel calcitonin and sCT induced activation of the receptors to a similar extent (data not shown), thus sCT was chosen as comparator to KBP-042 due to our previous proof of concept studies for oral sCT in DIO and ZDF rats (14; 15). In general, KBP-042 demonstrated a more potent activation of the amylin and calcitonin receptors than sCT, resulting in more pronounced induction of cAMP and \(\beta\)-arrestin signaling. Additionally, KBP-042 did not activate the CGRP-receptor, which contrasts native amylin and analogues observed to exert off-target activation of the CGRP-receptor (data not shown and (33)). Activation of CGRP-receptors exerts detrimental effects on insulin secretion (43), as well as vasodilatation, which has been linked to migraines (6), thus the more restricted agonist profile of KBP-042 could be of therapeutic importance, although this awaits further investigation.

The short-term dose-response study in DIO rats, a polygenic obese prediabetic animal model (28), confirmed biological activity of oral KBP-042 \textit{in vivo} and demonstrated dose-dependent reductions in food intake, body weight and improved glucose tolerance, consistent with previous data obtained for oral sCT in DIO rats (15). To explore whether the anorectic and leptogenic actions of oral KBP-042 were sustainable and superior to oral sCT, we performed a longer-term intervention study in DIO rats. Oral KBP-042 induced a sustained reduction in food intake throughout the study period, which was superior to that of oral sCT. Furthermore, the reduction also appears to exceed that observed in previous studies of both oral sCT (15), and injectable amylin analogues (32). We speculate this pronounced reduction in food intake could partly be attributed to the rather unique prolonged or almost irreversible binding of sCT and analogues to both amylin receptors located in area postrema/nucleus tractus solitarii (AP/NTS) of the brain stem (Hilton et al, 2000)(31).
further support by a blunting of the anorectic effect of sCT when lesions are introduced in AP/NTS (30). Importantly, oral KBP-042 was superior to both oral sCT and pair-feeding in inducing substantial weight loss in DIO rats, which could indicate an additive effect on energy expenditure, as reported for amylin agonism under certain situations (36; 45), although this was not formally investigated. Supporting previous findings for oral sCT (14; 15), we found a concomitant reduction in hyperinsulinemia and hyperleptinemia in conjunction with improved glucose homeostasis, which could indicate a direct alleviation of insulin and leptin resistance to improve metabolic and glycemic control. In support, amylin administration was recently demonstrated to improve the insulin-sensitizing effect of leptin in DIO mice (22), and to enhance the effect of leptin on energy balance and glycemic control in insulin-resistant diabetic mice (26). Hence, accumulating evidence now highlights the importance of peptides with amylin-like properties in modulation of adiposity signals and energy homeostasis (29). Of interest, KBP-042 reduced GSIS when added directly to isolated islets and, furthermore, that oral KBP-042 reduced the hyperinsulinemic response to glucose, albeit concomitantly alleviated glucose intolerance during OGTT in DIO rats. Interestingly, a previous study indicated that human calcitonin possessed the ability to reduce glucose-stimulated insulin secretion by isolated islets (22), a finding that, due to the calcitonin receptor specific nature of human calcitonin, could indicate that activation of the calcitonin receptor is an important part of the overall response, although more studies are needed to clarify this. Thus, we speculate, that oral KBP-042 directly relieves the β-cell stress in an insulin-resistant state by enhancing insulin action, a concept also recently demonstrated for the insulin-sensitizer Pioglitazone (23; 24).

To further explore whether oral KBP-042 could exert anti-diabetic efficacy, as reported for oral sCT (14), we performed a multiple dose head-to-head study in ZDF rats, a monogenic obese animal model of type 2 diabetes (39). Oral KBP-042 attenuated diabetic hyperglycemia and dose-
dependently reduced fasting and non-fasting blood glucose in conjunction with HbA1c levels. Furthermore, oral KBP-042 markedly improved glucose tolerance and insulin action/sensitivity. As observed in DIO rats, superiority of KBP-042 to sCT, was demonstrated at a treatment dose of 1 mg/kg of KBP-042, which resembled the efficacy of sCT at 2 mg/kg dose. As expected from our previous study (14), the anti-glycemic effect of this class of therapeutic peptides was associated with a prevention of hypoinsulinemia, which indicates preserved pancreatic β-cell function and, importantly, resulted in increased pancreatic insulin content. Furthermore, the pronounced hyperglucagonemia in the ZDF rat was markedly reduced by oral KBP-042 and concomitantly pancreatic glucagon content was normalized towards levels observed in control rats. While the effects on pancreas in the ZDF rats appear contradictory to those in the DIO rats, these are, previously published (18, 20), related to the highly different phenotype of the two animal models. The reason for the increase in fasting plasma insulin in the ZDF lies within the protection against β-cell loss, and therefore is an indirect effect (20). In support of a directly mediated glucagonostatic action, we confirmed that KBP-042 reduced excessive glucagon secretion during arginine-exposure in isolated islets, which has been previously reported for amylin at high doses (2), although a centrally mediated effect most likely also is prevalent for amylin-like peptides (40). Irrespectively, the findings suggest that oral KBP-042 preserves pancreatic secretory function by decelerating the α-cell and β-cell stress induced by hyperglycemia and impaired insulin action. During the study of the glucoregulatory mode of action, we found strong evidence towards enhanced insulin action and increased glucose disposal during IVITT, thus introducing an insulin-sensitizing effect, and again KBP-042 was superior to sCT. Although short-term IVITT is highly correlated with clamp (45), a more formal investigation will have to clarify whether this insulin-sensitizing effect can be attributed to enhanced hepatic and/or peripheral action.
In conclusion, oral KBP-042 is a novel DACRA with prominent pharmacological effects on energy balance and glucose homeostasis, including a dual modulation of insulin action and glucagonostatic effects in animal models of obesity and type 2 diabetes. Hence, the present studies introduce oral KBP-042 as a novel pharmacological intervention in obesity and type 2 diabetes.
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Conflict of interest


Author contribution

K.V.A., M.F., M.A.K., and K.H designed the study.
K.V.A., M.F., and K.H. drafted the manuscript.
K.V.A., M.F., J.E.H., H.B.N., M.A.K., and K.H revised the manuscript.
FIGURE LEGENDS

FIG. 1. KBP-042 is a specific in vitro dual amylin and calcitonin receptor agonist and improves energy balance and glucose tolerance during short-term treatment in DIO rats.

Dose-range KBP-042 induction of β-Arrestin in (A) calcitonin receptor (CT-R), (B) amylin receptor (AMY-R), and (C) calcitonin gene-related peptide receptor (CGRP-R) over-expressing cell lines. Short-term treatment effect of oral dose-range KBP-042 in DIO rats in regards to (D) body weight, (E) food intake, and (F) glucose tolerance and (G) incremental area under the glucose curve (iAUC) during OGTT. n = 10 rats per group. *P < 0.05, **P < 0.01, ***P < 0.001. Statistical analysis between groups was evaluated by one-way ANOVA (D, E, F and H), and two-way ANOVA (A,B,C,G) post hoc analyses. Data are means ± SEM.

FIG. 2. Longer-term administration of oral KBP-042 improves energy and glucose homeostasis and adiposity hormones in DIO rats. A: Body weight during 4 weeks intervention with oral doses (1 mg; b.i.d.) of salmon calcitonin (sCT) and KBP-042. A pair-feeding group to KBP-042 (Pair-fed) was included and dosed similar to oral vehicle (5-CNAC, b.i.d.). B: Weekly food intake. C: Change in fasting plasma glucose at study end. D: Homeostatic model assessment - insulin resistance (HOMA-IR). E: Fasting plasma leptin and F: Plasma insulin at study end. n = 9-10 rats per group. *P < 0.05, **P < 0.01, ***P < 0.001 vs vehicle. †P < 0.05, ††P < 0.01, †††P < 0.001 vs sCT. ‡P < 0.05, ‡‡P < 0.01, ‡‡‡P < 0.001 vs Pair-fed. §P < 0.05 vs sCT (Student t test). Statistical analysis between groups was evaluated by one-way ANOVA (B, C, D, E, and F) and two-way ANOVA (A) post hoc analyses. Data are means ± SEM.
FIG. 3. Longer-term administration of oral KBP-042 improves glucose tolerance and pancreatic glucoregulatory hormones during OGTT in DIO rats. A: Plasma glucose response and B: iAUC values (0-240 min) during OGTT. C: Plasma insulin response and D: iAUC values (0-120 min) during OGTT. E: Plasma glucagon response and F: iAUC values (0-30 min) during OGTT. n = 9-10 rats per group. *P < 0.05, **P < 0.01, ***P < 0.001 vs vehicle. †P < 0.05, ††P < 0.01, †††P < 0.001 vs Pair-fed. ‡P < 0.05 vs sCT. Statistical analysis between groups was evaluated by one-way ANOVA (B, D, F) and two-way ANOVA (A, C, E) post hoc analyses. Data are means ± SEM.

FIG. 4. Chronic administration of oral KBP-042 attenuates diabetic hyperglycemia and improves glucose tolerance in ZDF rats. Overnight fasting (A) and non-fasting (B) blood glucose, and glycosylated hemoglobin (HbA1c) (C) following 7 weeks of multiple dose (b.i.d.) oral salmon calcitonin (sCT) or KBP-042. OGTTs performed after overnight fasting in treatment groups administered 2 mg/kg (D) of sCT or KBP-042 for 5 weeks. Incremental area under the glucose curve (iAUC)(E) during OGTT for treatment groups administered 0.25 mg/kg, 0.5 mg/kg, 1 mg/kg and 2 mg/kg of oral sCT or KBP-042. n = 8-10 rats per group. Statistical analysis between groups was evaluated by one-way ANOVA (A, B, C, E) and two-way ANOVA (D) post hoc analyses. *P < 0.05, **P < 0.01, ***P < 0.001 vs vehicle. ‡P < 0.05, ‡‡P < 0.01 vs sCT 1 mg/kg. §P < 0.05 vs sCT 1 mg/kg (Student t test). Data are means ± SEM.
FIG. 5. Chronic administration of oral KBP-042 modulates pancreatic glucoregulatory hormones in ZDF rats. Fasting plasma insulin (A), fasting plasma glucagon (B), pancreatic insulin content (C), and pancreatic glucagon content (D) after 7 weeks of multiple dose (b.i.d.) oral salmon calcitonin (sCT) or KBP-042. \( n = 8-10 \) rats per group. Acute in vitro effect of KBP-042 and sCT (10 nM) on glucose-stimulated insulin secretion (E), and arginine-stimulated glucagon secretion (F) in isolated islets from lean healthy rats. (8 different wells in 2-3 separate experiments). Statistical analysis between groups was evaluated by one-way ANOVA post hoc analyses. *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \) vs vehicle. #\( P < 0.05 \) vs sCT 1 mg/kg. §\( P < 0.05 \) vs sCT 1 mg/kg (Student t test). Data are means ± SEM.

FIG. 6. Chronic administration of oral KBP-042 enhances insulin action in ZDF rats. Blood glucose response to IPITTs in treatment groups administered 0.25 mg/kg, 0.5 mg/kg, 1 mg/kg (A) and 2 mg/kg of oral salmon calcitonin (sCT) or KBP-042 for 6 weeks. Blood glucose tAUC values for treatment groups administered 0.25 mg/kg, 0.5 mg/kg, 1 mg/kg and 2 mg/kg of oral salmon calcitonin (sCT) or KBP-042 IPITT (B), and rate constant for blood glucose disappearance (Knitt) (C) during short-term IVITT after 7 weeks of treatment. \( n = 8-10 \) rats per group. Statistical analysis between groups was evaluated by one-way ANOVA (B, C) and two-way ANOVA (A) post hoc analyses. *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \) vs. vehicle. Data are means ± SEM.
Table 1: Amino acid sequence comparison of sCT, KBP-042 and eCT. In addition, KBP-042 has an acetyl-modification at the N-terminal end for improved peptide stabilization.

| Salmon Calcitonin | - | 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 | NH2 |
| 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 | NH2 |
| Eel Calcitonin   | - | 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 | G | T | P | NH2 |

Table 2: In Vitro Receptor Binding and Activity of sCT and KBP-042

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<th>Ligand</th>
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<tr>
<td></td>
<td>cAMP EC50</td>
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<tr>
<td>sCT</td>
<td>1.7 ± 1.3 (3)</td>
<td>6.7 ± 1.0 (3)</td>
<td>3.9 ± 1.1 (3)</td>
<td>14.4 ± 2.0 (3)</td>
<td>1.8 ± 1.3 (3)</td>
<td>5.1 ± 1.1 (3)</td>
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<tr>
<td>KBP-042</td>
<td>0.8 ± 1.5 (3)*</td>
<td>4.8 ± 1.0 (3)***</td>
<td>3.2 ± 1.2 (3)</td>
<td>8.4 ± 1.9 (3)</td>
<td>4.5 ± 1.1 (3) **</td>
<td>2.9 ± 1.2 (3)*</td>
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<td>Fold difference (KBP-042/sCT)</td>
<td>2.2</td>
<td>1.4</td>
<td>1.2</td>
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<td>0.4£</td>
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Table 2: In vitro screening data of sCT and KBP-042
EC50 values on cAMP production and β-arrestin recruitment as well as IC50 values from competitive binding with 0.25 nM 125I-sCT. All parameters measured on cells expressing human CTR or human AMY-R. The data are presented as mean ± SD. The values in parentheses are the number of individual experiments performed. In most instances, no difference in EMAX for sCT and KPB-042 was observed.

* p < 0.05, ** p < 0.01, *** p < 0.001 KPB-042 compared to sCT.
£ Lower observed EMAX for sCT compared to KPB-042
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MK, Fineman M, Porter L and Schernthaner G. Exenatide once weekly versus liraglutide once daily


