Original Article

MOLECULAR METABOLISM

KBP-066A, a long-acting dual amylin and calcitonin receptor agonist, induces weight loss and improves glycemic control in obese and diabetic rats

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ABSTRACT

Objective: Dual amylin and calcitonin receptor agonists (DACRAs) are novel therapeutic agents that not only improve insulin sensitivity but also work as an adjunct to established T2DM therapies. DACRAs are currently administered once daily, though it is unknown whether DACRAs with increased plasma half-life can be developed as a once-weekly therapy.

Methods: The *in vitro* potencies of the KBP-066A and KBP-066 (non-acylated) were assessed using reporter assays. Acylation functionality was investigated by a combination of pharmacokinetics and acute food intake in rats. *in vivo* efficacies were investigated head-to-head in obese (HFD) and T2D (ZDF) models.

Results: In *in vitro*, KBP-066A activated the CTR and AMY-R potently, with no off-target activity. Acylation functionality was confirmed by acute tests, as KBP-066A demonstrated a prolonged PK and PD response compared to KBP-066. Both compounds induced potent and dose-dependent weight loss in the HFD rat model. In ZDF rats, fasting blood glucose/fasting insulin levels (tAUC) were reduced by 39%/50% and 36%/47% for KBP-066 and KBP-066A, respectively. This effect resulted in a 31% and 46% vehicle-corrected reduction in HbA1c at the end of the study for KBP-066 and KBP-066A, respectively.

Conclusions: Here, we present pre-clinical data on an acylated DACRA, KBP-066A. The *in vivo* efficacy of KBP-066A is significantly improved compared to its non-acylated variant regarding weight loss and glycemic control in obese (HFD) and obese diabetic rats (ZDF). This compendium of pre-clinical studies highlights KBP-066A as a promising, once-weekly therapeutic agent for treating T2DM and obesity.

Keywords DACRA; Once-weekly; T2DM; Obesity; HFD rats; ZDF rats

1. INTRODUCTION

Advances in the therapeutic peptide development of metabolic conditions now guide peptide-based inventions toward a prolonged plasma half-life and once-weekly dose regimen [1—4]. However, the transition from a once-daily to a once-weekly treatment modality is a pharmacological challenge, and not all peptide hormones necessarily benefit from an increase in *in vivo* exposure [5,6]. Hence, this development requires careful investigation. In contrast, the benefits of therapeutics with protracted plasma half-lives are not only seen as an improvement in patient compliance [7—9] but also as improved treatment efficacy and safety [10—12].

Dual amylin and calcitonin receptor agonists (DACRAs) are somewhat unique in the field of novel treatment modalities for T2DM, as DACRAs improve insulin sensitivity [13], and, like other amylin agonists, also work as an adjunct to other established T2DM therapies, such as metformin and GLP-1 analogs [14—17]. These traits are coveted, as the field lacks novel insulin sensitizers, and combination treatments have seen an increased interest in T2DM drug development as a path to improve efficacy further [18].

Until now, the DACRAs used *in vivo* have required daily administration to exert their effects. Animal studies have demonstrated that DACRAs possess metabolic efficacy (beyond amylin) on several classical amylin-related effects, including body weight loss and the improvement of postprandial glucose control [14,19—22]. Furthermore, DACRAs elicit positive effects in animal models of T2DM by markedly improving glycemic status and HbA1c levels [14,19,20]. Consequently, it remains unknown whether DACRAs with a longer plasma half-life can be developed for once-weekly administration.

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Abbreviations: AMY-R, Amylin receptor; CTR, Calcitonin receptor; DACRA, Dual amylin and calcitonin receptor agonist; HFD, High-fat diet; OGTT, Oral glucose tolerance test; T2DM, Type 2 diabetes; ZDF, Zucker diabetic fatty; KBP, KeyBioscience Peptide

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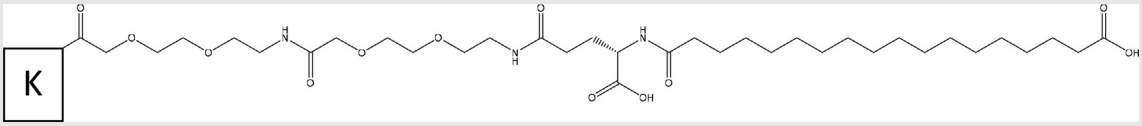
Table 1 — DACRA Peptide Sequences.

KBP-066: ***Ac***-CSNLSTC***X***LGRLSQDLHRLQTYPKTDVGANAP-***NH2***
KBP-066A11.03: ***Ac***-CSNLSTC***X***LG***K***LSQDLHRLQTYPKTDVGANAP-***NH2***
Ac-: Acetylation of the N-terminus
X: AiB (2-Aminoisobutyric acid)
K: OEG-OEG-γGlu-C18 diacid acylation complex conjugated to the lysine side chain
-NH2: Amidation of the C-terminal COOH group
OEG-OEG-γGlu-C18 diacid



To address the potential benefits of protracted plasma half-life on DACRAs, KBP-066A was developed based on an extensive screening process focusing on optimizing acylation size and position as a function of ligand activity (See patent application [23]).
This article characterizes KBP-066A through a series of *in vitro* and *in vivo* studies, both in acute and chronic settings. KBP-066A was compared head-to-head with its once-daily, non-acylated variant KBP-066 in rat models of obesity and T2DM to establish efficacy.

2. MATERIALS AND METHODS

2.1. Peptide therapy

Synthetic KBP-066A and KBP-066 (SynPeptide, Shanghai, China. Purity >95%) were dissolved in saline (NaCl 0.9%) for subcutaneous (s.c.) delivery. Peptide sequences and modifications are summarized in Table 1. The doses chosen for KBP-066 are based on previous comparable DACRA studies in animal models of both obesity and T2DM [15,24,25].

2.2. Cell lines

The following receptor cell lines overexpressing human receptors were used in *in vitro* potency screening: Calcitonin receptor (CTR; U2OS CALCR, 93-0566C3 DiscoverX), Amylin 3 receptor (AMY3R; CHO K1 CALCR RAMP3, 93-0268C2, DiscoverX), CGRP receptor (CHO K1 CALCRL RAMP1, 93-0269C2, DiscoverX), Adrenomedullin 2 receptor (AM2R; CHO K1 CALCRL RAMP3, 93-0252C2, DiscoverX).

2.3. β-arrestin quantification

The assay was conducted in white 384-well plates (784080, Greiner Bio-One). 2,500 cells/well were seeded in a 10 μL cell-type-specific medium the day prior to the experiment. The quantification of the GPCR receptor-mediated β-arrestin signal was measured according to the manufacturer's instructions, using PathHunter ® Detection Kit (93-0001, DiscoverX).

2.4. Cell-based competitive ligand binding

The competitive ligand binding was conducted in the AMY3R cell line using ^{125}I-(Tyr22)-sCT (NEX423, Perkin Elmer) as a hot ligand. 15,000 cells/well were seeded into 96-well culture plates the day prior to the experiment. Initial cold concentrations were 200 nM KBP-066 and 25 μM KBP-066A. Both curves are 5-fold dilution steps down and a vehicle. The competitive binding assay was performed as previously described [26].

2.5. KBP-066 and KBP-066A detection sandwich ELISA

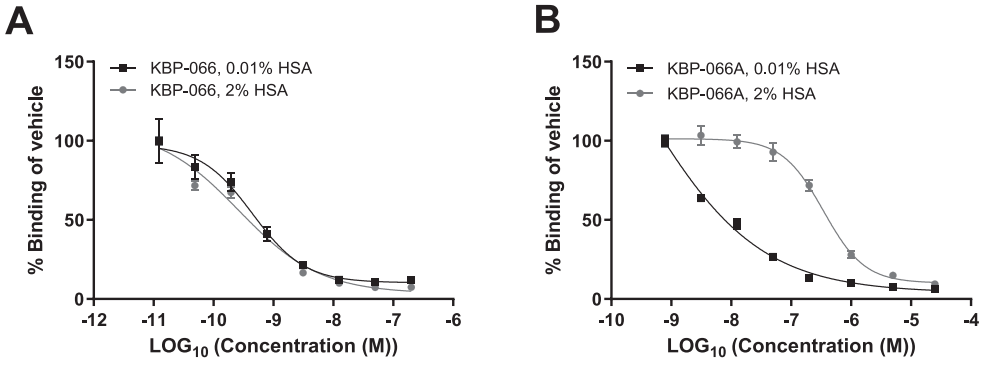
To detect KBP-066 and KBP-066A in rat plasma, two in-house sandwich ELISAs were developed using two in-house antibodies (ab) targeting the N-terminal (HRP-labeled detector ab) and C-terminal end (Biotinylated coater ab) of KBP-066/KBP-066A and then optimized for measurement in rat plasma. Abs had previously been used to detect KBP-042, which shares N- and C-terminus with KBP-066/KBP-066A [24]. KBP-066 or KBP-066A was used as calibrators for their respective assays. The calibrator was diluted in plasma-EDTA collected from in-house lean rats to mimic a sample matrix. The KBP-066 ELISA used a 500 ng/mL coater and detector and 20-0 nM calibrator range (2-fold dilution), whereas the KBP-066A ELISA used a 300 ng/mL coater and detector and 100-0 nM calibrator range (2-fold dilution). Both ELISAs were performed as previously described [24] regarding non-peptide materials, volumes, and times. T_{max} (time for compound peak) and $T^1/_2$ were used as parameters to quantify data. $T^1/_2$ was calculated as $T^1/_2 = Ln2/K$, with K calculated in GraphPad Prism 8 based on the PK curves (not shown).

2.6. Animal studies

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). Animals were housed pairwise in standard type IV cages (Scanbur A/S, Karlslunde, Denmark) under a controlled temperature (21-23 °C, 55-65% relative humidity) and normal 12-hour light—dark cycle with *ad libitum* access to food and water.
The dosing of the rats every 3rd day (q.3.d.) was used as a surrogate for "Once-weekly dosing in man" to compensate for the higher metabolic rate of rats, a strategy previously used in the development of once-weekly treatment modalities preclinically [3,27].

2.6.1. Acute studies in Sprague Dawley rats

Acute studies were conducted in obese male Sprague Dawley rats (Envigo, Venray, The Netherlands) obtained at 5-6 weeks of age and fed a 60 kcal% fat high-fat diet (HFD; #D12492, Research Diets, New Brunswick, NJ) from their arrival and throughout the studies to induce obesity. Rats were considered obese at >500 g. Prior to the tests, rats were allocated into treatment groups based on body weight (n = 4—10, n is the same in each experiment). In all acute studies, rats received a single s.c. injection of the test compounds at time 0 (details about compounds and concentrations are stated in the result section and figures). In the study recording both pharmacokinetic and pharmacodynamic compound profiles, body weight and food intake were measured daily throughout the study prior to sampling blood for the pharmacokinetic profile. To assess the acute effect of the compounds on gastric emptying rate, overnight fasted rats received an s.c. injection of the compound and a bolus of glucose (Sigma—Aldrich, Copenhagen, Denmark) and acetaminophen



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Compound	Plasma Half-life T½ (hours)	Time for peak plasma concentration T_{max} (hours)
KBP-066 (4 nmol/kg)	0.44 ± 0.10 (6)	0.33 (4)
KBP-066A (4 nmol/kg)	23.4 ± 2.0 (6)	8.0 (4)

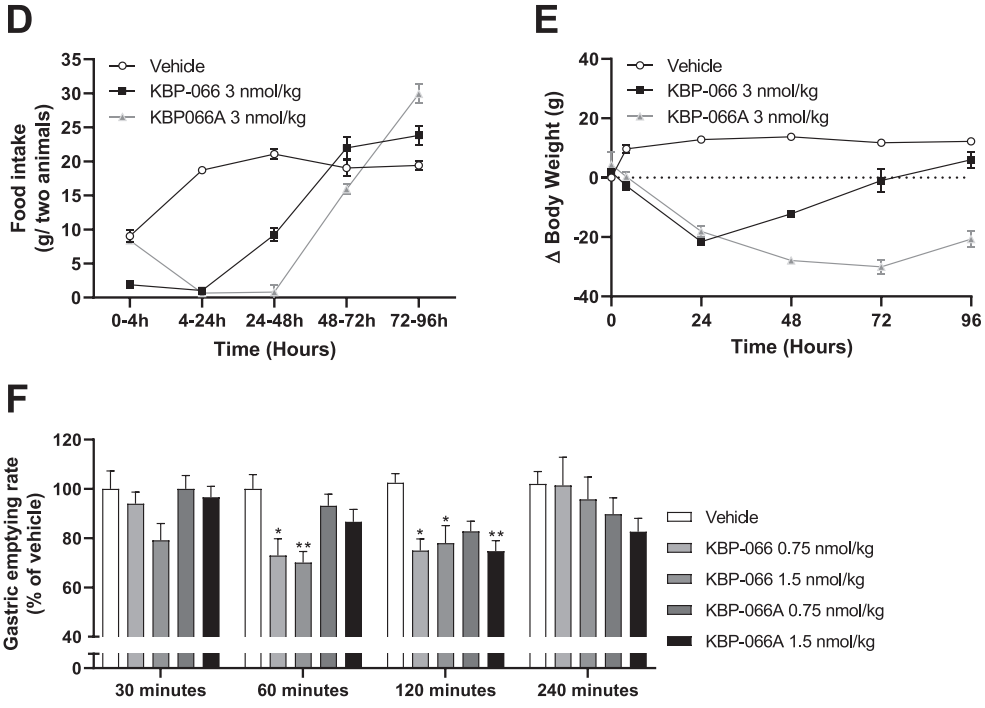

Figure 1: PD and PK characteristics of KBP-066 and KBP-066A *in vitro* and in an acute setting in HFD SD rats. Competitive ligand binding assay using 0.25 nM radio-labeled salmon calcitonin (^{125}I-sCT) with KBP-066 (A) or KBP-066A (B) as competitors in the presence of 2% or 0.1% human serum albumin (HSA). Assay was conducted in triplicates. (C) $T^1/_2$ and Tmax PK values in hours for KBP-066 or KBP-066A after s.c. injection (4 nmol/kg) in HDF SD rats, n = 4 (KBP-066) or n = 6 (KBP-066A) rats per group. 96-hour PD profile for 3 nmol/kg s.c. KBP-066 and 3 nmol/kg s.c. KBP-066A on food intake (D) and body weight change (E) in HFD SD rats, n = 4 rats per group. Acute effect of KBP-066 and KBP-066A on gastric emptying shown as % of vehicle (F), n = 8 rats per group. Statistical analysis between groups (E) was performed as one-way ANOVA and Tukey's multiple comparison test (60 and 240 min) and as Kruskal–Wallis test and Dunn's multiple comparisons test (30 and 120 min) with the following annotations: *P < 0.05, **P < 0.01 versus vehicle. All data are presented as mean ± SEM.

(Sigma–Aldrich, Copenhagen, Denmark; p.o.) at time 0. EDTA blood samples were collected from the tail vein before the administration (0 min) and the following 30, 60, 120, and 240 min. The level of acetaminophen in the plasma was used as an estimate of the gastric emptying rate.

2.6.2. Chronic study in high-fat diet-fed Sprague Dawley rats

Thirty male Sprague Dawley rats (Envigo, Venray, The Netherlands) were obtained at 5-6 weeks of age and fed a 60 kcal% fat high-fat diet (HFD; #D12492, ResearchDiets, New Brunswick, NJ) from their arrival and throughout the study. Rats were allowed to gain body weight for

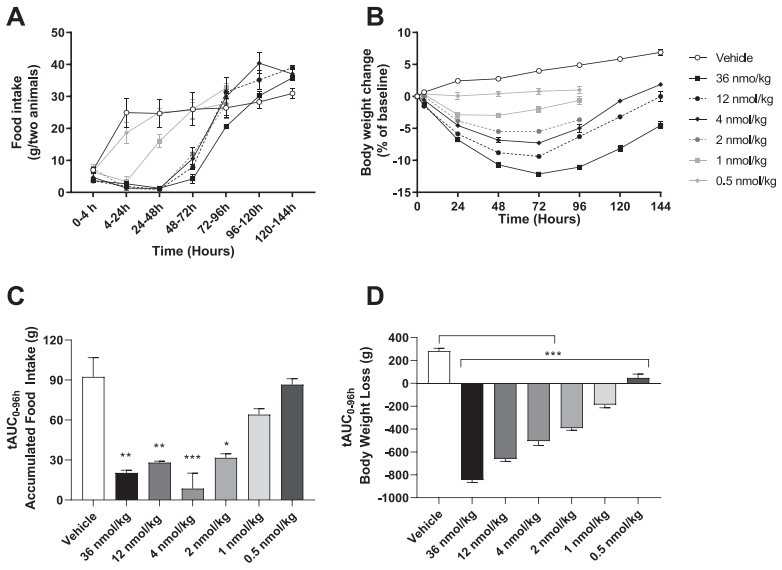

Figure 2: Acute effect of KBP-066A on food intake and body weight in a broad dose response Change in daily food intake (A) and body weight (B) as a function of a single s.c. injection of KBP-066A of either 36, 12, 4, 2, 1 or 0.5 nmol/kg tAUCs (0–96 h) for food intake (C) and body weight loss (D) were calculated to quantify effect. Graphs are composites and consist of several studies ranging from 96 to 144 h in length, n = 6–10 rats per group. Statistical analysis between groups was performed as one-way ANOVA and Tukey's multiple comparison test with the following annotations: *P < 0.05, **P < 0.01, ***P < 0.001 versus vehicle. All data are presented as mean ± SEM.

20 weeks before they were randomized into treatment groups according to body weight (n = 6 rats/treatment group). At the study start, the rats weighed 528.2 ± 27.9 (SD) g. The vehicle group received s.c. injections of saline (0.9%), whereas the treatment groups received s.c. injections of either KBP-066 1.5 nmol/kg once-daily (q.d.) or KBP-066A 1 nmol/kg, KBP-066A 2 nmol/kg, or KBP-066A 4 nmol/kg every third day (q.3.d.; to simulate weekly dosing in humans) for 4 weeks. Body weight and food intake were monitored daily throughout the study. At the study end, an oral glucose tolerance test (OGTT; 2 g/kg, 4 mL/kg) was performed in overnight fasted rats followed by euthanization by exsanguination. Epididymal, perirenal, and subcutaneous inguinal fat depots were surgically removed and weighed. Homeostasis model assessment of insulin resistance (HOMA-IR) analysis was calculated and used to estimate insulin resistance using the formula HOMA-IR=FI (μU/ml) * FBG (mM)/22.5, where FI and FBG is the fasting insulin and fasting blood glucose, respectively.

2.6.3. Intervention study in ZDF rats

Fifty male Zucker diabetic fatty (*fa/fa*; ZDF; Charles River Laboratories, Lyon, France) were obtained at 5-6 weeks of age and were fed a Purina Laboratory Diet (#5008, LabDiet, St. Louis, MO, USA) from their arrival and throughout the study. Rats were left idle to develop diabetes before being allocated into treatment groups primarily according to fasting blood glucose levels (6 h) and secondarily according to body weight (n = 10 rats/treatment group). At the study start, the rats weighed 334.1 ± 15.5 (SD) g and had a fasting blood glucose level of 14 ± 5.1 (SD) mM. The vehicle group revived s.c. injections of saline (0.9%), whereas the treatment groups received s.c. injections of KBP-066 1.5 nmol/kg once-daily (q.d.), KBP-066A 0.75 nmol/kg, KBP-066A 1.5 nmol/kg, or KBP-066A 3 nmol/kg once-weekly (q.3.d.) for eight weeks. Body weight and food intake were monitored daily for the initial two weeks of the study and then once weekly throughout the study. Fasting blood glucose levels (6 h) were measured weekly, fasted plasma insulin every other week, and HbA1c levels at baseline and study end. After seven weeks of treatment, an oral glucose tolerance test (OGTT; 1 g/kg, 2 mL/kg) was performed in rats fasted for 11 h. At the study end, rats were fasted 6 h, blood was sampled, and rats were euthanized by exsanguination. Epididymal, perirenal, and subcutaneous inguinal fat depots were surgically removed and weighed, and pancreases were surgically removed and stored to analyze insulin content.

2.7. Glucose tolerance test

Oral glucose tolerance tests (OGTT) were performed in overnight fasted (14 h in HFD and 11 h in ZDF) rats, 24 h post-dosing. A glucose bolus (Sigma–Aldrich, Copenhagen, Denmark) was administered p.o. gavage at time 0. EDTA blood samples were collected from the tail vein before the glucose challenge (0 min) and following 15, 30, 60, and 120 min post-glucose challenge. Blood glucose was monitored at times 0, 15, 30, 60, 120, and 180 min post-glucose challenge.

2.8. Biochemical analysis

Blood samples were collected in EDTA tubes and centrifuged at 5000 rpm for 10 min at 4 °C, and plasma was kept at −20 °C until further analysis. Blood glucose was monitored by an Accu-Check® Avia monitoring system (Roche Diagnostics, Rotkreuz, Switzerland). HbA1c levels were measured by a DCA Vantage Analyzer (Siemens, Erlangen, Germany). Plasma levels of insulin (Mercodia Rat Insulin ELISA, Mercodia AB, Uppsala, Sweden) were analyzed according to the manufacturers' instructions. The gastric emptying rate was determined by measuring the acetaminophen in the plasma (Acetaminophen Forensic ELISA kit, Neogen Toxicology, KY, USA).

2.9. Statistical analysis

All analyses were performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA). For time-dependent data, total area under the curve (tAUC) or incremental area under the curve (iAUC) was calculated, and statistical analyses were conducted on the AUCs to describe group differences during the entire time period. Group differences were assessed using a one-way ANOVA followed by Tukey's

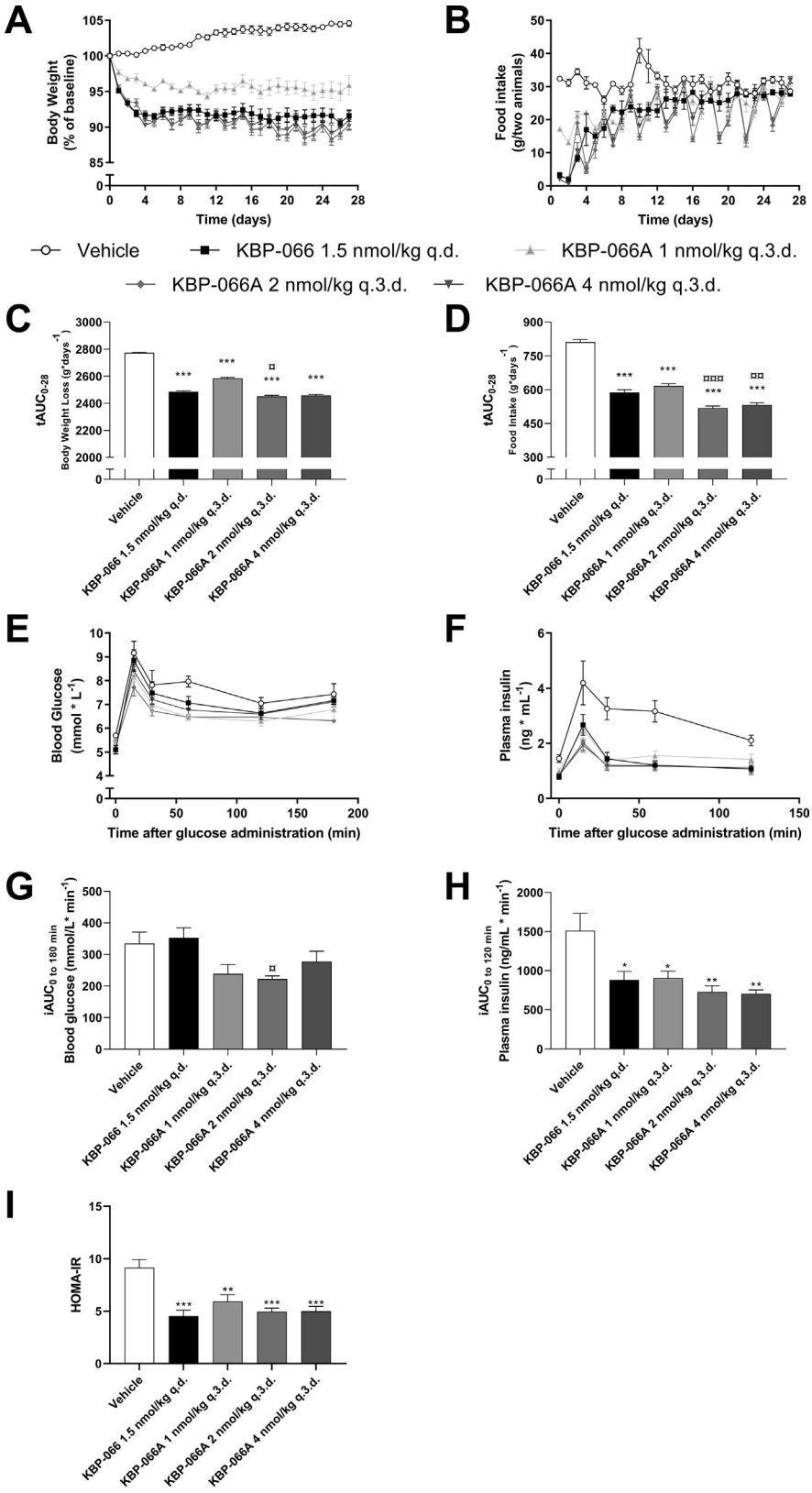

Figure 3: Four-week study in HDF SD rats to investigate DACRAs' effect on food intake and body weight as well as glycemic control. Body weight shown as % change from baseline (A) and food intake (g/two animals) during the study (B). The total area under the curve (tAUC) is shown for body weight loss (C) and food intake (D). Blood glucose (D) and plasma insulin levels (E) during the OGTT performed at study end after four weeks of treatment. The incremental area under the curve (iAUC) is shown for glucose (G) and plasma insulin (H). Insulin resistance calculated as HOMA-IR (I). n = 6 rats per group. Statistical analysis between groups was performed as one-way ANOVA and Tukey's multiple comparison test with the following annotations: *P < 0.05, **P < 0.01, ***P < 0.001 versus vehicle and ¤P < 0.05 versus KBP-066 (1.5 nmol/kg q.d.). All data are presented as mean ± SEM.

Table 2 — Additional HFD and ZDF study parameters. tAUC values of body weight (BW) loss and total food intake (FI) over the course of the studies, and weights of three different types of adipose tissue (AT) depots at study end in HFD rats (Figure 3) and ZDF rats (Figure 4 + 5). HFD (n = 6), ZDF (n = 10).

(nmol/kg)	HFD Rats					ZDF Rats				
	Vehicle	KBP-066 (1.5)	KBP-066A (1)	KBP-066A (2)	KBP-066A (4)	Vehicle	KBP-066 (1.5)	KBP-066A (0.75)	KBP-066A (1.5)	KBP-066A (3)
tAUC, BW ($g*days^{-1}$)	2773 ± 4	2485 ± 7***	2584 ± 7***	2452 ± 6***¤	2458 ± 8***	6475 ± 103	6454 ± 81	6180 ± 67	6405 ± 89	6409 ± 122
tAUC, FI ($g*days^{-1}$)	811 ± 11	588 ± 12***	617 ± 10***	518 ± 6***¤¤¤	532 ± 10***¤¤	4453 ± 123	3327 ± 105***	3815 ± 135**¤	3134 ± 111***	2898 ± 61***
Inguinal AT (g)	3.2 ± 0.5	3.3 ± 1.0	2.8 ± 0.3	1.9 ± 0.5	2.9 ± 1.4	4.4 ± 0.3	4.9 ± 0.3	4.8 ± 0.4	5.8 ± 0.5	6.3 ± 0.7
Epididymal AT (g)	5.4 ± 1.0	4.0 ± 0.8	4.4 ± 1.0	3.9 ± 1.1	4.2 ± 1.2	4.0 ± 0.2	4.5 ± 0.2	3.7 ± 0.1	4.1 ± 0.2	4.7 ± 0.2
Perirenal AT (g)	4.5 ± 0.6	2.5 ± 0.4***	3.5 ± 0.9	2.7 ± 0.7**	2.8 ± 1.0**	6.5 ± 0.3	7.2 ± 0.3	6.0 ± 0.3	6.1 ± 0.3	6.7 ± 0.4

Statistical analysis between groups was performed as one-way ANOVA and Tukey's multiple comparison test with the following selected annotations: **P < 0.01, ***P < 0.001 versus vehicle; ¤P < 0.05 versus KBP-066 (1.5 nmol/kg q.d.). All data are presented as mean ± SEM.

post-hoc test for multiple comparison of the parametric data. For non-parametric data, Kruskal—Wallis test with Dunn's post-hoc test was applied. The normality of the data distribution was determined by Anderson-Darling normality test. EC50 values were calculated in a GraphPad Prism 8 using the *"Equation: log(agonist) vs. response – Variable slope"* graph option. All figure data are represented with standard error of the mean (SEM), except body weight and FBG data at randomization, which is described with standard deviation (SD). In general, a value of $P < 0.05$ was considered statistically significant.

3. RESULTS

3.1. Acylation of KBP-066 affects albumin affinity, and ligand receptor potency, but not receptor activation profile

To characterize the potency of acylated KBP-066A compared to the non-acylated KBP-066, the ligands were compared head-to-head in an *in vitro* setting. To detect any off-target activity on two related calcitonin-like receptors, CGRP-R, and AM2-R and to assess their potency, both ligands were assessed on two calcitonin family receptors, CTR and AMY3R (Supplementary Table S1). In terms of potency, KBP-066 demonstrated a two- and eight-fold lower EC_{50} than KBP-066A for the CTR and the AMYR, respectively (Supplementary Table S1). KBP-066 demonstrated similar lower EC_{50} than KBP-066A in the absence of serum, although the absence of serum affected the cells, resulting in a general increase in EC_{50} (Supplementary Table S2). Both ligands were clearly more potent than the endogenous ligands, calcitonin and amylin, on the CTR and AMYR, respectively. As expected, KBP-066 was also more potent on both receptors than the natural DACRA, sCT (Supplementary Table S1) [19]. KBP did not demonstrate any off-target activity of significance on the CGRP-R or AM2-R (Supplementary Table S1). To investigate the effectiveness of the acylation, an *in vitro* competitive binding study in the presence of low (0.01%) and high (2%) human serum albumin (HSA) was conducted to confirm HSA binding. KBP-066 was not affected by the difference in HSA concentration and produced similar competition curves and IC_{50} values (Figure 1A). In contrast, the IC_{50} of KBP-066A increased 100-fold when the competition assay was conducted in 2% HSA compared to 0.01% HSA (Figure 1B).

3.2. Acylation of KBP-066 has pronounced acute effects on pharmacokinetics and pharmacodynamics *in vivo*

To address exposure, an *in vivo* pharmacokinetics study of KBP-066 and KBP-066A in HFD rats was conducted. PK profiles of KBP-066 and KBP-066A were generated after a single s.c. injection of 4 nmol/kg with either ligand. Injection with KBP-066 resulted in a T_{max} of 0.33 h and a $T_{1/2}$ of 0.44 h (Figure 1C). In contrast, KBP-066A demonstrated a vastly different time frame resulting in a T_{max} of 8 h, a $T_{1/2}$ of 23.4 h (Figure 1C).

KBP-066 markedly reduced food intake 4 h post-injection, an effect that lasted 24 h before it started to return toward the vehicle level (Figure 1D). This effect was reflected in the body weight, which was most pronounced after 24 h and completely returned to the baseline after 72 h (Figure 1D). Conversely, KBP-066A markedly reduced food intake for 48 h post-injection and returned to the vehicle level after 72 h (Figure 1D), whereas the body weight was still markedly reduced even 96 h post-injection (Figure 1E). The acute effect of KBP-066A on the gastric emptying rate was assessed head-to-head with KBP-066. KBP-066 (1.5 nmol/kg) reduced the gastric emptying rate at 30 min, whereas both doses (0.75 and 1.5 nmol/kg) significantly did so at 60 and 120 min. The effect of KBP-066A had a later onset, as it significantly reduced the gastric emptying rate at 120 min and the effect of the highest dose (1.5 nmol/kg) tended to last up to 240 min, where the effect of KBP-066 had stopped (Figure 1F).

To investigate the dose range for further *in vivo* testing, the acute effect of KBP-066A was assessed in a broad dose-range. The treatment effect on both food intake and body weight was clearly dose-dependent, with the effect of the highest dose KBP-066A (36 nmol/kg) lasting 120 h on food intake and 144 h on body weight, whereas the lowest dose (0.5 nmol/kg) only had a minor effect on both parameters (Figure 2A—B). These observations were mimicked in the corresponding tAUCs, demonstrating an even dose response on body weight loss (Figure 2D), whereas the tAUC on food intake (Figure 2C) reached a lower plateau using concentrations of 2 nmol/kg or higher. These data led us to narrow down the KBP-066A doses used in the chronic *in vivo* studies to a range from 0.75 to 4 nmol/kg (~3—~17 ng/kg) depending on the animal model.

3.3. Acylated KBP-066 dosed every third day improves body weight and glucose tolerance in obese rats

To investigate the effect of KBP-066A on body weight, food intake and glucose control in obese rats, HFD rats (baseline 528.2 ± 27.9 [SD] g) received treatment with increasing doses of KBP-066A (1, 2 and 4 nmol/kg q.3.d.), KBP-066 (1.5 nmol/kg q.d.), and vehicle (saline) for 4 weeks.

All treatments resulted in a significant and sustained reduction in body weight (Figure 3A), which lead to decreased tAUCs compared to

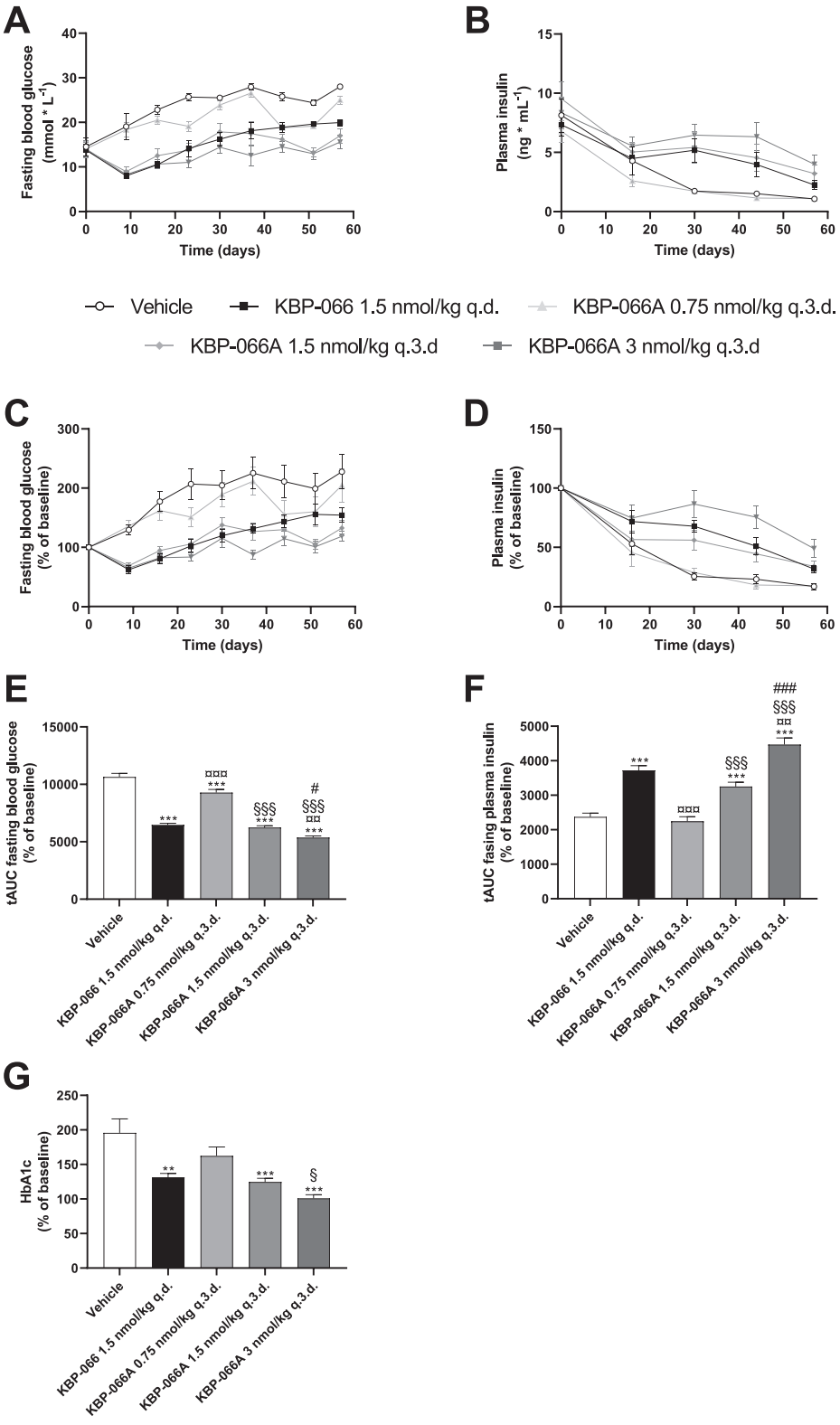

Figure 4: Eight-week study in diabetic ZDF rats investigating the effect on glucose control. Fasting blood glucose (A) and plasma insulin levels (B) during the study and shown as % change from baseline (C and D). Values at baseline: FBG 14 ± 5.1 (SD) mM and plasma insulin 8.0 ± 3.9 ng/mL (SD). The calculated total area under the curve (tAUC) is shown for fasting blood glucose (E) and plasma insulin (F). HbA1c levels shown as % change from baseline (G). n = 10 rats per group. Statistical analysis between groups was performed as one-way ANOVA and Tukey's multiple comparison test with the following annotations: **P < 0.01, ***P < 0.001 versus vehicle; ¤¤P < 0.01, ¤¤¤P < 0.001 versus KBP-066 (1.5 nmol/kg q.d.); §P < 0.05, §§§P < 0.001 versus KBP-066A (0.75 nmol/kg q.3.d.) and #P < 0.05, ###P < 0.001 versus KBP-066A (1.5 nmol/kg q.3.d.). All data are presented as mean ± SEM.

vehicle (Figure 3C, Table 2). Furthermore, the 2 nmol/kg dose of KBP-066A q.3.d. significantly improved body weight compared to KBP-066 q.d (Figure 3A and Table 2). Treatment with KBP-066A q.3.d. resulted in continuous fluctuations in body weight, reflecting the dosing regimen (Figure 3A). The effects on body weight were also reflected in the adiposity at the study end, though only the perirenal adipose tissue depots were significantly affected (Table 2). Furthermore, in the initial phase of the study, all treatments also resulted in a noticeable reduction in food intake, which leveled off after approximately one week of treatment (Figure 3B). All treatments significantly attenuated accumulated food intake, resulting in reduced tAUC compared to vehicle (Figure 3D, Table 2). Interestingly, the two highest doses of KBP-066A q.3.d. were also significantly reduced compared to KBP-066 q.d. Though the effect of KBP-066 q.d. was minor throughout the rest of the study, KBP-066A q.3.d., like body weight loss, resulted in continuous fluctuations in food intake, reflecting the dosing regimen (Figure 3B).

The treatment effect on glucose tolerance was assessed by an OGTT after four weeks of treatment. All treatments tended to improve oral glucose tolerance (Figure 3E). However, when calculating the iAUC, the effect was most pronounced in groups treated with KBP-066A q.3.d., though not statistically significant compared to vehicle (Figure 3G). All treatments resulted in significantly reduced plasma insulin levels during the test when calculating the iAUC (Figure 3F,H). In addition, the calculation of HOMA-IR (homeostasis model assessment of insulin resistance) showed that all treatments resulted in significantly reduced HOMA-IR (Figure 3I).

3.4. Acylated KBP-066 dosed every third day is superior to daily dosing in improving glycemic status in ZDF rats

To investigate the effect of KBP-066A on fasting blood glucose, plasma insulin and HbA1c levels, diabetic ZDF (baseline FBG 14 $\pm$ 5.1 [SD] mM and plasma insulin 8.0 $\pm$ 3.9 ng/mL [SD]) rats received treatment with increasing doses of KBP-066A (0.75, 1.5, and 3 nmol/kg q.3.d.) compared to KBP-066 (1.5 nmol/kg q.d.) or vehicle for 8 weeks.

Importantly, all treatments significantly lowered fasting blood glucose levels compared to vehicle, independent of weight loss (Table 2 and Figure 5G). However, the effect of the lowest dose of KBP-066A (0.75 nmol/kg) was minor (Figure 4A,C, and E). Interestingly, a molar equivalence (1.5 nmol/kg) KBP-066A was equally efficacious to KBP-066 even if dosed every third day. The highest dose of KBP-066A (3 nmol/kg) significantly improved fasting blood glucose levels compared to KBP-066 q.d. (Figure 4E). These effects were reflected in the plasma insulin levels assessed during the study. KBP-066 q.d. and the two highest doses of KBP-066A significantly increased plasma insulin compared to vehicle, whereas the low dose of KBP-066A (0.75 nmol/kg) did not (Figure 4B,D and F). Again, the highest dose of KBP-066A (3 nmol/kg) significantly preserved plasma insulin at higher levels compared to KBP-066 q.d. (Figure 4F). KBP-066 q.d. and the two highest doses of KBP-066A q.3.d. (1.5 and 3 nmol/kg) resulted in significantly improved HbA1c levels compared to vehicle. Of note, there was no significant difference between these treatments (Figure 4G).

The treatment effect on oral glucose tolerance was assessed by an OGTT after seven weeks of treatment. KBP-066 q.d. and KBP-066A (0.75 and 3 nmol/kg) both improved oral glucose tolerance, resulting in significantly reduced iAUC compared to vehicle (Figure 5A,C). This improvement was obtained with preserved insulin levels, though the differences between treatment groups were not significant due to large variation (Figure 5B,D). The beneficial effect on oral glucose tolerance is mainly driven by the improved fasting glucose levels prior to the OGTT, reflected in the calculated tAUC. When assessing tAUC, all treatments significantly reduced blood glucose compared to vehicle (Figure 5E), and KBP-066A (1.5 and 3 nmol/kg) was significantly better in tolerating glucose than the low dose of KBP-066A (0.75 nmol/kg) and KBP-066 q.d. (Figure 5E). Furthermore, KBP-066 q.d. and the two highest doses of KBP-066A (1.5 and 3 nmol/kg) had elevated insulin levels compared to vehicle, though only KBP-066A (3 nmol/kg) was significant (Figure 5F). All treatment significantly reduced food intake (Figure 5H and Table 2), with no concurrent reduction in body weight or adiposity at the study end (Figure 5G and Table 2), consistent with previous studies of DACRAs/amylin in the ZDF model [14,28–30].

4. DISCUSSION

In this article, we demonstrated that acylation of the DACRA KBP-066 alters the PK profile of the peptide both *in vitro* and *in vivo,* making it suitable for dosing every third day (a surrogate for weekly dosing in humans), indicating that the long-acting KBP-066A might be suitable for clinical development as a once-weekly therapy. We found that dosing every third day (surrogate for once-weekly dosing in humans) with acylated KBP-066, KBP-066A, was more potent than daily dosing with non-acylated KBP-066 in terms of inducing body weight loss in obese rats (HFD) and in terms of improving the overall glycemic status in both obese (HFD) and obese diabetic rats (ZDF). Overall, these findings highlight the benefit of weekly dosing in terms of convenience and efficacy on glycemic control.

Regarding *in vitro* activation, KBP-066A did not show any off-target activation of the tested calcitonin-like family of receptors, confirming its specificity for CTR and AMYR, as previously described for other non-acylated DACRAs [19,20,31]. Importantly, the *in vitro* data demonstrated that the addition of the acyl-side chain reduced the potency of KBP-066A compared to the non-acylated KBP-066, and thus, underscored the need for determining the chronic doses based on acute *in vivo* tests rather than equimolar concentrations or exposure.

The characterization data demonstrated that the acylation worked as intended, improving the $T^1/_2$ and T_{max} of KBP-066A to a similar range to semaglutide in rats, which has the same type of acylation [1,32]. Of note, during the development of KBP-066A, different acyl-side chains, linkers, and positions were tested in order to obtain the peptide, which retained the best activity while prolonging the plasma half-life [23]. The corresponding acute effects on food intake and body weight were also reflected in the observed differences in T_{max} and $T^1/_2$. KBP-066A had a later onset of action and a prolonged effect compared to KBP-066. Importantly, these acute pharmacodynamic studies, and not the PK profile alone, formed the basis for the doses used in the chronic *in vivo* studies. The correlation between the PK profile in the acute *in vivo* effects is in line with what has previously been published on the development of other once-weekly compounds [1,3,27,33], observing patterns in *in vivo* effects similar to what is observed in the present studies. These findings support the used dosing frequency and underscore the potential of KBP-066A for weekly administration. Of note, the difference in the onset of action was also reflected in the effect on gastric emptying, though the treatment effect was relatively short-lived compared to the effect on food intake.

The HFD study demonstrated that KBP-066A dosed every third day induced a body weight loss similar to daily dosing with KBP-066, and interestingly, KBP-066A was superior to daily KBP-066, suggesting that weekly KBP-066A as a treatment for obesity more than matches what has previously been shown with daily DACRAs [13,19,20,24,25,34]. Furthermore, oral glucose tolerance was improved with lowered plasma insulin levels by both KBP-066 q.d. and

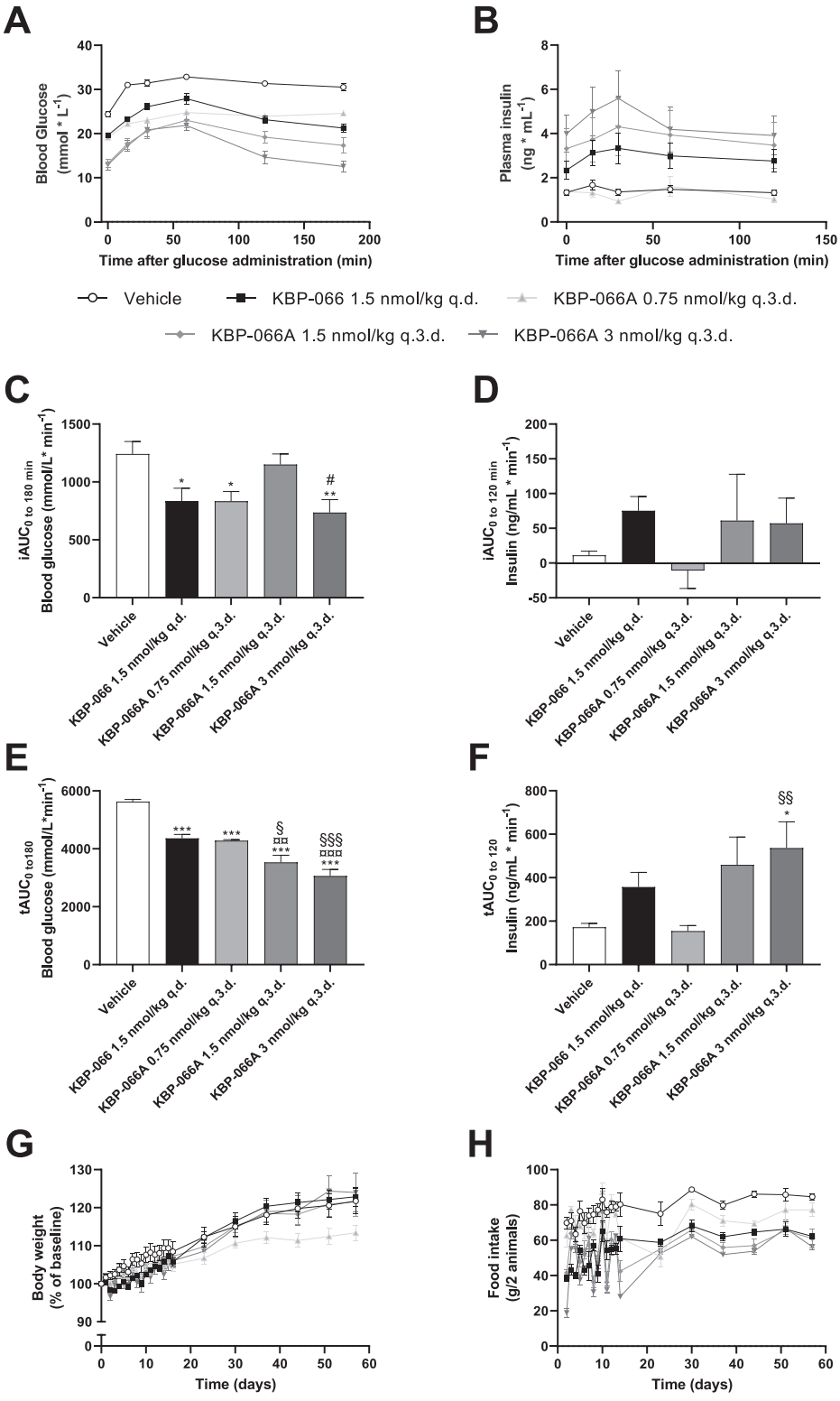

Figure 5: Eight-week study in diabetic ZDF rats. Blood glucose (A) and plasma insulin levels (B) during the OGTT performed after seven weeks of treatment. The incremental area under the curve (iAUC) is shown for blood glucose (C) and plasma insulin (D), and the total area under the curve (tAUC) is shown for blood glucose (E) and plasma insulin (F). Body weight shown as % change from baseline (G) and food intake (g/two animals) during the study (H). n = 10 rats per group. Statistical analysis between groups in (C and E) was performed as one-way ANOVA and Tukey's multiple comparison test and statistical analysis between groups in (D and F) was performed as a Kruskal—Wallis test and Dunn's multiple comparisons test with the following annotations: *P < 0.05, ***P < 0.001 versus vehicle; ¤¤P < 0.01, ¤¤¤P < 0.001 versus KBP-066 (1.5 nmol/kg q.d.), §P < 0.05, §§P < 0.01, §§§P < 0.001 versus KBP-066A (0.75 nmol/kg q.3.d.) and #P < 0.05 versus KBP-066A (1.5 nmol/kg q.3.d.). The statistical analyses of changes in body weight and food intake calculated as AUC can be found in Table 2. All data are presented as mean ± SEM.

KBP-066A q.3.d. in HFD rats. The positive effect on glucose tolerance could also partly be attributed to the induced delayed gastric emptying rate by even the low doses of the peptide.

In the ZDF rat study, KBP-066A successfully improved the overall glycemic status by improving fasting blood glucose, plasma insulin, HbA1c levels, and oral glucose tolerance. Importantly, the highest dose of KBP-066A was superior to KBP-066 q.d. in reducing fasting blood glucose and preserving plasma insulin levels, also reflected in the improved HbA1c levels. This effect highlights that KBP-066A, in addition to its anti-obesity effect, is effective as an anti-diabetic and a superior once-weekly alternative to the daily DACRAs previously reported to improve glycemic status in rat models of diabetes [14,15,19,25,29].

Treatment with weekly KBP-066A resulted in fluctuations in both body weight and food intake as a function of the dose and dosing regimen. These continuous fluctuations have previously been observed with non-acylated DACRAs doses performed every other day instead of daily [15,34], which led to improved DACRA-mediated body weight loss [34]. Interestingly, KBP-066A was better than KBP-066 (per molecule), as the weekly accumulated dose of KBP-066A at the highest concentration was lower than the weekly accumulated dose of KBP-066, resulting in better efficacy. It has been previously shown that the calcitonin receptor is important in the DACRA-mediated improvement of glucose control [29], and the superiority of once-weekly KBP-066A was most pronounced on glycemic control. These findings may suggest an improved *in vivo* efficacy via the calcitonin receptor.

The overall superiority of KBP-006A on glycemic parameters suggests that KBP-066A is an excellent candidate as a once-weekly therapy of T2DM and other complications related to metabolic syndrome.

Studies with weekly GLP-1 and amylin analogs demonstrated a similar pattern, with the once-weekly compounds being superior to the corresponding daily/bid compound in improving glycemic status in patients with T2DM [10,22,35–37]. This finding suggests a genuine efficacy advantage of weekly compounds, though any mechanisms contributing beyond protracted plasma half-life remain elusive.

Perhaps, another overlooked aspect of progressing peptide therapeutics with prolonged plasma half-life is the prospect of developing them for daily use in an oral formulation setting. This aspect became evident with the clinical approval of oral formulated semaglutide [38]. One of the major challenges of oral peptide development is the inherent daily variation in bioavailability [39,40], and acylated peptides with protracted plasma half-life may be pivotal in solving that issue.

In summary, we have developed and characterized an acylated DACRA, KBP-066A, and through a series of *in vitro* and *in vivo* studies, both acute and chronic, we have established the efficacy of KBP-066A *in vivo* to be overall superior to its non-acylated variant, KBP-066, in terms of weight loss and glycemic control in obese (HFD), and obese diabetic rats (ZDF). This collection of pre-clinical studies suggests that KBP-066A is an intriguing candidate for a once-weekly therapy of T2DM, obesity, and other co-morbidities associated with metabolic syndrome.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

AUTHOR CONTRIBUTION

K.V.A. and A.T.L. did most of the experiments, analyzed and interpreted data, and wrote the manuscript. NS and KEM assisted with conducting some experiments and interpreting data. KH. and M.K. assisted with data processing and analysis and with manuscript preparation. K.H. analyzed and interpreted data and was involved in the manuscript creation and preparation.

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CONFLICT OF INTEREST

Morten A. Karsdal and Kim Henriksen own stock in Nordic Bioscience A/S. All authors are employed by Nordic Bioscience A/S.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2021.101282.

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