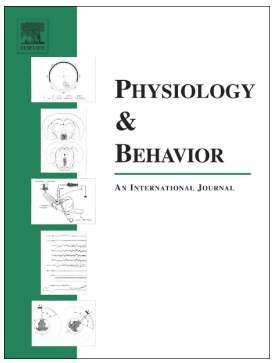

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Novel GLP-1/GLP-2 co-agonists display marked effects on gut volume and improves glycemic control in mice

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Abstract

Aim

Analogues of several gastrointestinal peptide hormones have been developed into effective medicines for treatment of diseases such as type 2 diabetes mellitus (T2DM), obesity and short bowel syndrome (SBS). In this study, we aimed to explore whether the combination of glucagon-like peptide-1 (GLP-1) and glucagon-like peptide-2 (GLP-2) into a potent co-agonist could provide additional benefits compared to existing monotherapies.

Methods

A short-acting (GUB09-123) and a half-life extended (GUB09-145) GLP-1/GLP-2 co-agonist were generated using solid-phase peptide synthesis and tested for effects on food intake, body weight, glucose homeostasis, and gut proliferation in lean mice and in diabetic *db/db* mice.

Results

Sub-chronic administration of GUB09-123 to lean mice significantly reduced food intake, improved glucose tolerance, and increased gut volume, superior to monotherapy with the GLP-2 analogue teduglutide. Chronic administration of GUB09-123 to diabetic mice significantly improved glycemic control and showed persistent effects on gastric emptying, superior to monotherapy with the GLP-1 analogue liraglutide. Due to the short-acting nature of the molecule, no effects on body weight were observed, whereas a marked and robust intestinotrophic effect on mainly the small intestine volume and surface area was obtained. In contrast to GUB09-123, sub-chronic administration of a half-life extended GUB09-145 to lean mice caused marked dose-dependent effects on body weight while maintaining its potent intestinotrophic effect.

Conclusion

Our data demonstrate that the GLP-1/GLP-2 co-agonists have effects on gut morphometry, showing a marked increase in intestinal volume and mucosal surface area. Furthermore, effects on glucose tolerance and long-term glycemic control are evident. Effects on body weight and gastric emptying are also observed depending on the pharmacokinetic properties of the molecule. We suggest that this novel co-agonistic approach could exemplify a novel concept for treatment of T2DM or SBS.

Keywords:

GLP-1, GLP-2, co-agonist, diabetes, obesity, short bowel syndrome, intestinal growth, glucose homeostasis.

1. Introduction

Circulating levels of the gut hormones GLP-1 and GLP-2, which are both processed from proglucagon in the intestine and secreted in equimolar amounts, have consistently been reported to be upregulated following Roux-en-Y gastric bypass (RYGB). Accordingly, GLP-1 and GLP-2 are considered to play important roles in the powerful effects of RYGB on body weight and T2DM remission [1–5]. Interestingly, one of the anatomical hallmarks of RYGB surgery is a marked hypertrophy and cell hyperplasia of the gut, leading to increased glucose utilization hereby indicating a putative role of intestinal turnover in the beneficial effects of RYGB surgery [6,7].

GLP-1 has been extensively studied in relation to T2DM and weight management [8,9]. Specifically, GLP-1 is a so-called incretin that stimulates the secretion of insulin from pancreas subsequent to food intake. In addition, GLP-1 inhibits the release of glucagon, delays gastric emptying, and decreases food intake [10–12]. Therefore, several GLP-1 receptor agonists have been developed by the pharmaceutical industry and are now approved for the treatment of both diabetes and obesity [9,13]. Besides the metabolic effects, a role for GLP-1 in modulating the enteric immune response has been demonstrated by Yusta and colleagues [14], who identified GLP-1 receptor (GLP-1R) expression on intestinal intraepithelial lymphocytes regulating cytokine production. Also, absence of GLP-1 signaling in *Glp1r*$^{-/-}$ mice led to abnormal representation of microbial species as well as an increased sensitivity to intestinal injury, indicating a role for GLP-1 in the protection of intestinal integrity [14]. Interestingly, GLP-1 has recently been shown to exert proliferative and anti-inflammatory effects in rodents [14–16], and administration of pharmacological doses of GLP-1 has been described to lead to intestinotrophic effects both in the small and large intestine through a mechanism likely involving FGF-7 [15,16]. Finally, GLP-1 analogues have been tested in SBS patients, alone [17,18] or in combination with GLP-2 analogue treatment [19]. Here, GLP-1 analogues were shown to induce additive effects on several parameters of intestinal function such as increased nutrient absorption and decreased diarrhea, hereby indicating a strong potential for the use of GLP-1 analogues in treatment of SBS.

GLP-2 increases epithelial proliferation and nutrient absorption [20–22], and these marked intestinotrophic properties of GLP-2 have led to development of teduglutide (Gattex), a GLP-2 analogue, which is available for the treatment of patients suffering from SBS and intestinal failure [23–26]. GLP-2 is hypothesized to be the hormone that connects nutritional status with control of mucosal proliferation [27]. I.e., GLP-2 enhances the absorption of lipids and free fatty acids (FFAs), amino acids and carbohydrates, decreases gastric acid secretion and emptying [28–31] and engages anti-inflammatory pathways in the intestinal mucosa [32]. Moreover, Baldassano and colleagues showed an improvement in glucose homeostasis in high fat diet (HFD) fed mice after treatment with teduglutide, independent from its effects on gastric emptying [33]. Other studies have indicated a role for GLP-2 in protection against hepatic inflammation, endotoxemia and islet dysfunction in HFD mice [27,32]. In addition, mice with a specific GLP-2 receptor (GLP-2R) KO in arcuate pro-opiomelanocortin (POMC) neurons display decreased hepatic insulin sensitivity and glucose intolerance [34] whereas mice on HFD receiving the GLP-2R antagonist, GLP-2 (3-33), showed reduced insulin sensitivity

and decreased glucose tolerance [35]. All in all, these data indicate a potential metabolic benefit of GLP-2 analogue treatment, specifically in the HFD situation [33,35–37].

Taken together, both GLP-1 and GLP-2 have been described in the literature to have both distinct and overlapping effects of proliferative and metabolic nature. Recently, there has been an increased focus on synthetic combinations of peptide hormones into single-molecule co-agonists. Such combinatorial approaches have shown not only additive, but also synergistic effects, on metabolic outcomes [38–40]. To gain further insight into the potential benefits of a GLP-1/GLP-2 co-agonist, we developed a compound (GUB09-123) with potent agonistic effects on both GLP-1R and GLP-2R. The intestinal efficacy was tested in wildtype (*wt*) mice and compared to treatment with teduglutide. Hereafter followed an assessment of compound efficacy in *db/db* mice compared to treatment with liraglutide. Finally, we assessed the effects of a lipidated version of the compound (GUB09-145), with prolonged systemic half-life, on body weight and gut proliferation.

2. Materials and methods

2.1 Animals

All animals were offered domestic quality tap water and housed in a temperature-, humidity-, and light-controlled room (22±1°C; 50±10% relative humidity; 12-hour light-dark cycle, lights on/off at 4AM/4PM hour). The Danish Animal Experiments Inspectorate approved all experiments which were conducted using internationally accepted principles for the use of laboratory animals under the personal license of Jacob Jelsing (2013-15-2934-00784).

2.2 Compounds

Liraglutide was acquired commercially (Victoza® pens, Hoersholm Pharmacy, Denmark). The peptides were prepared by automated solid-phase peptide synthesis (SPPS) using the Fmoc/tBu strategy on either pre-loaded PHB TentaGel resin (native GLP-2) or TentaGel S Rink amide resin for native GLP-1 and the GLP-1/GLP-2 co-agonists (Rapp polymere GmbH, Tuebingen, Germany). The couplings were performed using Fmoc-N^{α}-protected amino acids, N,N-diisopropylcarbodiimide and ethyl cyanoglyoxylate-2-oxime (oxyma) in N,N-dimethylformamide (DIC) (Iris Biotech GmbH, Marktredwitz, Germany) for 2 × 2 h. The N^{α} -deprotections were performed using 40% piperidine in N-methyl-2-pyrrolidione (NMP) (Iris Biotech GmbH, Marktredwitz, Germany) for 3 min followed by 20% piperidine in N-methyl-2-pyrrolidione (NMP) for 17 min. Finally, the peptide was simultaneously side-chain deprotected and released from the solid support by a trifluoro acetic acid (TFA) cocktail containing trifluoro acetic acid (TFA) (Iris Biotech GmbH, Marktredwitz, Germany), triethylsilane (TES) (Sigma–Aldrich, Brøndby, Denmark) and H_2O (95/2.5/2.5) as scavengers for 2 h at room temperature. The peptides were subsequently precipitated by the addition of diethylether (Sigma–Aldrich, Brøndby, Denmark). Finally, the peptides were purified by RP-HPLC (LC-150 system, Waters, Denmark) and identified by LC-MS (Acquity UPLC and SQD2 MS systems, Waters, Denmark). The final products were obtained with >95% purity determined by UV at 215 nm.

Co-agonist compound EC_{50} values on the GLP-1R and GLP-2R were determined by a cAMP homogeneous time resolved fluorescence (HTRF™) functional assay. HTRF employs the use of a long-lived fluorescence donor (coupled to an anti-cAMP antibody) and fluorescence resonance energy transfer (FRET) to an acceptor (coupled to synthetic cAMP) [41]. Under baseline conditions, FRET occurs between the donor and the acceptor. However, when endogenous cAMP is produced in response to GLP-1R or GLP-2R activation, this will compete with the labelled cAMP and result in an eradication of the FRET signal. Compounds were tested for agonist activity on the GLP-1R and GLP-2R in dose-response in duplicates in a CHO-K1 recombinant cell line overexpressing either the GLP-1R or the GLP-2R. EC_{50} values can be found in Table 1.

2.3 Phamacokinetics

Male C57BL/6J mice, 7 weeks of age (Taconic, Denmark) were randomized into two groups (n=27 in each group). Compounds GUB09-123 and GUB09-145 were dissolved in PBS buffer containing 3% mannitol and 0.6% L-His (pH 9.0) and administrated subcutaneously with a dose of 800nmol/kg in a volume of 5 mL/kg.

Blood samples were collected by decapitation of the animals at nine different time points after administration (0.25, 0.5, 0.75, 1, 2, 4, 6, 10 and 24 hours, n=3 for each time point). Approximately 200 µL of blood were collected, centrifuged at 4°C and the obtained plasma was frozen on dry ice and stored at -80°C.

Plasma samples were prepared for analysis by treatment of 50 µL plasma sample with 150 µL 8M guanidine·HCl followed by dilution with 200 µL water and purification by solid phase extraction (Oasis HLB µElution 96-well Plate 30 µm, Waters). The samples were loaded into the plate, washed with 200 µL 5% methanol in water and eluted with 2x50 µL 10% acetonitrile in methanol. The solvent was removed with a flow of N_2 gas.

The isolated peptides were cleaved into smaller peptide fragments with trypsin. The evaporated peptide samples were dissolved in 80 µL 25 mM ammonium bicarbonate buffer containing 1 mM $CaCl_2$, pH 8.5. Trypsin (2 µg) was added and incubated at 37 °C for 4h. The samples were diluted with 50 µL acetonitrile before analysis.

All samples were analyzed by LC-MS/MS using a Waters Acquity UPLC chromatography system connected to a XevoTQ triple quadrupole mass spectrometer. Samples were separated by reversed phase chromatography using a Waters Acquity UPLC BEH C18, 1.7 µm, 2.1 x 50 mm column, with a 4-min gradient of 5%-80% acetonitrile in water containing 0.1% HCOOH and a flow of 0.3 mL/min. The signal of each compound was detected by 2 multiple reaction monitoring (MRM) transitions.

2.4 Sub-chronic treatment with teduglutide and co-agonists in C57BL/6J mice

Male C57BL/6J mice (Taconic, Denmark), 10 weeks of age, were offered regular chow diet (Altromin 1324, Brogaarden A/S, Denmark). Before study start, mice were randomized by body weight into three individual study groups (n=10 per group). Group 1: Vehicle (SC, BID). Group 2: teduglutide (800nmol/kg, SC, BID). Group 3: GUB09-123 (800nmol/kg, SC, BID). Compounds were dissolved to a dosing volume of 5 ml/kg in PBS buffer containing 3% mannitol and 0.6% L-His (pH 9.0). On experimental day 7 the animals were subjected to an oral glucose tolerance test (OGTT) and on day 8, animals were fasted for 4 hours before being euthanized. The intestines were dissected out, their lengths were measured, they were flushed with saline and finally weighed before being placed in 10% natural buffered formalin.

2.5 Chronic treatment with liraglutide and co-agonists in db/db mice for 8 weeks

Male *db/db* (BKS.Cg-m +/+ Leprdb/J) mice (Janvier, France), 8 weeks of age, were offered regular chow diet (Altromin 1324, Brogaarden A/S, Denmark). Before study start, mice were randomized by body weight into four individual study groups (n=10 per group). Group 1: Vehicle (SC, BID). Group 2: liraglutide (50 nmol/kg, SC, BID). Group 3: GUB09-123 (50 nmol/kg, SC, BID). Group 4: GUB09-123 (250 nmol/kg, SC, BID). Compounds were dissolved to a dosing volume of 5 ml/kg in PBS buffer containing 3% mannitol and 0.6% L-His (pH 9.0) or PBS buffer with 0.1% BSA for liraglutide (pH 7.4). Body weight and food and water intake was measured daily during the study. 4-hour fasted blood glucose was measured on day 54, while HbA1c levels were measured on day 28 and 54. The animals were furthermore subjected to an OGTT combined with a gastric emptying test (GE-OGTT) on day 56. On experimental day 57 the animals were fasted for 4

hours before being euthanized. The intestines were dissected out, their lengths were measured, they were flushed with saline and finally weighed before being placed in 10% natural buffered formalin.

2.6 Treatment with non-lipidated and lipidated co-agonists in wt mice for 7 days

Male C57BL/6J mice (Taconic, Denmark), 10 weeks of age, were offered regular chow diet (Altromin 1324, Brogaarden A/S, Denmark). Before study start, mice were randomized by body weight into seven individual study groups (n=6 per group). Group 1: Vehicle (SC, BID). Group 2: GUB09-123 (50 nmol/kg, SC, QD). Group 3: GUB09-123 (10 nmol/kg, SC, BID). Group 4: GUB09-123 (50nmol/kg, SC, BID). Group 5: GUB09-145 (50 nmol/kg, SC, QD). Group 6: GUB09-145 (10 nmol/kg, SC, BID). Group 7: GUB09-145 (50nmol/kg, SC, BID). Compounds were dissolved to a dosing volume of 5 ml/kg in PBS buffer containing 3% mannitol and 0.6% L-His (pH 9.0). On experimental day 8 animals were fasted for 4 hours before being euthanized. The intestines were dissected out, their lengths were measured, they were flushed with saline and finally weighed before being placed in 10% natural buffered formalin.

2.7 Gastric emptying and oral glucose tolerance test

The animals were fasted for 2 hours prior to the GE-OGTT or the OGTT. At time -45 min compounds were administered and at time 0 min 2 g/kg glucose (200 mg/ml, Fresenius Kabi, Sweden), with 100 mg/kg acetaminophen added for the GE-OGTT (10mg/ml, Sigma-Aldrich, St. Louis, USA), were administrated orally. At 15, 30, 60 and 120 min after glucose administration, blood samples were collected into heparinized glass capillary tubes and immediately suspended in glucose/lactate system solution buffer (EKF-diagnostics, Germany). Blood glucose was measured using a BIOSEN c-Line glucose meter (EKF-diagnostics, Germany) according to the manufacturer's instructions. In case of the GE-OGTT, plasma acetaminophen concentrations were determined using a commercially available acetaminophen kit (Multigent, 2K9920, Abbot Laboratories, USA). After the last blood sample, the animals were re-fed [42].

2.8 Histology and stereology

After a minimum of 24 hours of storage in formalin, intestines were sampled using stereological principles of systematic uniform random sampling (SURS), providing 8-10 biopsies from the small intestine and 4-5 from colon (see Figure 1A). After paraffin infiltration, the biopsies were systematically embedded in paraffin blocks in a way that enabled later identification of individual biopsies. 5 µm thick top-sections were cut form all blocks providing 8-10 transverse sections of small intestine biopsies, thus covering the full rostro-caudal extension. For the colon biopsies, additional sampling in the block ensured that also 8-10 transverse sections of colon biopsies were obtained here. All slides were stained with hematoxylin-eosin and digitally scanned. Stereological volume estimations were performed by point-counting using the newCAST system (Visiopharm, Denmark), here illustrated with 7x7 point probes, each point representing 6543um^2 (Figure 1B). Total gut volume was estimated by point counting using a grid system. All points hitting the structure of interest (mucosa, submucosa and muscularis) were counted. The number of points was converted into volume by the following mathematical relationship:

$$Volume_{ref} = \sum points \times area(point) \times t$$

Where ∑P is the total number of points hitting the structure of interest, area(point) is the area per point and t is the distance between sections. Estimations of surface density and surface area were performed by counting intersections between linear probes and the luminal side of the gut, here illustrated by line probes of 6 groups with 3 segments and a length/point of 52,76um (see Figure 1C). The number of intersections was converted into surface density by the following mathematical relationship:

$$Surface_v = \frac{2 \times \sum Intersections}{I_P \times \sum points}$$

Where ∑Intersections is the number of intersections of the test line probes with the epithelium of the tunica mucosa, I(p) is the test line probe length associated with a point of the grid and ∑Point is the number of test points hitting the reference volume. Finally, the absolute surface area was estimated by multiplying the surface density with the reference volume. Estimates of surface density usually require isotropic or vertical sectioning protocols. However, due to the normal isotropic occurrence of villus (no preferred orientation of villus in 3D space) the present study was for all practical purposes performed on transverse sections only.

2.9 Statistics

All data were analyzed using Graph Pad Prism 5.0 software, applying either student's t-test or one-way analysis of variance (ANOVA) with Tukey's post-hoc test. Results are presented as mean ± SEM (standard error of the mean). A p-value less than 0.05 was considered statistically significant.

3. Results

3.1 GUB09-123 is superior to teduglutide in reducing glucose excursions and increasing small intestine volume.

GUB09-123 displayed similar potency as the native peptides on both the GLP-1R and GLP-2R (Table 1). In *wt* mice, teduglutide and GUB09-123 led to a slight increase in body weight (Figure 2A) reaching statistical significance as compared to vehicle when expressed as relative body weight during the 7-day study period (Figure 2B). GUB09-123 reduced cumulative food intake (Figure 2C) but had no effect on water intake (Figure 2D). During the oral glucose tolerance test on day 6, GUB09-123 showed marked reductions in glucose excursions (Figure 2E) which also became apparent when plotting the glucose area under the curve (calculated above 0, Figure 2F). Treatment with teduglutide did not affect blood glucose levels during the OGTT (Figure 2E and 2F). GLP-1 and GLP-2 co-agonism had a marked intestinotrophic effect on total gut volume (Figure 2G). This was most pronounced in the small intestine (Figure 2H), where GUB09-123 treatment increased small intestine volume by 90±16% as compared to vehicle (927.8±78.6 mm^3 GUB09-123 vs 487±29mm^3 vehicle, $p<0.001$) and by 53±13% as compared to teduglutide (606.9±36.8 mm^3, $p<0.001$). Teduglutide itself increased total intestine volume by 31±8% compared to vehicle ($p=0.02$, when analyzed by students t-test) (Figure 2G). The effect of GUB09-123 was slightly less pronounced in the colon (Figure 2I) leading to a 68±18% increase compared to vehicle (292±32 mm^3 GUB09-123 vs. 173±19 mm^3 vehicle, $p<0.05$) and 29±14% to teduglutide (226±19 mm^3 teduglutide).

3.2 GUB09-123 is equipotent to liraglutide in reducing glucose excursions in db/db mice.

Treatment with liraglutide led to a substantial reduction in body weight during the 8-week study period (Figure 3A and 3B), resulting in a 22±2.6% body weight reduction at termination as compared to vehicle (38±1.2g liraglutide vs. 48±1.8g vehicle, $p<0.001$, Figure 3A). GUB09-123 in the low or high dose did not reduce body weight (Figure 3A and 3B). Both treatment with liraglutide and GUB09-123 reduced cumulative food and water intake, being most pronounced for liraglutide (Figure 3C and 3D). On day 56 the animals were subjected to an GE-OGTT. All three treatment groups exhibited statistically significant lower glucose excursions compared to vehicle treated animals (Figure 3E) being most pronounced for the high dose of GUB09-123. Liraglutide led to a 66±3% reduction in total glucose AUC (3.1±0.3mmol/L*min liraglutide vs. 9.2±0.9 mmol/L*min vehicle, $p<0.001$), whereas GUB09-123 low dose and high dose reduced total glucose AUC by 61±5% (3.5±0.5mmol/L*min, $p<0.001$) and 73±1.3% (2.5±0.1mmol/L*min, $p<0.001$), respectively (Figure 3F). Furthermore, eight weeks of treatment with liraglutide led to a small, non-significant decrease in gastric emptying (2.84±0.11mg/ml*min liraglutide vs. 3.07±0.32 mg/ml*min vehicle, $p=0.26$). In contrast, both doses of the co-agonist led to a marked decrease in gastric emptying of 41±12% (1.82±0.37 mg/ml*min GUB09-123 50nmol/kg, $p<0.01$) and 60±7% (1.19±0.20 mg/ml*min GUB09-123 250nmol/kg, $p<0.001$, Figure 3G and 3H). The effects of treatment on glucose homeostasis were also reflected in fasted blood glucose levels. On day 54, both liraglutide and the high dose of GUB09-123 significantly reduced fasting blood glucose levels (Figure 3I). Finally, liraglutide and both doses of GUB09-123 significantly reduced mean HbA1c on day 54. Liraglutide reduced HbA1c 1.73 %points ($p<0.001$) the low dose of GUB09-123 1.15 %points ($p<0.01$) and the high dose of GUB09-123 1.80 %points ($p<0.001$) (Figure 3J and 3K).

3.3 GUB09-123 is superior to liraglutide in increasing intestinal volume in db/db mice.

Treatment with liraglutide did not led to any changes in small intestine or colon volume compared to vehicle (Figure 4A and B). On the other hand, treatment with both doses of GUB09-123 led to marked and statistically significant increase in small intestine volume of nearly 80% as compared to vehicle (2022±99mm^3 50nmol/kg, 2043±62mm^3 250 nmol/kg vs. 1143±52mm^3 vehicle, $p<0.001$, Figure 4A). In contrast, no chronic intestinotrophic effects were seen on colon volume (Figure 4B). The increase in small intestinal volume led to concomitant, and even more pronounced, increase in the absorptive mucosal surface area of nearly 90% for GUB09-123 (349±11cm^2 GUB09-123 50nmol/kg, 344±21 cm^2 GUB09-123 250nmol/kg vs. 181±12 cm^2 vehicle, $p<0.001$). No significant effect was observed in colon surface area even though GUB09-123 high dose tended to increase surface area slightly compared to vehicle treatment.

3.4 The lipidated GLP-1/GLP-2 co-agonist (GUB09-145) has improved plasma half-life and reduced body weight while preserving intestinotrophic effects.

Lipidation of GUB09-123 into a long-acting analogue (GUB09-145) led to a slight decrease in potency on both receptors compared to the non-lipidated version (Table 1). However, lipidation also markedly increased plasma half-life from 0.9 hours to 2.7 hours (Figure 5). The appetite and body weight regulatory efficacy of GUB09-145 is shown in Figure 6A to 6D. As compared to vehicle and the non-lipidated GUB09-123, lipidation led to a robust and highly significant effect on both body weight, food intake and water intake in a dose-dependent matter. Lipidation did, however, not affect the intestinotrophic properties of GLP-1 ad GLP-2 co-agonism and both GUB09-123 and GUB09-145 led to a marked up to 70% increase in small intestinal mass (GUB09-123 50nmol/kg BID 60±6.9%, GUB09-145 50nmol/kg BID 72±7.9%, Figure 6E), whereas no significant effect was observed in colon volume (Figure 6F). Bi-daily dosing was needed to retain significant intestinotrophic effects of GU09-123, with 50 nmol/kg BID being more efficacious than 10 nmol/kg BID. In contrast, GUB09-145 showed a consistent and reproducible effect on gut volume, irrespective of dose and dosing frequency.

4. Discussion:

Monotherapy with GLP-1R agonists have become the therapeutic gold standard in T2D diabetes, due to the strong incretin and weight loss effects [10–12] and several GLP-1 based therapies are now approved for T2D or obesity, including exenatide (Byetta), lixisenatide (Lyxumia), albiglutide (Tanzeum), liraglutide (Victoza/Saxenda), and dulaglutide (Trulicity) [9,40]. However, bariatric surgery, in particular RYGB, is to date the most effective treatment of obesity, with concomitant effects on diabetes resolution [43]. Notably, it is consistently reported that RYGB surgery causes a marked hypertrophy of the gut, especially the alimentary limb [44–46], with a concomitant increase in the number of enteroendocrine cells, hereby providing mechanistic support for an increased secretory capacity [1–3,47,48]. Accordingly, it is well-known that the surgery is accompanied by an increased level of a plethora of gut hormones, including GLP-1, GLP-2, glucagon, secretin (SCT), cholecystokinin (CCK), neurotensin (NT), and neuropeptide Y (NPY), whereas other hormones, like glucose-dependent insulinotrophic polypeptide (GIP) and somatostatin, are unaltered or even decreased [49–53]. The search for a RYGB mimetic peptide has spurred a focus on synthetic combinations of peptide hormones into single co-agonists often based on GLP-1 as a backbone in combination with other gastrointestinal hormones including GIP, GCG and SCT [39,54–57]. Here, due to the well-known intestinotrophic properties of GLP-2, and the glucose lowering and body weight reducing effects of GLP-1, we hypothesized that GLP-1/GLP-2 co-agonism could mimic the beneficial metabolic and intestinotrophic effects of RYGB surgery.

The GLP-1 and GLP-2 co-agonist, GUB09-123, was developed from the GLP-1 sequence with GLP-2 activity dialed in by substituting important GLP-2 amino acids while still retaining essential GLP-1 residues. An Aib substitution in position 2 protected GUB09-123 from DPPIV mediated degradation. All in all, this resulted in a co-agonist with GLP-1 and GLP-2 receptor potency in the therapeutically advantageously low nanomolar range, and with a prolonged half-life compared to the corresponding native peptides. In *db/db* mice, GUB09-123 showed markedly improved basal and challenged blood glucose levels, as well as reducing HbA1c, to a similar or even superior degree as seen for the long-acting GLP-1 analogue liraglutide. Our data are in line with a previous report on another short-acting GLP-1/GLP-2 co-agonist, ZP-GG-72, which was reported to improve insulin sensitivity and increase intestinal growth in diet induced obese (DIO) mice [58]. The chronic effects of GUB09-123 on glucose homeostasis suggest a strong GLP-1 receptor agonism with the known effects on insulin and glucagon secretion. However, a part of the effect could also be ascribed to the marked and consistent effect on gastric emptying being reduced by up to 60% compared to vehicle treated mice. A reduction in gastric emptying delays entry of glucose into the intestine and blood stream [59]. It is well-known that native GLP-1, and short-acting GLP-1R agonists, have profound and consistent effects on gastric emptying whereas a full 24-h exposure led to receptor desensitization/tachyphylaxis [60], as also shown here for liraglutide. As of recent, liraglutide is approved for treatment of obesity [13] affecting food intake at least partly by a central mechanism involving POMC/cocaine-and amphetamine regulated transcript (CART) expressing neurons in the hypothalamic arcuate nucleus [12]. In contrast to liraglutide, GUB09-123 did not affect food intake or body weight, which may be ascribed to a difference on pharmacokinetic properties (half-life of 0.9 hours versus approx. 4.4

hours for liraglutide [57]). It is also possible that a glucose sparing effect is relatively pronounced in the GUB09-123 treated diabetic mice (less calories excreted via the urine because of improved glucose homeostasis), which may mask any GUB09-123-mediated body weight loss.

The intestinal adaptation associated with co-agonist treatment is still an unknown aspect. It is possible that enhanced absorption from the enlarged gut may result in enhanced nutrient absorption. However, it could also potentially provide a positive feedforward mechanism enabling larger glucose disposal and enhanced secretion of gastrointestinal hormones due to the increased number of enteroendocrine cells. This putative explanation is supported by a clinical study where a positive correlation was found between levels of gastrointestinal hormones after RYGB surgery and degree of weight loss [61].

Finally, the difference in effect may be ascribed to the lipidation of liraglutide not present in GUB09-123. Accordingly glycosylation and lipidation not only improve peptide stability, but possibly also enhanced blood brain barrier transport and access to central receptors [12,62,63]. To further investigate the role of lipidation we developed a protracted form of our GLP-1/GLP-2 analogue, GUB09-145, for assessing the putative effects on appetite regulation. Interestingly, bi-daily dosing with GUB09-145 for 7 days in lean *wt* mice showed a robust dose-dependent reduction on food intake and body weight of nearly 10 percent. Further studies in a DIO model would be of interest to evaluate the full potential of GUB09-123 vs. GUB09-145 in obesity and the role of lipidation vs. non-lipidation.

In addition to the well-known metabolic effects of GLP-1, GLP-2-mediated metabolic actions have been indicated, although such metabolic actions are still not fully established. Previous studies have revealed a central role of native GLP-2 of food intake [37,64], whereas a peripheral effect on food intake and glucose homeostasis still needs to be elucidated [65,66]. Furthermore, GLP-2 may reduce intestinal inflammation and improve barrier function hereby causing increased insulin sensitivity [32]. Due to its very short plasma half-life, native GLP-2 is an unattractive clinical candidate and therefore in 2013, a DPPIV protected GLP-2 analogue teduglutide (Gattex) was approved for the treatment of SBS [67–69]. Teduglutide has not previously been reported to show beneficial effects on metabolic outcomes and did in the present study not affect glucose levels or food intake when administered for 7 days to lean *wt* mice. In this context however, it should be noted that Baldassano and colleagues [33] recently demonstrated glucose reducing effects in obese mice using a degradation-resistant GLP-2 analogue, independent from its effects on gastric emptying. This indicates beneficial metabolic effects of GLP-2 although these are highly diet dependent and thus these data still need to be confirmed from other work groups.

The beneficial effects of GLP-2R agonism in SBS is well described [25,68,70,71], and includes besides a profound enterotrophic action, also favorable effects on nutrient absorption, gastric acid secretion and gastric emptying (Meier 2006, Drucker et al. 1996, Brubaker et al. 1997). Teduglutide is applicable for once daily dosing, and has in preclinical studies been shown to induce changes in gut mass, gut volume, villus height etc. of 30-50 % [16,20,70,72]. In the current study, we demonstrated a teduglutide-mediated enterotrophic effect of ~30%, whereas both the short acting (GUB09-123) and long-acting (GUB09-145) analogue showed significantly more profound effects on gut volume and total mucosal surface area, indicating a potential add-on intestinotrophic effect of GLP-1 agonism. We and others have previously demonstrated an hypertrophic

effect of pharmacological doses of GLP-1 on gut volume in mice [15,16]. However, a clear assessment of GLP-1/GLP-2 co-agonism on gut growth is difficult to address unambiguously. Using KO models or peptide antagonists of GLP-1 or GLP-2 may provide additional information and is a subject for further experimentation. GLP-1/GLP-2 co-agonism is, however, considered of putative benefit in the treatment of SBS. The increased mucosal surface could lead to a vital increase in nutrient uptake, potential decrease in malnutrition, and hence a reduction in the need for parenteral nutrition. Moreover, the strong combined gastric emptying inhibitory and iliac break properties of a short-acting GLP-1/GLP-2 co-agonist could provide increased absorption and improved quality of life for SBS patients who exhibit a highly increased rate of gastric emptying [73,74]. Especially SBS patients lacking a functional ileum would benefit from the addition of GLP-1 therapy since GLP-1 is one of the important ileal break hormones [10]. The food intake inhibitory effects of GLP-1 may also be of value, as SBS patients often report a constant hunger sensation that cannot be subdued by ingestion of food. Preliminary data suggest that the uncomfortable hunger sensation is removed by GLP-1 analogue treatment, but importantly, without causing weight loss in these patients [19]. However, further studies are needed to fully address the beneficial role of food intake (lipidated version) and gastric emptying (non-lipidated version) for SBS treatment.

Finally, an interesting preliminary finding is that the combination of GLP-1 and GLP-2 might improve the adverse effects seen from treatment with each peptide alone, i.e. the nausea caused by GLP-1 treatment and the abdominal discomfort arising from GLP-2 analogue treatment [19]. This finding needs to be further elucidated, first of all in rodent models of conditioned taste aversion, taste reactivity, pica behavior etc. and secondly in clinical studies of longer duration and with a free diet intake.

A cardinal challenge when working with co-agonists is the clear deduction of specific receptor effects, especially in the case of overlapping functions. Accordingly, it has been shown to be extremely important how a specific co-agonist stimulate its specific target receptors [38,39]. In the current study, it was not possible to unambiguously demonstrate a positive add-on effect of GLP-2 co-agonism on the metabolism, or a positive add-on effect of GLP-1 co-agonism on gut growth. The strong effects by GUB09-123 and GUB09-145 on either parameter may be ascribed to receptor co-agonism, but from the current data we cannot rule out whether our findings simply result from an enhanced and/or altered signaling for the specific receptors, or differential effects on on-off rates of the receptors. Lessons from the development of GLP-1/glucagon co-agonists have already proven the difficulty in designing the correct balance when striving to achieve optimal glucagon induced weight loss without targeting the adverse effects on blood glucose levels [38,39]. In this respect, it is also worth emphasizing that preclinical rodent studies are not necessarily translatable to humans, leaving valid drug outcomes to be confirmed only through expensive clinical trials [75,76].

5. Conclusion:

Here, we present for the first time a full structure and report of GLP-1/GLP-2 co-agonists. We demonstrate, that a long-acting co-agonist, GUB09-145, dose-dependently decreased food intake and body weight, while maintaining a potent intestinotrophic effect. Also, the short-acting co-agonist GUB09-123 significantly reduced food intake, improved glucose homeostasis and increased gut volume, to a higher degree than the

GLP-2 analogue teduglutide. In addition, 8 weeks of chronic administration with GUB09-123 to diabetic *db/db* mice significantly improved glucose tolerance, reduced fed blood glucose and rescued HbA1c, to a similar degree as the GLP-1 analogue liraglutide, irrespective of body weight reducing effects. These metabolic effects were accompanied by a marked and robust intestinotrophic effect most pronounced in the small intestine. More in-depth studies in models of gastrointestinal diseases and obesity are still warranted to validate the full potential of GLP-1/GLP-2 co-agonism, however our data suggest that GLP-1/GLP-2 co-agonism, depending on pharmacokinetic properties, may provide a viable approach for the treatment of T2D and obesity, as well as SBS.

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Declaration of interest:

P.W., S.L.P., G.H., K.M., P.J.P and K.F are employees of Gubra Aps. N.V. and J.J. are owners of Gubra Aps.

Author contribution:

P.W., S.L.P., G.H., K.M. and P.J.P. performed the experiments and evaluated the data. P.W., S.L.P., G.H., K.M., P.J.P., P.B.J., N.V., K.F. and J.J. made substantial contributions to study design, evaluation of data and editing of the manuscript. P.W. and J.J. wrote the manuscript.

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Compound	On-letter abbreviation	GLP-1R, EC_{50} (nM)	n	GLP-2R, EC_{50} (nM)	n
GLP-1	H-HAEGTFTSDVSSYLEGQAAKEFIAWLVKGR-NH_2	0.028	2	>1000	2
GLP-2	H-HADGSFSDEMNTILDNLAARDFINWLIQTKITD-OH	ND	-	0.80	2
GUB09-123	H-HGDGSFTSELSTYLDALAARDFIAWLIQTKITD-NH_2	0.07	2	0.09	2
GUB09-145	H-HGDGSFTSELSTYK(C16-yE-)DALAARDFIAWLIQTKITD-NH_2	0.17	2	1.06	2

Table 1: GLP-1R and GLP-2R EC_{50} values for native peptides (human) and co-agonists.

Figure captions:

Figure 1: Stereological sampling, volume and surface probes.

Stereological SURS sampling overview (A), illustration of point probes for volume estimation (B) with 7x7 probes, each point representing 6543um2. Illustration of line probes for surface area estimation (C), with 6 groups each containing 3 segments, length of 50% and length/point 52,8 um.

Figure 2: GUB09-123 is superior to teduglutide in reducing glucose excursions and increasing small intestine volume.

The effect of teduglutide and GUB09-123 in wt mice on body weight (A), body weight change (B), cumulative food intake (C), cumulative water intake (D), day 7 oral glucose tolerance test (OGTT) (E), area under the curve (AUC) of the OGTT (F), total intestine volume (G), small intestine volume (H) and colon volume (I). Intestinal volumes were measured by stereology.

Figure 3: GUB09-123 is equipotent to liraglutide in reducing glucose excursions in db/db mice.

The effect of liraglutide and two doses of GUB09-123 in db/db mice on body weight (A), body weight change (B), cumulative food intake (C), cumulative water intake (D), day 54 oral glucose tolerance test (OGTT) (E), area under the curve (above 0) (AUC) of the OGTT (F), acetaminophen gastric emptying (G), AUC (above 0) of gastric emptying (H) 4-hour fasted blood glucose on day 54 (I) HbA1c on day 28 (J) and HbA1c on day 54 (K).

Figure 4: GUB09-123 is superior to liraglutide in increasing intestinal volume in db/db mice.

The effect of liraglutide and two doses of GUB09-123 in db/db mice on small intestine volume (A), colon volume (B), small intestine mucosal surface area (C) and colon mucosal surface area (D). Intestinal volumes and surface area were measured by stereology.

Figure 5: Lipidated GLP-1/GLP-2 co-agonist (GUB09-145) has improved plasma half-life.

Pharmacokinetic profile of GUB09-123 and GUB09-145 in wt mice.

Figure 6: Lipidated GLP-1/GLP-2 co-agonist (GUB09-145) reduces body weight while preserving intestinotrophic effects.

The effect of three doses of GUB09-123 and GUB09-145 in wt mice on body weight (A), body weight change (B), cumulative food intake (C), cumulative water intake (D), small intestine volume (E) and colon volume (F). Intestinal volumes were measured by stereology.

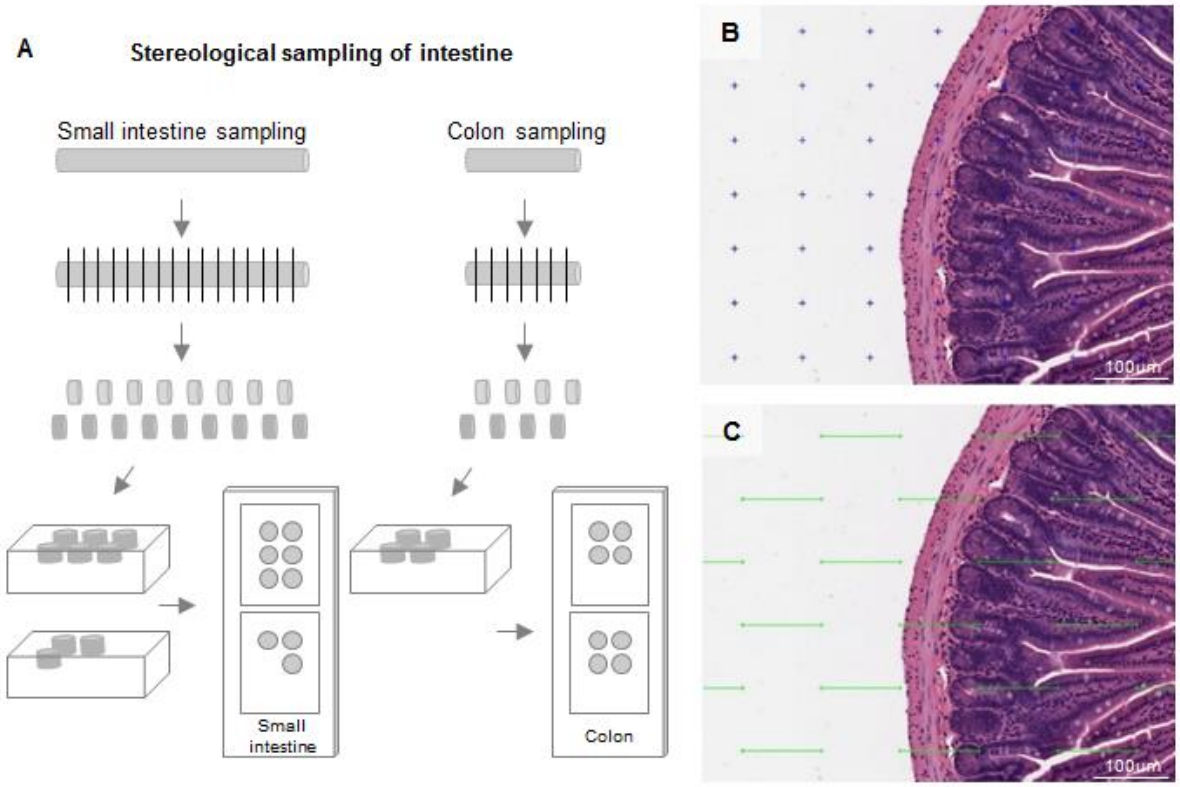

Fig. 1

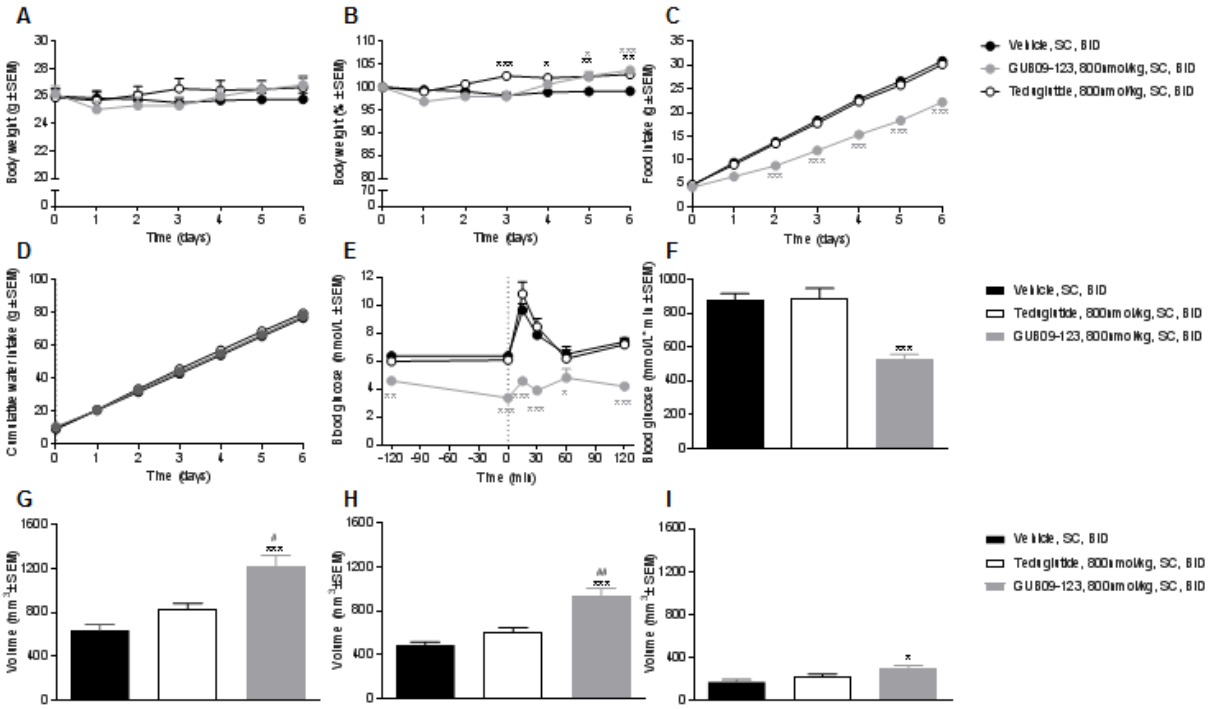

Fig. 2

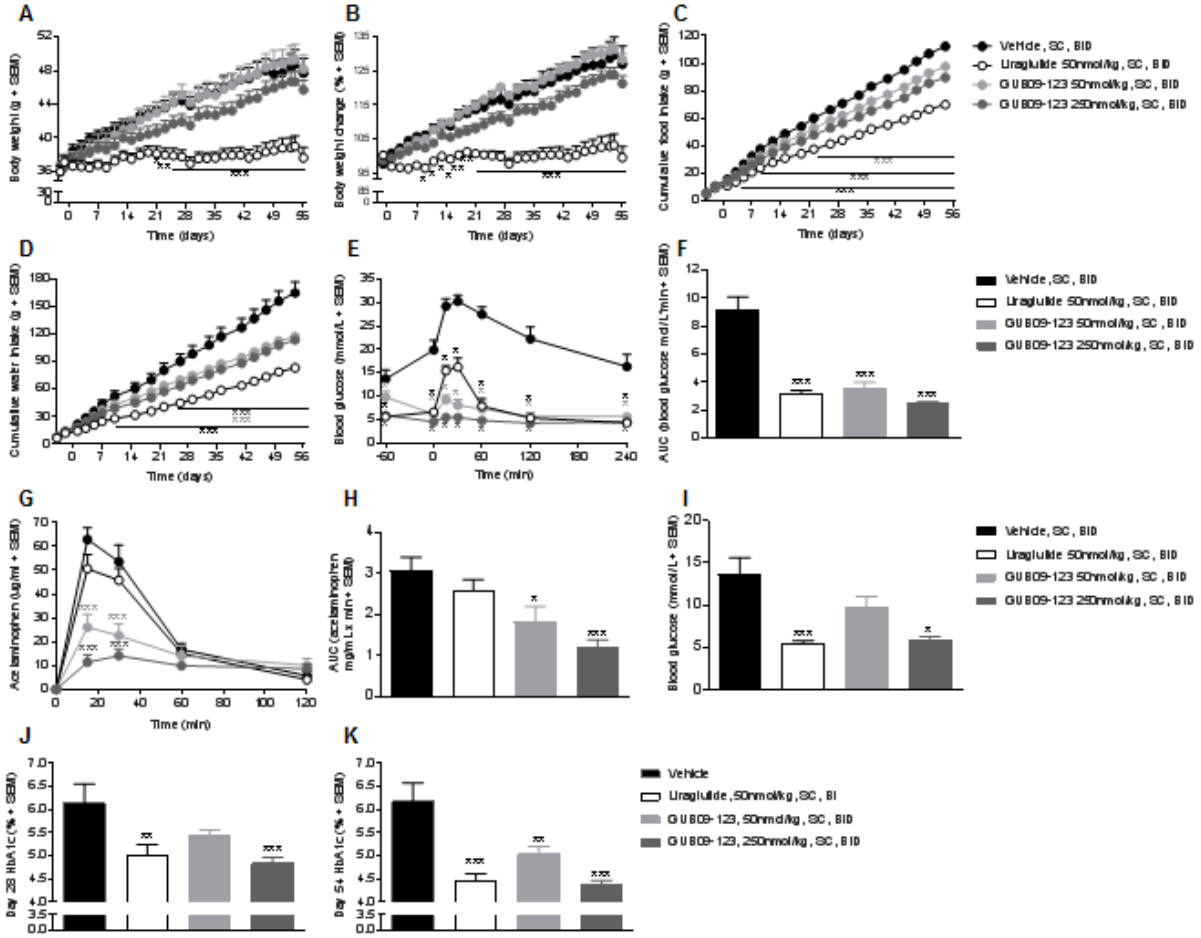

Fig. 3

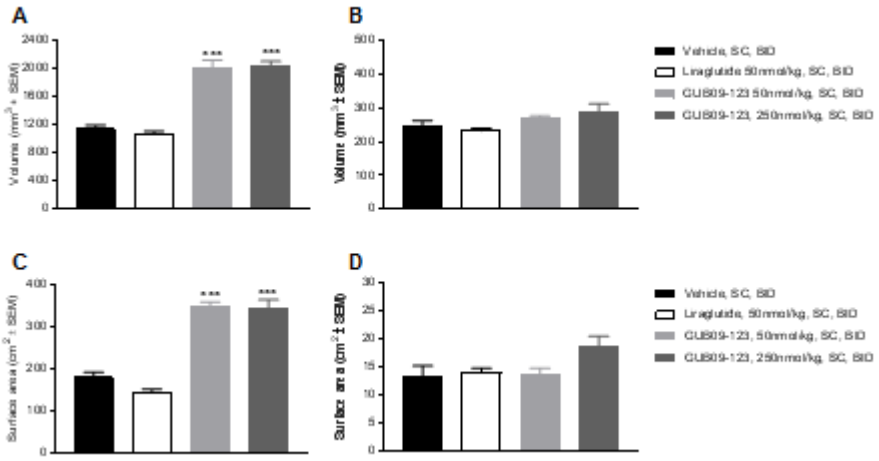

Fig. 4

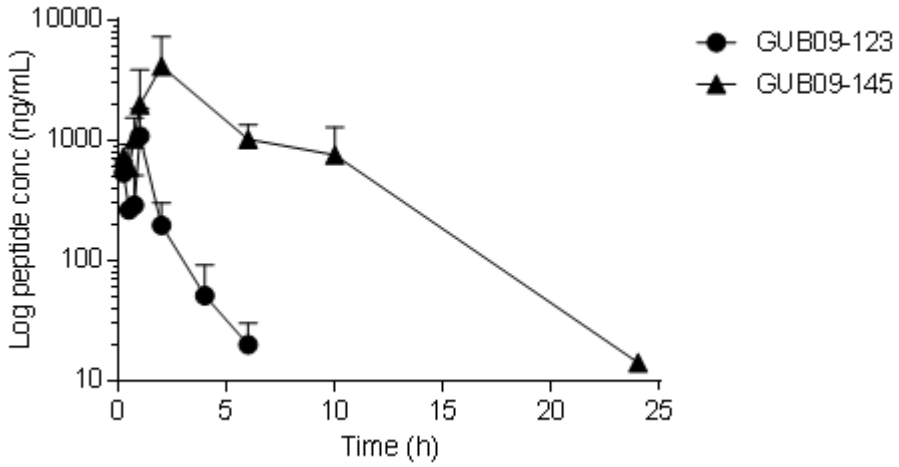

Fig. 5

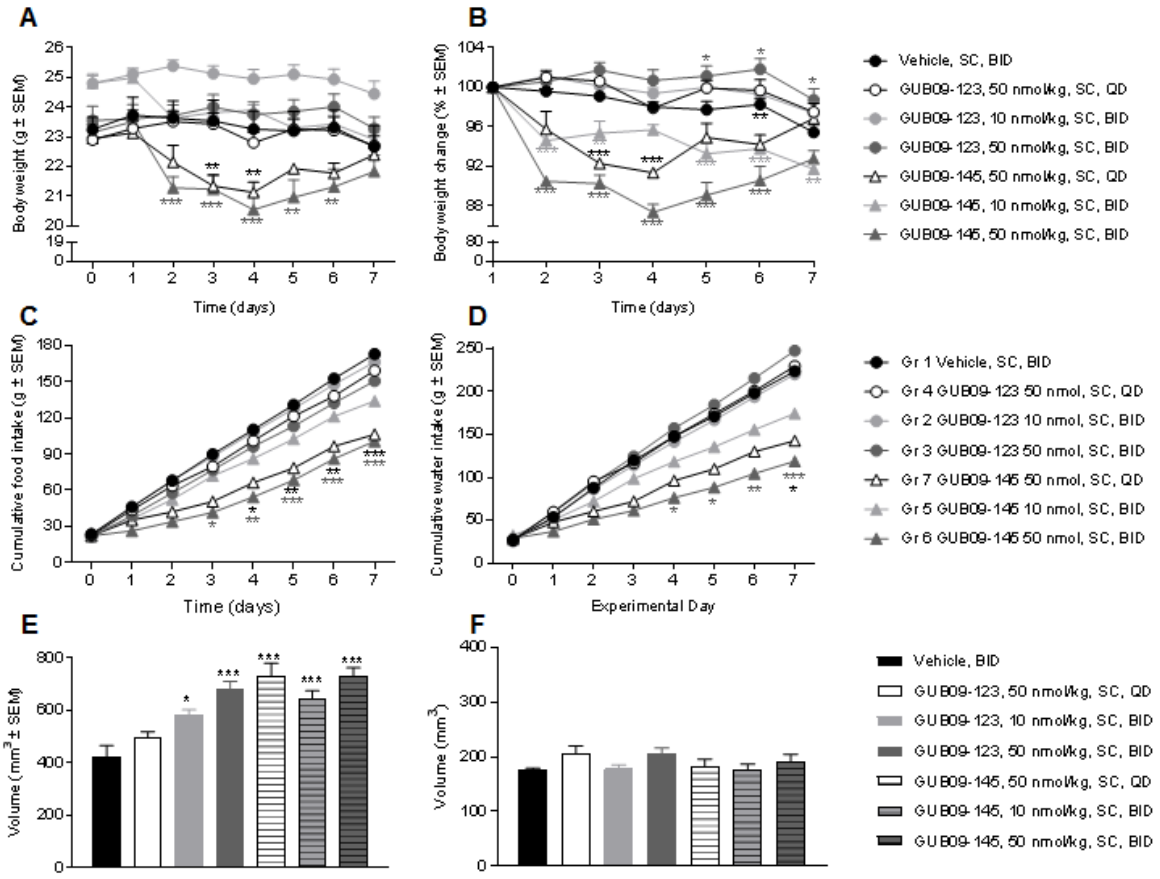

Fig. 6

Highlights:

- GUB09-123 is a novel GLP-1/GLP-2 co-agonist
- GUB09-123 improves glucose homeostasis and increases gut volume
- GUB09-145 is a pharmacokinetically improved lipidated GLP-1/GLP-2 co-agonist.
- GUB09-145 reduces body weight while maintaining intestinotrophic effects