Original article

MOLECULAR METABOLISM

GIPR:GCGR co-agonism restores normal weight in obese rodents

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ABSTRACT

Objectives: Functional co- and tri-agonists at the receptors for GLP-1, GIP and glucagon effectively decrease body weight and hyperglycemia but are associated with adverse gastrointestinal effects related to GLP-1R agonism. Here we report the discovery that obesity can be reversed in the absence of a functional GLP-1R. It propelled the identification of a unimolecular GIPR:GCGR co-agonist lacking GLP-1 activity that corrects obesity in obese mice and rats.

Methods: Selective, dual, and triple sustained-action agonists at GIPR, GCGR and GLP-1R were used to assess body weight and glucose management in diet-induced obese (DIO) wildtype (WT) and GLP-1R knock-out (KO) mice. Indirect calorimetry and pair-feeding studies were used to characterize the magnitude of weight lowering specifically to suppression of food intake relative to energy expenditure.

Results: When used in physical co-mixture, selective GIPR agonism interacts with selective GCGR agonism to correct obesity and enhance glycemia in DIO mice. Retatrutide a balanced GLP-1R:GIPR:GCGR triagonist normalized body weight in obese GLP-1R KO mice. BWB3054, a fatty acylated GIPR:GCGR co-agonist, was identified as comparably potent as retatrutide to induce cAMP production at the mGIPR, and 4-fold reduced at mGCGR, but notably more than 100-fold diminished at mGLP-1R. Despite minimal relative GLP-1R potency, BWB3054 reduces excess body weight in obese DIO-mice to a similar degree as that observed for retatrutide in obese GLP-1R KO mice.

Conclusions: Correction of obesity and glycemia in mice without employing GLP-1 agonism was demonstrated by three independent methods (GLP-1R KO with retatrutide, GIPR:GCGR physical co-agonism mixture, and GIPR:GCGR covalent co-agonist) which advocate for the prospect that the adverse GI effects commonly associated with its use might be avoided.

Keywords Obesity; Type 2 diabetes; GIP; Glucagon; GLP-1 free; GIPR:GCGR co-agonism

1. INTRODUCTION

Unimolecular multi-agonists targeting the receptors for glucagon-like peptide-1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP) and glucagon (GCGR) have emerged as best-in-class therapy to manage obesity and type 2 diabetes [1,2]. When administered weekly for 72 weeks to obese individuals without any other co-disease, the GIPR:GLP-1R co-agonist tirzepatide exceeded the weight loss of the selective GLP-1R agonist, semaglutide by nearly 50% to achieve an absolute mean weight reduction of more than 20%. [3]. Building upon this precedent-setting achievement, the metabolic efficacy of GIPR:GLP-1R co-activation can be further enhanced by the addition of GCGR agonism. The counterintuitive inclusion of glucagon given its diabetogenic liability derives from its complimentary ability to promote energy expenditure and hepatic lipid utilization [4—6], but buffered by the anti-hyperglycemic capacity and appetite suppression of the incretins and glucagon [1,2,7—9]. The first highly potent, and balanced unimolecular triagonist was reported by us in 2015 to significantly outperform GIPR:GLP-1R co-agonism to correct obesity and diabetes in rodents [10]. The subsequent discovery of retatrutide as a sustained-action, balanced triple agonist validated this concept clinically [11—13] in the demonstration of decreased body weight of 24.2% through 48 weekly doses in non-diabetic individuals with obesity [11], and 16.9% after 36 weeks in obese diabetic patients [12].

The fractional contribution and liabilities of agonism at each receptor have been measured in a series of preclinical studies employing selective agonists and obese rodents deficient for individual receptors [10,14—18]. While glucagon's limitation notably resides in its hyperglycemic propensity, excess GLP-1R agonism is associated with gastrointestinal distress, particularly nausea and vomiting [19]. They are both reported to individually increase heart rate, and likely more so when used in combination [4]. The aversive effects of GLP-1R agonism are induced via activation of specific GLP-1 receptors in

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the caudal brainstem [20,21] and abolished upon pharmacological [22] or genetic [20,23] inhibition of GLP-1R. In clinical studies, incidences of nausea induced by GLP-1R agonism range from 22 to 40% in the SURMOUNT trial, and from 14 to 53% in the STEP trial [24]. The adverse effect is dose-dependent and with once-weekly dose-escalation of semaglutide from 2.4 mg to 7.2 mg increasing the prevalence of vomiting from 16.4% to 24.8%, during 72 weeks of treatment [25]. Similar occurrences are reported for use of CagriSema [26]. The adverse GI-effect is believed to be common to GLP-1R based therapeutics and a sizable obstacle to clinical application [27,28], that requires lengthy structured protocols to achieve target doses which many patients cannot tolerate [29].

Consequently, we have interrogated the antithetical prospect of whether correction of obesity can be achieved free of GLP-1 by GIPR:GCGR co-agonism. Our demonstration that retatrutide retains much of its body weight and appetite suppression in DIO GLP-1R KO mice was a seminal observation, subsequently substantiated and deepened by the discovery of a more than additive interaction in GIPR and GCGR agonism to decrease body weight in DIO mice. This observation spurred development of a GIPR:GCGR co-agonist (BWB3054), which possesses retatrutide-like in vitro properties at mouse GIPR and GCGR, but more than hundredfold reduced potency at GLP-1R. In DIO mice, BWB3054 dose-dependently decreased body weight, food intake and hyperglycemia.

2. MATERIALS AND METHODS

2.1. Peptide synthesis

Methods employed in peptide synthesis, purification, characterization, formulation and in vitro characterization are analogous to prior reports [10] and experimental results are reported in Supplemental Materials (Tables S1 and S2; Figures. S1−12).

2.2. Rodent studies

Animals, Diets and Treatments: All studies were approved by the Institutional Animal Care and Use Committee of the University of Cincinnati in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice were housed in an AAALAC-approved room with a 12-hour light and 12-hour dark cycle room held at 22°Cand with free access to food and water.

Male C57BL/6J mice (Cat. Number #000664; Jackson Laboratory, Bar Harbor, ME, USA), GLP-1R KO mice [31] or male Wistar rats (Inotiv, Indianapolis, IN, USA) were maintained grouped housed as 4 per cage and fed high-fat diet (HFD) (D12492; Research Diets Inc., New Brunswick, NJ, USA; 5.24 kcal/g of metabolizable energy; 20/60/20% from protein/fat/carbohydrates) to induce diet-induced obesity (DIO). To investigate body weight and food intake, mice received once-daily subcutaneous administration of either the GLP1R agonist semaglutide (Sema), a GCGR agonist (BWB2086), a GIPR agonist (BWB2065), a physical co-mixture of the GCGR and GIPR agonists, the single-peptide triple GLP-1R:GIPR:GCGR agonist retatrutide (RTT), or a single-peptide GIPR:GCGR co-agonist (BWB3054). The peptides were dissolved in vehicle (pH 7.4) containing 0.05% polysorbate-80, 50 mM sodium phosphate and 70 mM sodium chloride. Body weight and food intake were measured immediately prior to dosing each day, which occurred early in light cycle (1−4 h dosing window).

Glucose Tolerance Test: Mice were fasted for 4−6 h and then injected intraperitoneally (IP) with glucose (2 g/kg, 20% wt/vol D-glucose [Sigma−Aldrich] in phosphate-buffered solution). Blood glucose (BG) measurements were taken from tails directly prior to injection (0 min) and then at 15-, 30-, 60-, 90-, and 120-minutes post-injection. A handheld glucometer and corresponding test strips were used to take BG measurements (FreeStyle, Abbott Diabetes Care Inc.).

Body Composition: Nuclear magnetic resonance (echoMRI, Houston, TX) was used to determine body composition.

Determination of Energy Expenditure via Indirect Calorimetry: Mice were individually housed in sealed chambers (TSE systems, Chesterfield, MO, USA) with unrestricted access to food and water for 96 h to measure locomotor activity, respiratory exchange ratio (RER) and energy expenditure (EE).

Tissue Collection and Gene Expression Analysis: Liver, epididymal white adipose tissue (eWAT), inguinal white adipose tissue (iWAT), brown adipose tissue (BAT), and quadriceps (Quad) were collected immediately after decapitation, weighed, flash-frozen using liquid nitrogen, and then stored at −80 °C. Tissues were homogenized in the lysis buffer provided by the kit using tissue homogenizer (TissueLyser, Qiagen). RNA from the liver lysate was extracted using RNeasy mini kit (Qiagen, Cat #74106). RNA was extracted using RNeasy lipid tissue mini kit (Qiagen, Cat #74804) or RNeasy Fibrous tissue mini kit (Qiagen, Cat #74704) in the case of muscle. cDNA was generated using iScript cDNA synthesis kit (Bio-Rad, Cat #1708891). Gene expression was performed with TaqMan probes and TaqMan gene expression master mix (Cat #4370074) from Applied Biosystems using QuantStudio 5 realtime PCR system (Applied Biosystems).

Plasma Measurements: We used commercial assays for Insulin (Chrystal Chem, #90080), Cholesterol (Infinity Cholesterol, #TR13421), Glycerol (Sigma Aldrich, #MAK117), and Triglycerides (Infinity Triglycerides, #TR222421).

Tolerability and PK in lean Cynomolgus Monkeys: Plasma concentrations in healthy cynomolgus monkeys were measured over time following single-dose SC administration of retatrutide, tirzepatide or BWB3054 (Inotiv Labs) using fit-for-purpose bioanalytical procedures (liquid chromatography-mass spectrometry; dipotassium ethylenediaminetetraacetic acid was used as an anticoagulant for the monkey plasma samples). Concentrations were measured using using a Sciex triple quad 6500+ LC-MS/MS liquid chromatography-tandem mass spectrometry. Blood samples were collected from the femoral vein before dosing and at several timepoints after peptide administration. Within 1 h of collection, blood was centrifuged for 15 min at approximately 1500 rcf. The resultant plasma for each sample was transferred and stored in a tube. Plasma bioanalysis was (performed by Veloxicty Labs, LLC and the resulting pharmacokinetic data analysis was conducted by Inotiv Labs using noncompartmental methods (Phoenix® WinNonlin® Version 8.1).

Safety assessments: All AEs were recorded from the time study treatment was initiated through study completion on a case report form (Inotiv Labs). AEs that started during or after administration of the study drug and increased in severity after administration were recorded. Other safety assessments included routine clinical pathology tests, vital sign measures, food consumption, fecal observations, and physical examinations.

Statistical Analysis: The statistical analysis was performed using GraphPad Prism (v10). The data are expressed as mean and standard error of the mean. The number of replicates in each group is stated in the figure legends. Statistical differences between groups were determined using t-test (paired, unpaired, or nested) for pairwise comparisons. Two-, three-way, or repeated measurements (RM) ANOVA were followed by pairwise post-hoc comparison using Fischer's LSD or Tukey's multiple comparison test if statistically significant main effects were detected. $P < .05$ were considered statistically significant.

3. RESULTS

3.1. Effect of chronic administration of the GLP1R:GIPR:GCGR triple agonist retatrutide (RTT) in DIO GLP1R KO mice

We sought to determine the relative contribution of GLP-1R agonism to the BW lowering efficacy of RTT by comparing its dose-dependent effect in DIO wildtype (WT) and GLP-1R KO mice. Consistent with previous reports [32], RTT dose-dependently decreased body weight (Figure 1a,b) and food intake (Figure 1c) in DIO mice with great superiority over semaglutide as a selective GLP-1R agonist. When administered daily at equimolar doses of 3 nmol/kg, RTT further outperformed semaglutide to improve glucose control, as evidenced by greater decrease in blood glucose (Figure 1d) and further enhanced glucose tolerance (Figure 1e,f).

In DIO GLP-1R KO mice, RTT dose-dependently decreased body weight, food intake and blood glucose when administered daily at doses of 6, 12, 25 or 50 nmol/kg (Figure 1g–j). As expected, the ability of RTT to decrease body weight and food intake was less potent in GLP-1R KO compared to wildtype mice, with approximately 8-fold greater doses required to achieve a similar degree of body weight loss (Figure 1a,b vs. 1 g, h) and food intake suppression (Figure 1c vs.1i), and somewhat lower potency to improve glucose tolerance (Figure 1k,l). These results confirm the important contribution of GLP-1R agonism in body weight lowering of RTT, but they also demonstrate that similar weight loss can be equally achieved in a comparable duration with increased dose, through GIPR and GCGR combined agonism alone. Furthermore, it suggests that the inherent GLP-1R potency of RTT to completely suppress appetite may interfere in its chronic use at sufficient dose to achieve the full metabolic effect of its inherent GCGR and GIPR agonisms.

3.2. Effect of GCGR and GIPR agonist co-therapy on BW loss and glycemic control

We next assessed the body weight lowering effect of combined chronic GCGR (BWB2086) and a GIPR agonism (BWB2065) in DIO mice (Table S1, Figures. S1–3). When used individually as monotherapy, the GIPR agonist BWB2065 and the GCGR agonist BWB2086 only moderately reduced food intake and body weight in DIO mice (Figure 2a–d). However, when used in physical co-mixture, BWB2086 dose-dependently interacted in a more than additive fashion with BWB2065 to markedly accelerate body weight loss (Figure 2c,d) and food intake suppression (Figure 2a,b). Treatment with BWB2065 (30 nmol/kg/day) elicited a marked reduction in food intake at the onset of the study that tended to diminish overtime (Figure 2a), leading to a 39% food intake reduction over vehicle controls at the end of study (Figure 2b). While co-therapy of GCGR and GIPR at 30 + 30 nmol/kg/day suppressed food intake comparable to GIPR agonism alone (Figure 2a,b), dose-escalation to 100 + 30 nmol/kg/day profoundly enhanced food intake suppression, with comparably decreased food intake relative to single GIPR agonism at treatment initiation, but with persistently lower food intake and clear separation throughout the study (Figure 2b).

While body weight remained unchanged in mice treated with 30 nmol/kg/day of the GCGR agonist (Figure 2c,d), dose-escalation to 100 nmol/kg/day accelerated BW loss compared to vehicle controls (8.5%, Figure 2d). Mice treated with the GIPR agonist exhibited a similar BW loss (10.5%; Figure 2d), despite a more pronounced reduction in food intake (Figure 2b). Co-administration of GCGR + GIPR agonists at 30 + 30 or 100 + 30 nmol/kg/day doses elicited a statistically significant 18.8% and 37.1% BW loss relative to their controls, respectively (Figure 2c,d). It dramatically exceeded the

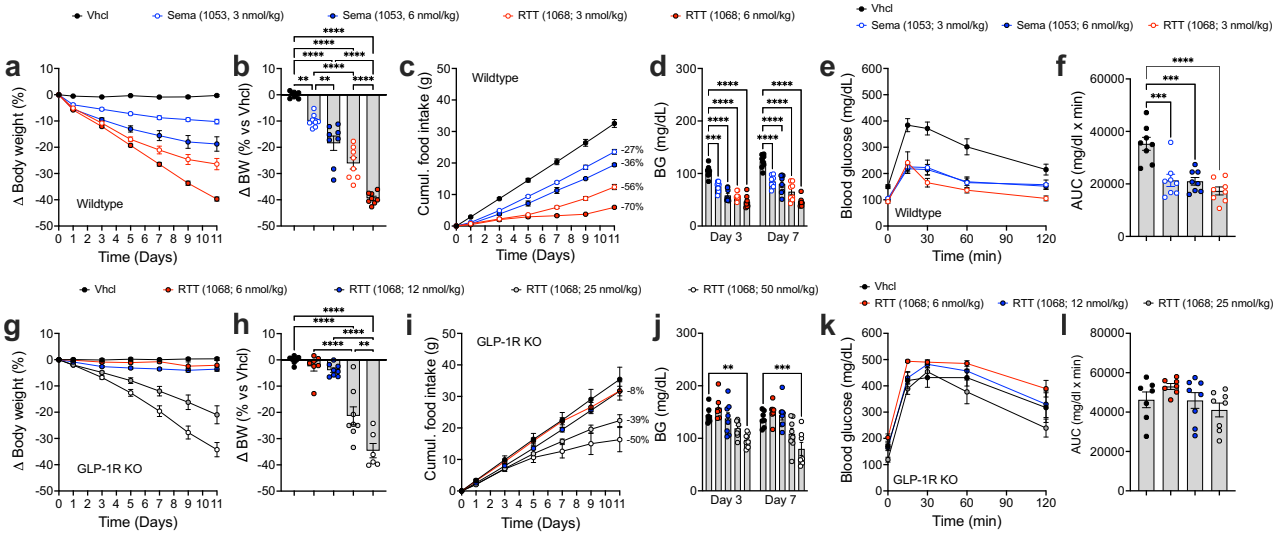

Figure 1: Treatment of DIO wildtype or GLP-1R KO mice reveals GLP1-independent BW lowering efficacy of the triple GLP1:GIP:GCG receptor agonist retatrutide. (a–f) Wildtype DIO mice (initial BW = 65.4g) were given subcutaneous injections once per day of semaglutide or RTT (each at 3 or 6 nmol/kg/d). (a) Body weight change as percentage from day 0. (b) Percentage body weight change relative to Vhcl control at day 11. (c) Cumulative food intake. The numbers next to the lines indicate percentage of cumulative food intake relative to the Vhcl control on day 11. (d) Baseline blood glucose was measured on days 3 and 7 immediately before the daily injection. (e) An IPGTT was performed after 13 days of daily treatment, 24-h after compound administration. (f) Area Under the Curve (AUC) of blood glucose during the IPGTT. (g–h) GLP-1R KO mice (initial BW = 56.6g), aged-matched and exposed to HFD for the same period of time as the wildtype DIO mice (a–f), received concurrent subcutaneous injections once per day of RTT (6, 12, 25 or 50 nmol/kg/d). (g) Percentage daily body weight change and (h) percentage body weight change relative to Vhcl control at day 11. (i) Cumulative food intake. (j) Baseline blood glucose on days 3 and 7. (e) IPGTT and (f) area Under the Curve (AUC) of blood glucose during the IPGTT. Data presented as mean ± s.e.m., n = 6–8, except for food intake (n = 2). (a, f, h, l) 1-way ANOVA, followed by Tukey's post hoc test. (d, j) 2-way ANOVA. Statistically significant differences vs Vhcl were calculated using the Tukey post hoc test. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$.

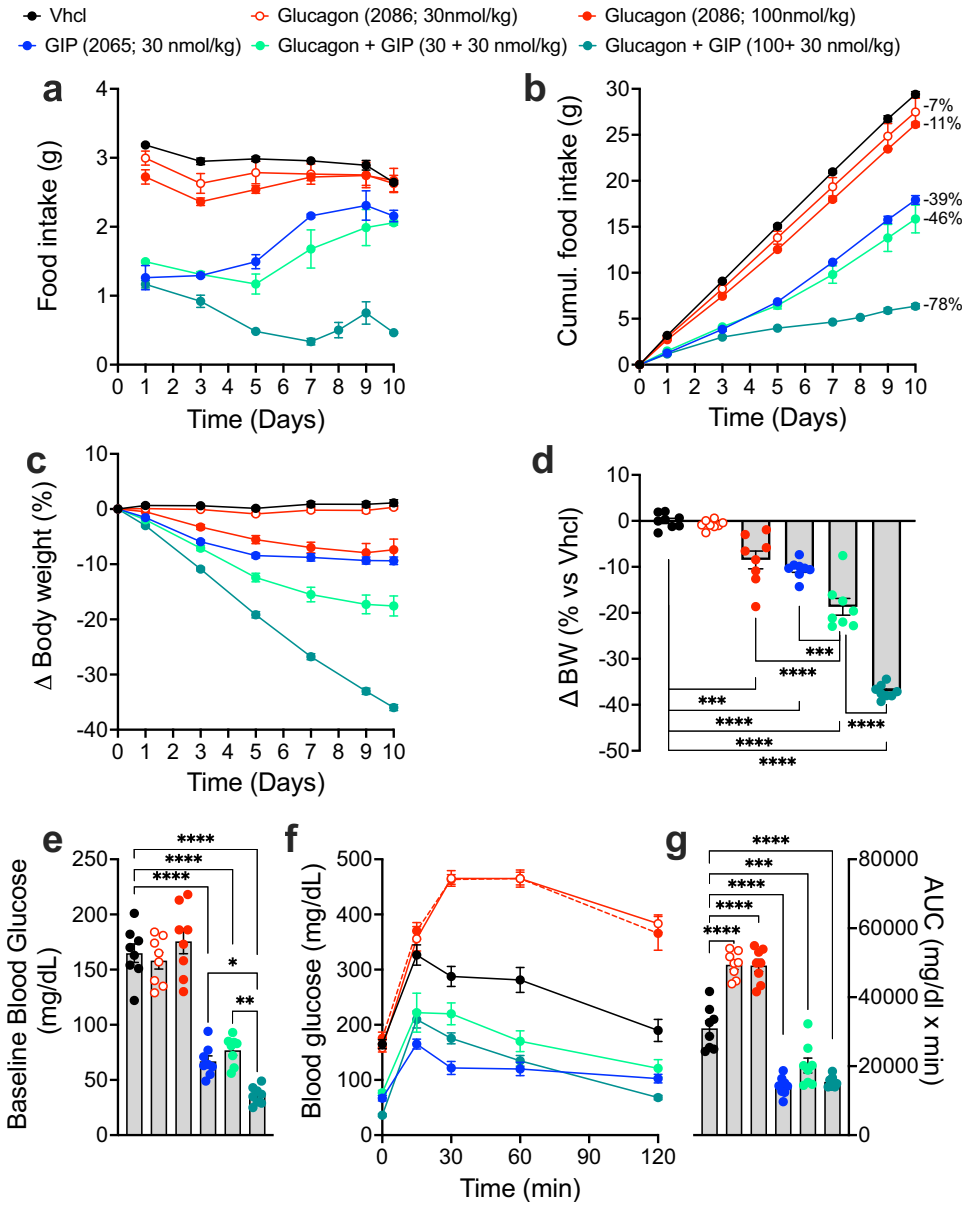

Figure 2: Co-administration of a GCG receptor agonist increases the weight lowering effect of chronic GIP receptor agonist treatment in DIO mice without impairing glycemic control. Wildtype DIO mice (initial BW = 57.8g) were given subcutaneous injections once per day of either a GCG receptor agonist (BWB2086; 30 or 100 nmol/kg), a GIP receptor agonist (30 nmol/kg) or a combination of both GCG + GIP (30 + 30 or 100 + 30 nmol/kg) for 10 days. (a) Daily food intake. (b) Cumulative food intake. The numbers next to the lines indicate percentage of cumulative food intake relative to the Vhcl control on day 10. (c) Body weight change as percentage from day 0. (d) Percentage body weight change relative to Vhcl control at day 10. (e) Baseline blood glucose immediately before a glucose bolus administration, 24-h after compound administration. (f) Blood glucose during the IPGTT, performed after 10 days of daily treatment. (g) Area Under the Curve (AUC) of blood glucose during the IPGTT. Data presented as mean ± s.e.m., n = 8, except for food intake (n = 2). (d,e,g) 1-way ANOVA, followed by Tukey's post hoc test. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$.

sum of the average BW loss seen in mice treated with the corresponding doses of agonists alone (i.e. 11.3% and 19%, respectively). Consistent with the observation that GCGR agonism interacts with GIPR agonism to accelerate weight loss and food intake suppression, co-therapy with BWB2086 and BWB2065 further decreased blood glucose compared with either monotherapy (Figure 2e). However, improvement in glucose tolerance with the co-therapy was slightly attenuated compared with GIPR agonism alone (Figure 2f,g). Collectively, these data in wildtype DIO mice support the findings with RTT in GLP-1R KO mice that simultaneous pharmacological engagement of GIPR and GCGR signaling is sufficient to elicit meaningful BW loss and improve glycemic control.

3.3. Synthesis and in vitro characterization of BWB3054, a single GIP: GCG receptor dual agonist

The integration of high potency, balanced GIPR and GCGR agonism in a unimolecular format suitable for extended duration of action was approached from three different chemical directions. They included the addition of GIPR or GCGR agonism to selective agonists that possess complementary receptor activity. Additionally, the selective

chemical ablation of GLP-1R activity from balanced triple agonists, including first generation [10] and second-generation triple agonists [11,12] was attempted. The ablation of the GLP-1 activity to a previously validated triagonist of comparable length [10] through four amino acid substitutions and inclusion of fatty acylation as observed in tirzepatide yielded the desired molecular profile. BWB3054 is uniquely differentiated by more than one-hundred-fold relative reduction in mGLP-1R potency (<1% potency) when compared to retatrutide but possesses high potency at the GIP and GCG receptors (Table S1, Figures. S1–3). This co-agonist maintains high aqueous solubility in physiological buffers and resistance to biophysical degradation comparable to competitive peptide-based drugs and candidates, such as semaglutide, tirzepatide and retatrutide.

3.4. Effect of chronic administration of the GIP: GCG receptor dual agonist BWB3054 to DIO mice and rats

Once-daily administration of BWB3054 dose-dependently decreased body weight and food in DIO mice (Figure 3a–d). Similar to our studies of RTT in the GLP-1R KO mice (Figure 1g–h), the GLP-1 free GIPR:GCGR co-agonist BWB3054 required an increased dose relative to RTT to fully promote the interaction effect to suppress food intake (Figure 3a,b). Consequentially, while BWB3054 was inferior to RTT (4 nmol/kg) to decrease body weight at daily doses of 10 nmol/kg, it yielded equal weight loss at 20 nmol/kg and superior weight loss and food intake suppression over RTT at daily doses of 30 and 40 nmol/kg (Figure 3c,d). Remarkably, and again consistent with our previous data (Figure 2a), we found in contrast to RTT food intake suppression by BWB3054 persisted throughout the study (Figure 3a). BWB3054 also dose-dependently decreased baseline blood glucose in DIO mice, with non-inferiority to RTT (4 nmol/kg) at daily doses of 10 and 20 nmol/kg and superiority at daily doses of 30 and 40 nmol/kg (Figure 3e). Furthermore, BWB3054 improved glucose tolerance in DIO, with non-inferiority to RTT at all tested doses (Figure 3f,g).

We also tested the efficacy of treatment with BWB3054 (30, 100 and 300 nmol/kg/d) for 11 days in DIO rats and compared it to the effect of RTT (3 or 10 nmol/kg/d) or a GIP mono-agonist (LY3537021 [33]; 300 nmol/kg), (Table S2, Figures. S1–3). In DIO rats, BWB3054 similarly decreased body weight and food intake in a dose-dependent manner, with great superiority over RTT (3 nmol/kg) at daily doses of 30, 100 and 300 nmol/kg, but comparable weight loss and food intake suppression upon dose-escalation of RTT to 10 nmol/kg (Figure 3h–k). In DIO rats, BWB3054 and RTT comparably decreased body fat and lean tissue mass (Fig l,m).

Baseline blood glucose did not differ among groups (Figure 3n) but plasma insulin was significantly reduced by the highest dose of RTT and the 100 nmol/kg dose of BWBW3054 (Figure 3o), although insulin levels did not differ among all BWB3054 doses and the highest dose of RTT (Figure 3o). Treatment with 300 nmol/kg of the GIPR agonist did not elicit changes in food intake, BW or body composition compared to vehicle controls.

Collectively, our data in DIO rats correlate with the results in DIO mice to demonstrate that a GLP-1 ablated (>100x), GIPR:GCGR co-agonism, when appropriately dosed, decreased body weight, persistently supressed food intake and managed glycemia comparable to retatrutide.

3.5. Characterization of energy balance in DIO mice after chronic GIPR and GCGR co-agonism and co-therapy

When administered at a daily dose of 30 nmol/kg in DIO mice, the GIPR:GCGR co-agonist BWB3054 decreased food intake with superiority over GCGR agonism, but with comparable efficacy to GIPR agonism and co-therapy with GIPR and GCGR agonism (30 + 30 nmol/kg) (Figure 4a,b). Nonetheless, despite similar food intake suppression, BWB3054 and the co-therapy outperformed both monotherapies to yield greater weight loss, hence demonstrating that BWB3054 and the co-therapy decreased body weight via food intake-dependent and independent mechanisms (Figure 4c,d). Consistent with this, weight loss induced by BWB3054 and the co-therapy was also enhanced relative to mice that were calorie-restricted (pair-fed) to match the food intake of the BWB3054 treated mice (Figure 4c,d), and this was further paralleled by a greater decrease in both fat and lean tissue mass (Figure 4e). A subset of mice receiving vehicle or the single agonists (alone or in combination) were placed in sealed cages during days 6–10 of the study to assess energy expenditure, respiratory exchange ratio and locomotor activity. No major effects were observed after treatment with either glucagon, GIP or their physical co-mixture, but the co-therapy increased dark phase expenditure relative to GIP alone (Figure 4f). Treatment with GIP reduced RER during the dark phase compared with vehicle controls, indicating increased fat utilization, and this was further accelerated by the co-therapy of GIP and glucagon (Figure 4g). None of the treatments altered locomotor activity during either the light or dark phase (Figure 4h).

When assessed at either baseline (18 h after last injection) or acutely (2 h) post injection, BWB3054 lowered blood glucose relative to vehicle controls and comparable to GIPR agonism and the co-therapy of GIPR agonism and GCGR agonism, whereas GCG monotherapy rather increased glycemia (Figure 4i). BWB3054 and their pair-feed controls potently decreased plasma insulin relative to mice treated with vehicle or the GIPR agonist (Figure 4j). All GIP containing groups tended to decrease plasma glycerol, but with significance reached only in the co-therapy (Figure 4k). The co-therapy and BWB3054 further reduced plasma triglycerides, cholesterol and liver triglycerides, with superiority of BWB3054 over its pair-fed controls (Figure 4l-n).

We performed gene expression analysis to gain preliminary insights on the impact of these treatments on metabolically relevant tissues. In the liver, the GCGR agonist increased expression of *glucose-6-phosphatase* (*G6pc),* while the co-therapy and BWB3054 decreased expression of *glucokinase* (*Gck*) and the key lipogenic genes *sterol regulatory element-binding transcription factor 1* (Srebf1), *fatty acid synthase, stearoyl-CoA desaturase 1* (Scd1) and *peroxisome proliferator activated receptor alpha (Ppara)* (Figure 4o-u). Interestingly, only BWB3054 increased expression of fibroblast growth factor 21 (*Fgf21)* (Figure 4v). In the interscapular brown adipose tissue (iBAT), BWB3054 increased expression of *uncoupling protein 1* (*Ucp1*), *peroxisome proliferator activated receptor gamma coactivator 1-alpha (Ppargc1a) and FGF21 (Figure 4w-z)* while BWB3054 uniquely increased expression of *lipoprotein lipase* (*Lipe)* and *Srebf1 in the* inguinal white adipose tissue *(iWAT)*, mimicking calorie restriction–associated WAT remodeling [34] (Figure 4b).

3.6. Comparative pharmacokinetic analysis and tolerability of BW3054 in cynomolgus monkey

The co-agonist BWB3054 was administered as a single subcutaneous dose to cynomolgus male monkeys (Figure 5, S4,5) to evaluate its tolerability and pharmacokinetic (PK) profile for potential use in treatment of obesity, as compared to tirzepatide and retatrutide. Each of the three peptides were administered at respective doses of 20 and 40 nmol/kg to a total of twelve lean monkeys, in groups of two. BWB3054 was distinguished by the absence of any sign of discomfort that was apparent in the two other treatments at the higher of the two

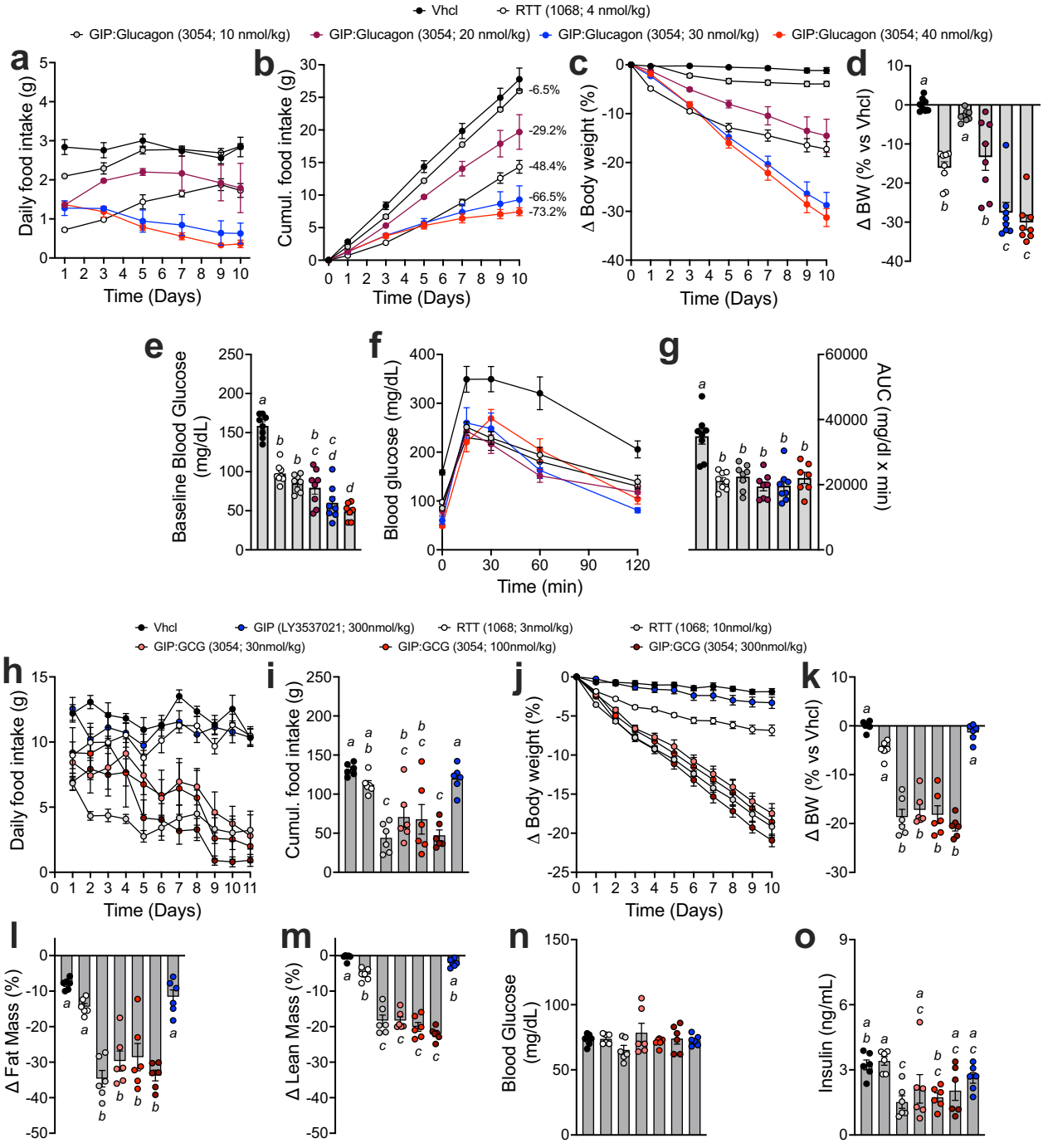

Figure 3: Treatment with a single molecule GIP:GCG receptor dual-agonist BW3054 reduces body weight and improves glycemic control in DIO mice and rats. (a–g) Wildtype DIO mice (initial BW = 64.4g) were given subcutaneous injections once per day of a GIP:GCG receptor dual-agonist (BWB3054; 10, 20, 30 or 40 nmol/kg) for 10 days. An additional group received injections of the GLP1:GIP:GCG receptor triple-agonist Retatrutide (RTT; 4 nmol/kg). (a) Daily food intake. (b) Cumulative food intake. The numbers next to the lines indicate percentage of cumulative food intake relative to the Vhcl control on day 10. (c) Body weight change as percentage from day 0. (d) Percentage body weight change relative to Vhcl control at day 10. (e) Baseline blood glucose immediately before a glucose bolus administration, 24-h after compound administration. (f) Blood glucose during the IPGTT, performed after 10 days of daily treatment. (g) Area Under the Curve (AUC) of blood glucose during the IPGTT. (h–o) Wildtype DIO rats (initial BW = 669.5g) were given subcutaneous injections once per day of a GIP:GCG receptor dual-agonist (BWB3054; 30, 100 or 300 nmol/kg) for 11 days. In addition, two groups received injections of the GLP1:GIP:GCG receptor triple-agonist Retatrutide (RTT, BWB 1068) at either 3 or 10 nmol/kg and an additional group received a GIP receptor mono-agonist (LY3537021) at 300 nmol/kg. (h) Daily food intake. (i) Cumulative food intake on day 11. (j) Body weight change as percentage from day 0. (k) Percentage body weight change relative to Vhcl control at day 11. (l) Fat and (m) lean mass change as percentage from day 0. (n) Baseline blood glucose and (o) plasma insulin, 24-h after compound administration on day 11. Data presented as mean ± s.e.m.,(a–g) n = 7–8, except for food intake (n = 2). (h–o) n = 6. (d,e,g,i,k-o). 1-way ANOVA, followed by Tukey's post hoc test. Groups that share the same letter are not statistically different (P ≥ .05).

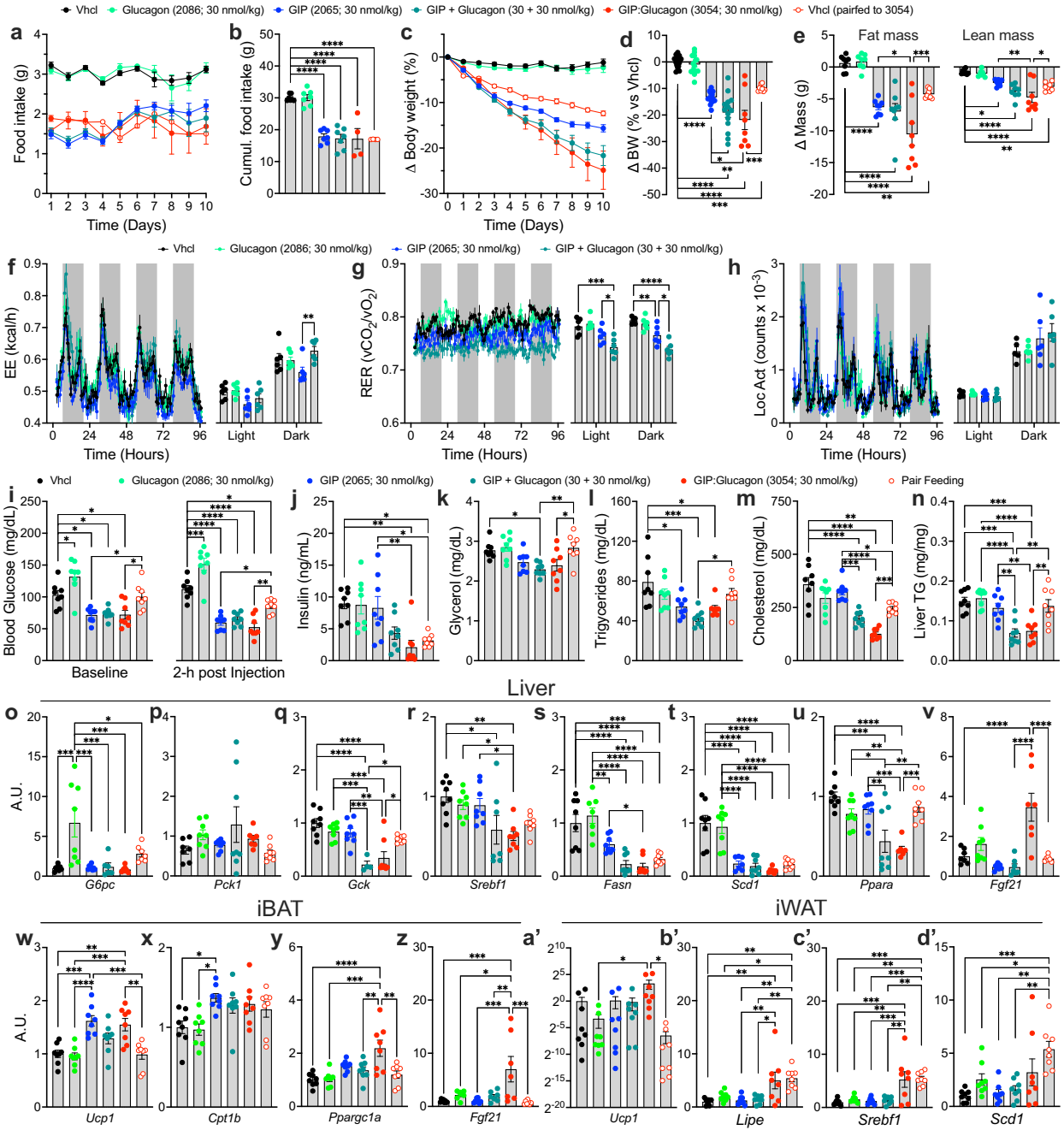

Figure 4: Effect on energy balance, plasma metabolites, and gene expression of chronic administration—either individually or in combination—of GIP or GCG receptor selective agonists, as well as GIP:GCG receptor dual agonist, in DIO mice. (Wildtype DIO mice (initial BW = 56.5g) were were given subcutaneous injections of 30 nmol/kg once per day of either a GIPR:GCGR dual agonist (BWB3054), a GCG receptor agonist (BWB2086), a GIP receptor agonist or a combination of both GCG + GIP (30 + 30 nmol/kg) for 10 days. (a) Daily food intake. (b) Cumulative food intake. (c) Body weight change as percentage from day 0. (d) Percentage body weight change relative to Vhcl control at day 10. (e) Change in fat and lean mass during the 10-day treatment. (f–h) A subset of mice (n = 6/group) receiving vehicle or the single agonists (alone or in combination) were placed in sealed cages during days 6–10 of the study to assess energy expenditure (f), respiratory exchange ratio (g) and locomotor activity (h). Hourly (lines, left) and light/dark phase average (f,g) or cumulative (h) (bars, right) energy expenditure (f), respiratory exchange ratio (g) and locomotor activity (h). Light/Dark phase activity is expressed as counts/10-5 (h, right). (i) Blood glucose on day 10, before (left) and 2-hours after a final treatment injection (left), measured at the time of tissue collection. (J) Plasma insulin, (k) glycerol, (l) triglycerides and (m) cholesterol, 2-hours after a final treatment injection. (n) Hepatic triglyceride content, expressed as milligrams per milligram of wet tissue. (o–v) Expression of genes involved in gluconeogenesis (i.e. (o) *G6pc*, (p) *Pck1*, (q) *Gck*) and lipid synthesis (i.e. (r) *Srebf1*, (s) *Fasn*, (t) *Scd1*), as well as of (u) *Ppara* and (v) *Fgf21*, in the liver. (w–z) Expression of genes involved in thermogenesis in iBAT (i.e. (w) *Upc1*, (x) *Cpt1b*, (y) *Ppargc1a* and (z) *Fgf21*. (a'-d') Expression of genes involved in thermogenesis and lipid metabolism in iWAT (i.e. (a') *Ucp1*, (b') *Lipe*, (c') *Srebf1* and (d') *Scd1*. Data presented as mean ± s.e.m. (a,b) n = 4–6; (c,d) n = 14–8, (f,g) n = 6; (h) n = 5–6; (e, i-d') n = 6–8. Outliers on gene expression analysis detected using the Grubb's test (Alpha = 0.05) were removed from statistical analysis. (b, d, e, i-d') 1-way ANOVA, followed by Tukey's post hoc test. (bar graphs on f, g, h) Repeated measurements ANOVA, followed by Tukey's post hoc test. * = P < 0.05, ** = P < 0.01, *** = P < 0.001, **** = P < 0.0001.

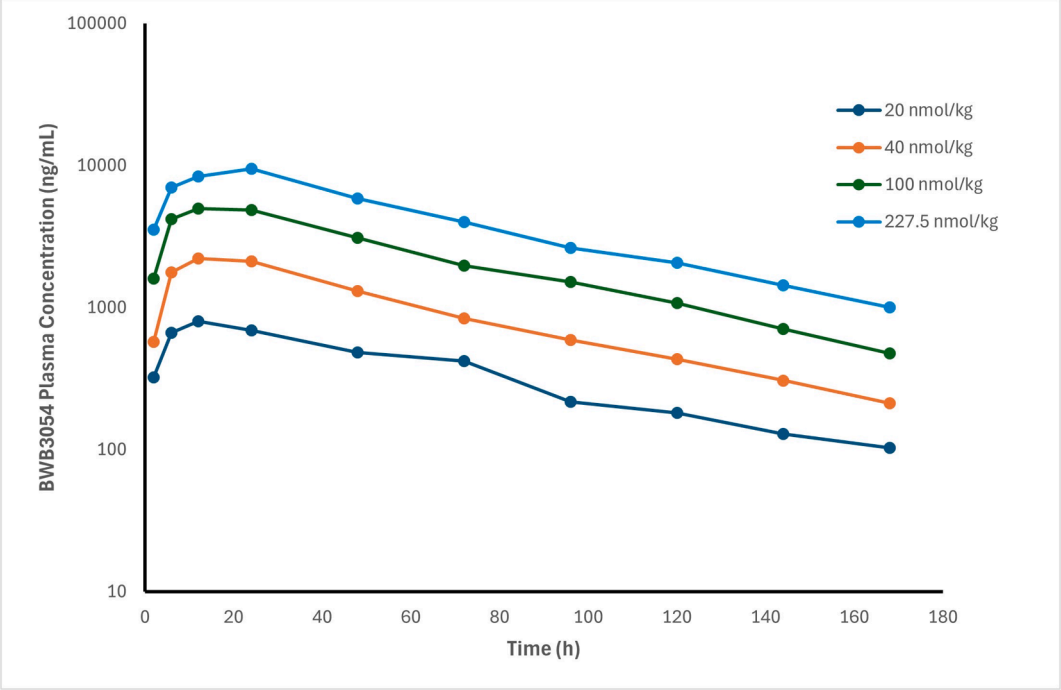

Figure 5: Pharmacokinetic profile of BWB3054. BWB3054 plasma concentrations (ng/mL) versus time profiles following a single subcutaneous administration to male cynomolgus monkeys (n-2/group).

administered doses (i.e., transient inappetence and fecal alterations corresponding to the timing of peak plasma exposure). Continued escalation in dosing of only BWB3054 included administration of 100 and 227.5 nmol/kg without any signs of distress or abnormal behavior. Tirzepatide plasma concentration increased in a dose proportional manner. Mean half-life ($t_{1/2}$), apparent clearance (CL/F), and apparent volume of distribution (Vz/F) ranged from 48.9 to 61.2 h, from 0.0150 to 0.0162 mL/min/kg, and from 0.0697 to 0.0741 L/kg, respectively. Retatrutide peptide exposures also increased in a dose proportional manner. Mean half-life ($t_{1/2}$), apparent clearance (CL/F), and apparent volume of distribution (Vz/F) ranged from 69.2 to 83.9 h, from 0.0109 to 0.0114 mL/min/kg, and from 0.0667 to 0.0789 L/kg, respectively. BWB3054 also increased in a generally dose proportional manner following a single SC dose of 20, 40, 100, or 227.5 nmol/kg. Mean half-life ($t_{1/2}$), apparent clearance (CL/F), and apparent volume of distribution (Vz/F) ranged from 45.4 to 62.9 h, from 0.0198 to 0.0246 mL/min/kg, and from 0.0794 to 0.129 L/kg, respectively. The Tmax for the three peptides was comparable and centered at 18h in a range of 12–24h.

All peptides had similar exposure (at equivalent doses) and disposition parameters. Exposure for the high dose of BWB3054 was ~4-fold higher than observed for 40 nmol/kg RTT and TZP, treatments that were associated with adverse observations that warranted against further dose escalation comparable to BWB3054 (Figure S13).

4. DISCUSSION

The steadily increased global burden of obesity and type 2 diabetes (T2D) over the past half century was medicinally managed with agents of modest efficacy or unacceptable safety profiles [2]. The advent of incretin-based therapies, anchored by GLP-1 agonism has initiated a watershed transition in the treatment of obesity and associated metabolic co-diseases [1,17,24]. The GLP-1R agonist semaglutide was first to demonstrate weight loss in the range of 15% in obese, non-diabetic individuals to herald the power of this single mechanism in weight management [25]. While far short of what can be attained by bariatric surgery, it has been transformative in establishing that excess weight can be medicinally managed in a similar manner to elevated blood pressure, glucose and cholesterol. A new generation of peptide-based therapeutics combining two (i.e., GLP1:GIP, GLP1:GCG, or GLP1+Amylin) or even three (i.e., GLP1:GIP:GCG) degrees of receptor agonism has emerged to transform clinical outcomes to what otherwise required surgical intervention [11–13,26,32]. However, the ceiling to therapeutic performance for these medicines is not necessarily defined by their maximum biological efficacy but rather by GI tolerability inherent to GLP1R agonism (i.e. nausea, vomiting, and diarrhea). It poses a significant, well-recognized barrier to dose optimization and long-term adherence [24,27–29].

Here, we provide evidence that therapies relying on combined GIP:GCG activity can provide comparable weight-lowering outcomes in rodent models of obesity to those dependent on GLP1R agonism. This promotes a novel, previously unappreciated approach to anti-obesity therapy given the central importance of GLP-1 as a proven mechanism of action, but complicated by its tolerability profile.

Our determination that retatrutide can normalize body weight in obese mice devoid of GLP-1 action is a seminal observation that anchors the subsequent demonstration of additivity in GIPR and GCGR co-agonism when chronically administered at doses commonly not employed due to the overwhelming appetite suppressive impact of GLP-1 pharmacology. The combination studies reveal an interaction resulting in suppression of food intake and consequent body weight loss that exceeds the sum of the individual effects. Given that this apparent synergistic interaction becomes more evident with increasing doses of GCG combined with a given dose of GIP, it is tempting to speculate that GCG-derived events increase the food intake-lowering efficacy of GIPR agonism. GIP's modulatory effects on feeding are largely

restricted to the brainstem [14,15], an area lacking GCGR expression [35], making a cell-autonomous interaction between GIPR and GCGR signaling in those neuronal centers unlikely. Whether cell-autonomous or not, the mechanistic underpinning of such interaction now emerges as a high priority for further study.

Our findings with the GCGR and GIPR selective agonists are consistent with the inherent feeding-suppressive activity of GIP [15,36] and, to a lesser extent, glucagon [7–9]. Two lines of evidence demonstrate that it is indeed the GIP:GCG interaction that is responsible for the superior efficacy. The first is the dose-dependent food intake suppression (and corresponding body weight loss) seen with RTT in GLP1R KO mice. Importantly, the ability for RTT to reduce food intake in GLP1R KO mice demonstrates that endogenous GLP1R signaling is not a necessary element mediating the food-suppressive effect of combined GIPR:GCGR co-agonism. The second is that the single-molecule GIPR:GCGR dual agonist BWB3054 promotes similar dose-dependent suppression of feeding despite significantly diminished GLP1R in vitro activity. It is important to note that measurement of food intake from group-housed mice limits our ability to draw statistically robust conclusions regarding observed differences. Additionally, the daily frequency of data collection does not allow us to determine whether the observed reduction in food intake is attributable to changes in meal size, meal frequency, or both. Lastly, the degree to which the residual 1% GLP1R may differentially contribute to the efficacy of BWB3054 across species, while likely small needs further characterization. The underlying mechanisms by which simultaneous activation of GIPR and GCGR signaling leads to a reduction in food intake that exceeded the effect of individual agonists remain unclear.

Our results are consistent with a contribution of energy expenditure to the weight-lowering effect seen with GIPR:GCGR co-agonism. This is clearly evidenced by the fact that mice receiving simultaneous GIPR:GCGR co-agonism, either as co-administration or treatment with the dual-agonist BWB3054, exhibited a larger reduction in body weight than vehicle-treated pair-fed mice constrained to the same caloric consumption. Although the analysis of energy expenditure did not reveal overall statistically significant differences relative to the control group, it did show that mice receiving co-treatment maintained higher energy expenditure during the active phase when compared to those receiving GIPR agonist alone, which tended to exhibit a slight reduction in energy expenditure, a counterregulatory response to the sustained negative energy balance [37]. This suggests that GIP and GCG co-administration may impair such a response, allowing for preservation of baseline energy expenditure, thus contributing to a larger net negative energy balance. The lower respiratory exchange ratio further suggests a more pronounced negative energy balance in the mice receiving GIP and GCG co-administration, as it indicates increased reliance on fatty acid oxidation as metabolic fuel due to mobilization of endogenous fat stores. Importantly, the mice receiving the GIP and GCG co-administration exhibited normal locomotor activity compared to the rest of the groups, despite sustained suppression of food intake and body weight loss. Malaise-induced anorexia is often accompanied by reduced locomotor activity [38,39]. Hence, normal locomotion argues against sickness contributing to the reduced caloric intake seen with GIP:GCG treatment. This finding in mice is also consistent with the favorable apparent tolerability seen in NHPs with BWB3054, at considerably higher relative doses.

Importantly, the anti-obesity benefits of GIPR:GCGR co-agonism do not come at the expense of glycemic control. This is supported by the improvement in glucose tolerance seen with the dual GIPR:GCGR agonist BWB3054 and by the preservation of improved glucose in mice receiving GIP + GCG physical co-administration. It is also noteworthy that chronic exposure to GIP:GCG receptor co-agonism, either due to co-administration or treatment with the dual agonist, failed to elicit an increase in hepatic expression of the gluconeogenic gene *G6pc*, which is under strong direct control of GCGR signaling. Collectively, these results suggest that sustained GIP agonism can offset the hyperglycemic activity of unopposed GCGR agonist treatment. It is noteworthy that similar GCG buffering capacity characterizes the metabolic benefits of treatment with single-molecule GLP1R:GCGR dual agonists [30]. Whether GCG buffering exhibited by GIP and GLP1 is due to a common (i.e., insulinotropic) or distinct mechanisms remains to be determined.

In addition to the weight and glucose-lowering benefits, GIPR:GCGR co-agonism elicited uniquely superior reduction in circulating cholesterol and hepatic triglyceride content, above and beyond the effect seen by body weight loss due to calorie restriction (i.e., pair-feeding) or selective GCG, or GIP treatment. These reductions were consistent with a reduction in hepatic expression of genes involved in lipogenesis, rather than with changes in the expression of genes involved in lipid catabolism. These gene expression findings do not rule out the possibility that enhanced hepatic fatty acid oxidation, resulting from increased GCGR signaling in the liver [40] contributes to the reduction in hepatic triglyceride content. Therefore, while recognizing the limitations inherent in relying solely on gene expression analysis, the data supports the potential benefits of GIP:GCG co-therapy possibly extending to fatty liver.

In summary, we demonstrate that while GLP-1 receptor agonism is a potent and proven method in reversing obesity and its metabolic consequence, it is not unique or essential in rodent models. In its absence co-treatment with GCG and GIP by physical and covalent combination demonstrates an interactive pharmacology not previously recognized to normalize body weight in obese rodents. It presents the translational prospect in integrating GIP and GCG pharmacology to achieve comparable effects on weight loss, energy balance, glycemic control, and lipid metabolism with improved tolerability. In a disease of this magnitude and heterogenous etiology it seems certain there will be a need for alternative approaches.

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Diego Perez-Tilve: Writing — original draft, Formal analysis, Conceptualization. **Fa Zhang:** Writing — original draft, Methodology, Formal analysis. **Yujin Zhang:** Writing — original draft, Methodology, Formal analysis. **Kaitlyn Lohman:** Investigation, Formal analysis. **Joyce Sorrell:** Investigation, Formal analysis. **Andrew Vick:** Writing — original draft, Methodology, Formal analysis. **Timo D. Müller:** Writing — original draft, Formal analysis, Conceptualization. **Matthias H. Tschöp:** Writing — original draft, Formal analysis, Conceptualization. **Richard D. DiMarchi:** Writing — original draft, Formal analysis, Conceptualization.

AUTHORS CONTRIBUTIONS

KL, JS performed research and analyzed data. **FZ, YZ, AV** designed research, analyzed data, and wrote the manuscript (formal analysis,

methodology, writing). **DPT, TDM, MHT, RDD** conceptualized the studies, designed research, analyzed data, and wrote the manuscript (conceptualization, formal analysis, writing).

DECLARATION OF COMPETING INTEREST

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: **D.P-T.** is an employee of the University of Cincinnati-College of Medicine, which has a research agreement with BWB and holds BWB stock. **F.Z.** and **Y.Z.** are employees of Indiana University, which has research and licensing agreements with BWB. **K.L.** and **J.S.** are employees of the University of Cincinnati-College of Medicine, which has a research agreement with BWB. **A.V.** is an employee of BWB. **T.D.M.** and **M.H.T.** are cofounders of BWB and hold BWB stock. **R.D.D.** is a cofounder of BWB and holds BWB stock. He is also an employee of Indiana University, which has research and licensing agreements with BWB. **F.Z.**, **M.H.T.** and **R.D.D.** have co-invention pending that pertains to GIP:GCG coagonism.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2026.102365.

DATA AVAILABILITY

Data will be made available on request.

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