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Novel cannabinoid receptor 1 inverse agonist CRB-913 enhances efficacy of tirzepatide, semaglutide, and liraglutide in the diet-induced obesity mouse model

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Abstract

Objective: Incretin receptor agonists are now standard of care in treating obesity. Their efficacy and tolerability might be further improved by combining them with compounds that offer orthogonal mechanisms of action. The cannabinoid type 1 receptor (CB1R) is a clinically validated therapeutic target in obesity, and several experimental CB1R inverse agonists have been shown to induce weight loss.

Methods: This study characterizes a novel CB1R inverse agonist (CRB-913) with similar preclinical potency to rimonabant but markedly reduced brain penetration. CRB-913 was tested as monotherapy and in combination with tirzepatide, semaglutide, or liraglutide in the diet-induced obesity (DIO) mouse model for body weight reduction.

Results: CRB-913 demonstrated enhanced plasma exposure (3.8-fold larger area under the curve$_{last}$) and reduced brain levels (9.5-fold lower area under the curve$_{last}$) than rimonabant. CRB-913 monotherapy yielded a dose-dependent decrease in body weight in DIO mice reaching −22% within 18 days. In further DIO studies in combination with tirzepatide, semaglutide, or liraglutide, CRB-913 (2.5 mg/kg) resulted in −32.6%, −28.8%, and −16.8% decreases in body weight on Day 18, respectively, with concomitant improvements in body fat content, liver triglycerides, and liver fat deposits.

Conclusions: CRB-913 in combination with incretin analogues could deliver meaningful improvements over current standards of care for obesity and related conditions.

INTRODUCTION

The discovery of the role of incretins in metabolic regulation and the subsequent development of their analogues for treating type 2 diabetes and obesity have given rise to an impactful therapeutic class of drugs for many patients [1–3]. However, an unmet need to improve the efficacy, tolerability, and convenience of these therapeutics remains. Combining incretin analogues with orthogonal therapeutic modalities could offer a path to achieving this important objective [4].

The cannabinoid receptor type 1 (CB1R) is a validated clinical target for obesity and related disorders [5]. CB1R is a G-protein coupled receptor (GPCR) that is abundant in the brain but also found in the liver, kidney, and pancreas, as well as adipose and muscle tissues [6]. In the early 2000s, numerous brain-penetrant CB1R inverse agonists were developed for obesity, producing significant and meaningful reductions in body weight and related clinical outcomes [7–10]. The most advanced of these drug candidates, rimonabant (Sanofi), was approved in 2006 for treating obesity in 37 countries, including the European Union [11]. In sharp contrast, the US Food and Drug

Administration (FDA) rejected its marketing authorization application in 2007, citing a potential risk of increased suicidal ideation. In January 2009, the European Medicines Agency withdrew authorization for rimonabant, leading to the worldwide withdrawal of rimonabant and abandonment of this drug class [12, 13].

There is now renewed attention on developing a new generation of CB1R inverse agonists that target peripheral CB1R while reducing brain exposure [14, 15]. Several such compounds have demonstrated weight loss in preclinical models of obesity as well as improved systemic and tissue-specific metabolic outcomes, including insulin and leptin resistance, dyslipidemia, nonalcoholic fatty liver disease, inflammation, β-cell loss, and diabetic nephropathy [16–20]. CRB-4001 (also known as JD-5037) has been one of the most extensively characterized compounds of this new class of CB1R inverse agonists [21, 22]. However, during our translational preclinical studies with CRB-4001, several pharmacokinetics (PK) and formulation challenges were encountered that were unfavorable to advancing this compound into the clinic. We therefore set out to design a chemically distinct drug candidate with improved bioavailability and with even lower brain exposure than CRB-4001 [21, 23, 24].

In this paper we characterize the *in vivo* pharmacology of CRB-913, our follow-on compound to CRB-4001. We demonstrate that CRB-913 exhibits similar binding to CB1R as rimonabant but has markedly reduced brain exposure. Like rimonabant, CRB-913 monotherapy in diet-induced obese (DIO) mice induces reductions in body weight, body fat content, food consumption, liver triglycerides, and liver fat deposits, in addition to improvements in insulin resistance and leptinemia.

Zizzari et al. demonstrated the ability of CRB-4001 to enhance the effects of semaglutide in the DIO mouse model [25]. We extend those results to show that combining CRB-913 with tirzepatide, semaglutide, or liraglutide yields additive effects across weight loss and related physiological end points. We propose that such combination therapy could significantly enhance the emerging antiobesity therapeutic standard of care.

Study Importance

What is already known?

- The cannabinoid receptor type 1 (CB1R) is a validated clinical target for obesity and related disorders.
- Peripherally restricted CB1R inverse agonists such as CRB-4001 reduced food intake, body weight, and adiposity in the absence of high brain CB1R occupancy common with rimonabant and other first generation CB1R inverse agonists.
- The coadministration of CRB-4001 with semaglutide achieved greater reduction in body weight and fat mass than monotherapies in diet-induced obese mice.

What does this study add?

- We have designed a novel CB1R inverse agonist (CRB-913) with markedly reduced brain exposure compared with rimonabant but with similar efficacy in inducing weight loss in the diet-induced obesity (DIO) mouse model.
- Combining CRB-913 with the incretin analogues tirzepatide, semaglutide, or liraglutide resulted in additive efficacy in the DIO mouse model that could translate to improved antiobesity therapy.

How might these results change the direction of research or the focus of clinical practice?

- Convergent physiological benefits of dual CB1R and incretin modulation mechanisms could translate to improved obesity management.
- CRB-913 could enhance the efficacy of current incretin analogue drugs and provide an oral treatment that could improve patient compliance and help address incretin tolerability issues.

METHODS

All animal experiments were conducted in accordance with the Institutional Animal Care and Use Committee standard animal procedures and in compliance with the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Office of Laboratory Animal Welfare.

DIO mouse generation

Five-week-old male mice (C57BL/6J [Gem Pharmatech] [two studies: CRB-913 monotherapy compared with rimonabant; CRB-913 monotherapy and in combination with tirzepatide or semaglutide] or C57BL/6NTac [Vivo Biotech] [one study: CRB-913 monotherapy and in combination with liraglutide]) were fed a high-fat pelleted diet (D12492i [Research Diets, Inc.]) with ad libitum fresh water and housed four mice per cage in a controlled environment (20–24 °C, 30%–70% relative humidity) with a 12-h dark/light cycle. After ≥14 weeks of feeding, the mice had obesity (mean starting weight ~ 46 g [range 44.0–48.8 g]), were mildly to moderately hyperglycemic (fasting blood glucose ≥ 120 mg/dL), and showed impaired glucose tolerance. Mice were housed singly for 2 weeks before and during the DIO studies, except for the CRB-913 in combination with liraglutide study, during which mice were housed four per cage. To habituate mice to dosing procedures and minimize stress-related body weight changes, mice were orally dosed with placebo (40 mg/kg hydroxypropyl methylcellulose [HPMC] E3 in 0.5% HPMC-E3), 5 mL/kg, for ~2 weeks before study start. Mice whose body weight had not stabilized by the end of dosing habituation were removed from the study.

Test materials and dosing

CRB-913 was produced by Corbus Pharmaceuticals; liraglutide was from Medchem Express; semaglutide, tirzepatide, and rimonabant were sourced from WuXi AppTec. CRB-913 and rimonabant formulations (10 mg/mL suspension in 0.5% HPMC-E3) were prepared weekly, stored at 2 to 8 °C, and diluted with 0.5% HPMC-E3 to reach desired treatment concentrations. Incretin dosing solutions were prepared at the desired final concentrations the day before dosing and stored at 2 to 8 °C. The vehicles were phosphate-buffered saline (PBS) (pH 7.4) for liraglutide and 20mM citrate buffer (pH 7.0) for tirzepatide and semaglutide. Incretins were administered subcutaneously (SC), with liraglutide with a short half-life requiring twice daily dosing, whereas both semaglutide and tirzepatide were administered every 3 days. CRB-913 and rimonabant were given by oral gavage. CRB-913 was administered twice daily at 8- and 16-h intervals. The once-daily rimonabant treatment group received an equivalent dose of placebo/vehicle (40 mg/kg HPMC-E3 in 0.5% HPMC) from 9:00 a.m. to 11:00 a.m. and active-treatment dosing between 5:00 p.m. and 7:00 p.m.

Body weight, food intake measurements, body composition analysis, and glucose metabolism

During the active-treatment period in DIO studies, each animal's body weight and food intake were recorded daily before morning dosing. Cumulative body weight changes were reported on Day 18, before fasting procedures required for the oral glucose tolerance test (oGTT). Lean and fat mass was measured using an EchoMRI-130 (Echo Medical Systems). Resting blood glucose was measured in DIO mice at baseline and before dosing on Days 7, 14, and 20. For the oGTT, mice were fasted for 16 h after the evening dosing on Day 20 (CRB-913 in combination with tirzepatide or semaglutide study) or Day 27 (CRB-913 in combination with liraglutide study). In the morning, basal fasting glucose was measured by tail vein nick before dosing. Glucose was administered 1 h later by oral gavage at 2 g/kg (dosing volume 5 mL/kg). Blood glucose levels were measured with Bayer Contour TS or LifeScan glucometer at 0 (predose), 15, 30, 60, and 120 min after glucose dosing. Following glucose measurement, ~30 µL of blood was collected by tail vein nick into EDTA tubes at 0, 15, and 30 min; plasma was collected from spun blood and stored at −80 °C for subsequent assay of insulin levels.

Plasma and tissue analyses

Plasma PK and brain samples in lean mice were collected at 0.25, 0.5, 1, 2, 4, 8, 12, and 24 h post dose after 1 and 10 days. Liraglutide DIO satellite PK samples on Day 21 were collected after the first dose by sparse sampling from six mice ($n = 3$/time point) at 1, 2, 4, 8, and 12 h post dose (50 µL blood collected). For twice daily dosing, all plasma collection was completed before the second dose. Samples were analyzed by liquid chromatography-mass spectroscopy.

On Day 35 (CRB-913 in combination with liraglutide study) or Day 29 (CRB-913 alone and in combination with tirzepatide or semaglutide study) after 5 hours of fasting and 1 h after dosing, mice were euthanized and bled via cardiac puncture and whole blood was collected into EDTA-2K tubes. Serum alanine aminotransferase, aspartate aminotransferase, high-density lipoprotein, low-density lipoprotein, triglyceride, and cholesterol levels were analyzed on the day of blood collection using ERBA EM360 clinical chemistry analyzer. Plasma leptin levels were measured using a mouse leptin enzyme-linked immunosorbent assay (ELISA) kit from Sigma (lot #3713197) or Millipore (EZML-82 K), reading at 450 and 590 nm.

Mouse brains and livers were collected for all groups. The brains were rinsed briefly with cold saline, blot dried, and halved along the sagittal midline. The right side was weighed and snap-frozen in liquid nitrogen for bioanalysis. Samples of blood (25 µL) or brain were precipitated with 200 µL of acetonitrile containing an internal standard, vortexed (850 rpm, 5 min), and centrifuged (2000*g*, 5 min) at 4 °C. The supernatant (110 µL) was diluted with 150 µL of methanol:water (1:1, volume per volume [v/v]) and analyzed by liquid chromatography-mass spectroscopy using a C18 column. CRB-913 concentrations were determined against a standard curve generated from ~12 standards prepared in a matching matrix (plasma or brain extract) over a range from 0.5 to 3000 ng/mL (plasma) or 1 to 3000 ng/mL (brain).

For liver biomarker measurements, the left lobe was fixed in formalin for Oil Red O staining for lipid deposition. An additional 30 mg of liver tissue was stored at −80 °C for triglyceride, total cholesterol, and free fatty acid detection. To analyze hepatic triglycerides, stored tissues were thawed and homogenized in 1 mL of 5% Triton X-100 to extract triglycerides. Homogenized tissues were heated to 80 °C in a water bath for 5 min then cooled and reheated to 80 °C to solubilize the triglycerides. Samples were centrifuged (2000*g*, 5 min), and the supernatant was collected for quantification using a commercially available kit from ERBA (lot #S062146) or Nanjing Jiancheng (TG Elisa Kit A110-1).

Receptor selectivity

CB1R and CB2R human cannabinoid GPCR binding, cell-based agonist arrestin, and inverse agonist cyclic AMP (cAMP) assays were done at Eurofins/CEREP.

Statistical analyses

Data are mean ± SEM and were analyzed using Prism 9 (GraphPad). ANOVA, followed by the appropriate post hoc test, was used as specified in the figure legends.

RESULTS

CRB-913 is a novel CB1R inverse agonist with differentiated brain exposure to rimonabant

We aimed to develop an improved CB1R inverse agonist with increased binding and selectivity to CB1R and minimal blood–brain

barrier penetration. In human CB1R and CB2R binding assays, CRB-913 bound selectively to CB1R (half-maximal inhibitory concentration [IC_{50}] = 1.2 nM) over CB2R ([IC_{50}] > 1000 nM) (Figure 1A). In two functional assays, CRB-913 displayed high potency in both cAMP inverse agonist (half-maximal effective concentration [EC_{50}] = 1.7 nM) and β-arrestin antagonist (EC_{50} = 0.3 nM) activities compared with the standard CB1R inverse agonist AM281 (Figure 1B,C).

CRB-913 PK following a single 10 mg/kg dose in lean mice was markedly different from rimonabant PK, demonstrating both enhanced plasma exposure (3.8-fold larger area under the curve [AUC] last [Figure 2A]) and reduced brain levels (25-fold lower maximum brain concentration [C_{max}] and 9.5-fold lower brain AUC_{last}; Figure 2B). In particular, CRB-913 brain concentrations were extremely low and similar across the time points evaluated (14.9, 22.3, 20.9, and 11.0 ng/g at 1, 2, 4, and 8 h, respectively). In addition, we confirmed the low brain exposure of CRB-913 relative to rimonabant in a second PK study following single and repeated daily dosing. After a single dose, CRB-913 brain C_{max} was 18-fold lower than rimonabant (31.4 ± 3.9 vs. 561 ± 74.5 ng/g; Figure 2C). Importantly, after repeated once daily dosing at 10 mg/kg for 10 days, CRB-913 resulted in minimally higher C_{max} (37.6 ± 12.5 ng/g, 1.2-fold) whereas brain accumulation was 1.8-fold by AUC_{last}, consistent with calculations estimated from the terminal brain half-life (10.7–13.1 h). Thus, CRB-913 has low brain penetration and accumulation properties.

As PK properties can be affected by fat content, we subsequently measured brain and peripheral exposure in DIO mice maintained on a high-fat diet at steady state (Day 21) following a twice daily dosing regimen at 5 and 10 mg/kg. Twice daily dosing was selected to minimize brain C_{max} and maintain peripheral exposure above CB1R EC_{90} (after accounting for plasma protein binding). Dosing at 5 mg/kg twice daily led to only a 1.6-fold increase in peak steady state brain levels (62 ng/g) of CRB-913 when compared with lean animals dosed at 10 mg/kg once daily (Figure 2D), driven by 2-fold higher plasma C_{max} and 5-fold higher $AUC_{0\text{-}24h}$ in the DIO mouse (data not shown). A similar 2.3-fold increase in DIO mouse brain levels (1282 ng/g) was found for rimonabant 10 mg/kg once daily. Dosing CRB-913 at 10 mg/kg twice daily (20 mg/kg/d) resulted in a dose-proportional increase in brain levels (122.7 ng/g), a ratio still 10-fold lower than with rimonabant. Importantly, coadministration of the incretin liraglutide (13 nmol/kg, SC, twice daily) had no effect on CRB-913 plasma levels (Figure 2D) or peak brain levels (measured 1 h after CRB-913 administration) at either 5 or 10 mg/kg twice daily doses (51 vs. 94.3 ng/g, respectively) compared with monotherapy.

CRB-913 monotherapy in the DIO mouse model demonstrates reduced weight, body fat content, and food intake

Given the markedly different brain and plasma levels of CRB-913 compared with rimonabant, we next examined the metabolic effects of CRB-913 in mice with high-fat diet-induced obesity/metabolic syndrome (DIO) at doses of 2.5, 5, and 10 mg/kg twice daily compared with 10 mg/kg once daily rimonabant [26]. Twice daily CRB-913 dosing was used to maximize peripheral exposure while minimizing brain C_{max}, and the 5 mg/kg twice daily dose allowed comparison to an equal total daily dose of rimonabant. Mouse body weight loss from baseline, evaluated on Day 18, showed a CRB-913 dose-dependent increase reaching −9.9% at 2.5 mg/kg twice daily and −22% at 10 mg/kg twice daily (Figure 3A). Animals administered the intermediate dose of 5 mg/kg twice daily lost −17.4% of their baseline body weight, comparable to rimonabant (−16.5%) at the same total daily dose. Changes in body weight were related to reduced daily and cumulative food intake (Figure 3B). At the same total daily dose, CRB-913 and rimonabant demonstrated equivalent reductions in cumulative food intake from vehicle-treated animals (−18.8 and −18.1 g, respectively) after 18 days. Decreased body fat accounted for 85% to 89% of the observed weight reduction in CRB-913 and rimonabant-treated groups with minimal changes in lean mass in either group, as

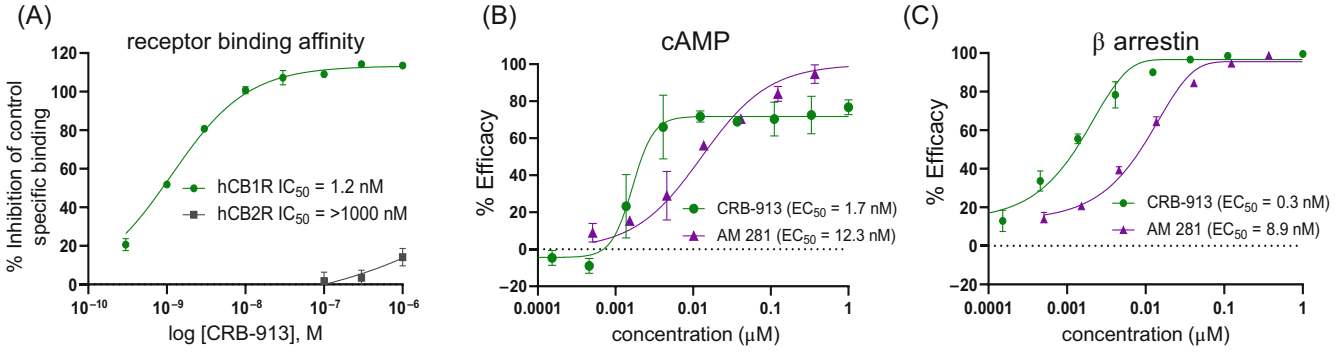

FIGURE 1 CRB-913 biochemical characterization. (A) Binding affinity of CRB-913 to human CB1R and CB2R (hCB1R-green, hCB2R-black) as determined by the displacement of a radiolabeled cannabinoid agonist, [^{3}H]CP 55940 (2 nM) for CB1R and [^{3}H]WIN 55212-2 (0.8 nM) for CB2R. (B) Inhibition by CRB-913 (green) or AM 281 (purple) using DiscoverX cAMP secondary messenger pathway assay preincubated with the sample followed by agonist challenge at the EC_{80} forskolin concentration. For the inverse agonist assay, data were normalized to the maximal and minimal response observed in the presence of control ligand and vehicle. (C) Inhibition by CRB-913 (green) or AM 281 (purple) using PathHunter β-arrestin assay preincubated with the sample in the presence of EC_{20} forskolin. For the agonist assay, data were normalized to the maximal and minimal response observed in the presence of control ligand and vehicle. CB1R, cannabinoid receptor 1; CB2R, cannabinoid receptor 2 [Color figure can be viewed at wileyonlinelibrary.com]

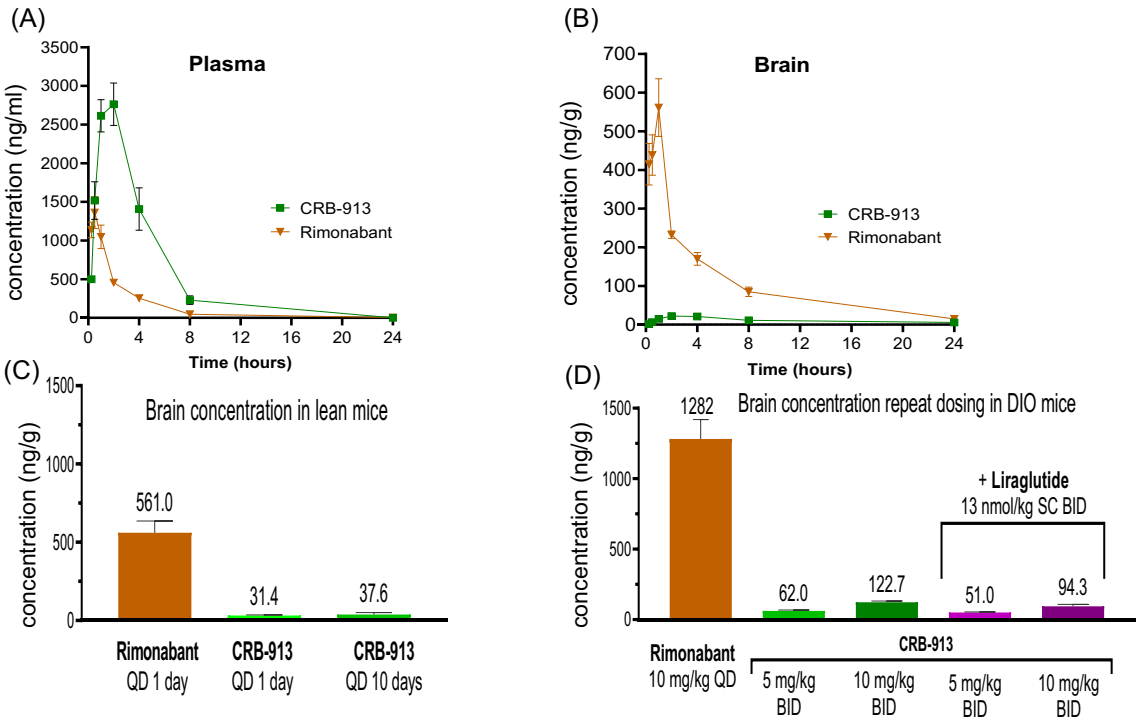

FIGURE 2 CRB-913 and rimonabant pharmacokinetics and brain exposure. (A) Plasma concentrations of CRB-913 (green square) and rimonabant (brown triangle), as determined by LC-MS, after a single 10 mg/kg oral dose in lean male mice. Plasma PK values based on noncompartmental fit to data were AUC_{last} = 13,323 h*ng/mL and C_{max} = 2763 ng/mL for CRB-913 and AUC_{last} = 3534 h*ng/mL and C_{max} = 1362 ng/mL for rimonabant. (B) Brain concentrations of CRB-913 (green square) and rimonabant (brown triangle), as determined by LC-MS, after acute oral dosing at 10 mg/kg in lean male mice. Brain PK values based on noncompartmental fit to data were AUC_{last} = 265 h*ng/g and C_{max} = 22.3 ng/g for CRB-913 and AUC_{last} = 2515 h*ng/g and C_{max} = 561 ng/g for rimonabant. (C) Brain C_{max} concentrations of rimonabant (single dose) and CRB-913 following single and repeat (once daily for 10 days) oral dosing at 10 mg/kg in lean male mice. T_{max} was 1 h for rimonabant and 2 h (Day 1) or 4 h (Day 10) for CRB-913. (D) Brain concentrations of CRB-913 1 h post dose, after 35 days repeat oral dosing in DIO male mice. In the case of co-dosing with liraglutide (13 nmol/kg, SC, twice daily), only CRB-913 brain levels were determined. The corresponding plasma concentrations were 674, 1249, 3031, 1139, and 1987 ng/mL for rimonabant, CRB-913 (5 and 10 mg/kg), and CRB-913 (5 and 10 mg/kg) plus liraglutide (13 nmol/kg). Data in panels A and B represent mean ± SEM from three mice/group. Data in panels C and D represent mean ± SEM from 10 mice/group. AUC, area under the curve; DIO, diet-induced obesity; LC-MS, liquid chromatography-mass spectroscopy; PK, pharmacokinetics; SC, subcutaneous [Color figure can be viewed at wileyonlinelibrary.com]

indicated by magnetic resonance imaging (MRI) measurements on Days 0 and 20 (Figure 3C). CRB-913 (5 mg/kg twice daily) showed almost identical fat loss (−35%) as rimonabant, despite dramatically different brain and plasma exposure. Vehicle-treated mice on a high-fat diet continued to increase body fat content (+13%) over this period.

CRB-913 combined with incretins further enhances weight loss and reduces food intake in DIO mice

We next examined the effect of treating DIO mice with CRB-913 (2.5 and 5 mg/kg twice daily) alone or in combination with tirzepatide, semaglutide, or liraglutide. To better understand the impact of the combinations in the DIO model, without inducing extreme weight changes that would not be tolerated in the animals, we selected doses that were lower than those yielding the maximum weight loss for the individual drugs [27]. Tirzepatide and semaglutide were administered SC every 3 days, liraglutide was administered SC twice daily, and CRB-913 was administered as an oral dose twice daily. As with the previous study (Figure 3), CRB-913 (2.5 mg/kg) monotherapy induced similar weight loss (−10.2%) in the combination studies (Table 1). Combining tirzepatide 5 and 10 nmol/kg, which yielded −11.9% and −21.7% weight loss as monotherapy, with CRB-913 (2.5 mg/kg) improved weight loss in an additive fashion to −24.7% and −28.9%, respectively (Figure 4A). Likewise, combining semaglutide 10 and 30 nmol/kg monotherapy (−7.8% and −14.3% weight loss) with CRB-913 (2.5 mg/kg) again increased weight loss in an additive fashion to −19.0% and −24.2%, respectively (Figure 4C).

At the higher CRB-913 dose (5 mg/kg), the monotherapy weight loss (−18.5%) was approximately additive with the 5 nmol/kg tirzepatide and 10 nmol/kg semaglutide monotherapy doses (−11.9% and −7.8%) to yield combination weight loss of −28.1% and −24.3%, respectively (Figure 4B,D). The effects were less than additive at higher semaglutide or tirzepatide doses as a dosing holiday was required for both combination groups due to rapid weight loss, −30% within 7 days, and markedly reduced food consumption (Figure 4F,H). CRB-913 was stopped on Day 6 and only after weight and food consumption

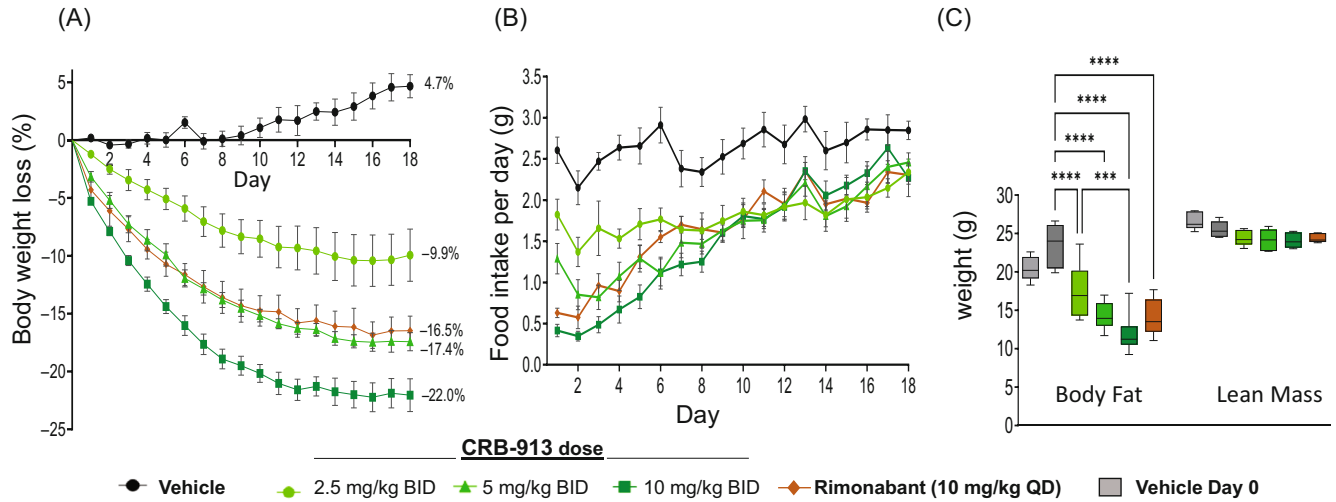

FIGURE 3 Efficacy of CRB-913 monotherapy in the DIO mouse model. (A) DIO mice were treated daily with vehicle (black curve), rimonabant (10 mg/kg oral dose, once daily, brown curve), or CRB-913 (2.5, 5, or 10 mg/kg, oral dose, twice daily, green curves) for 20 days. Body weight changes were analyzed by two-way ANOVA repeated measurements with the Tukey multiple comparison test for individual time points (interaction: F [72, 475] = 10.04; $p < 0.0001$; difference by day: F [18, 475] = 76.33; $p < 0.0001$). Posttest results were statistically significant ($p < 0.0001$) for vehicle vs. CRB-913 (10 mg/kg) from Day 2; vehicle vs. rimonabant from Day 3; vehicle vs. CRB-913 (5 mg/kg) from Day 3; and vehicle vs. CRB-913 (2.5 mg/kg) from Day 6. Posttest results were statistically significant ($p < 0.0001$) for CRB-913 (2.5 mg/kg) vs. rimonabant from Day 10; CRB-913 (2.5 mg/kg) vs. CRB-913 (5 mg/kg) from Day 9; and CRB-913 (2.5 mg/kg) vs. CRB-913 (10 mg/kg) from Day 3. Posttest results for CRB-913 (5 mg/kg) vs. rimonabant were not significant for all days. All data are mean ± SEM from six mice/group. (B) Food intake for each animal was recorded before dosing in the morning. DIO mice on high-fat diet are denoted by light gray color. Food intake per day changes were analyzed by two-way ANOVA repeated measurements with the Tukey multiple comparison test for individual time points (treatment effect: F [72, 475] = 2.946; $p < 0.0001$; difference by day: F [18, 475] = 34.9; $p < 0.0001$). Posttest results were statistically significant ($p < 0.0001$) for vehicle vs. CRB-913 (5 and 10 mg/kg) from Day 1; vehicle vs. rimonabant from Day 1; and $p < 0.01$, for vehicle vs. CRB-913 (2.5 mg/kg) from Day 1. All data are mean ± SEM from six mice/group. (C) Lean and fat mass measured by EchoMRI-130 body composition analyzer after dosing on Days 0 (predosing) and 20 (end of study; box and whisker plot). Fat and lean mass change was examined by two-way ANOVA followed by Dunnett test for individual time points (treatment effect: F (4, 50) = 10.92; $p = 0.0007$). Posttest results were statistically significant (****$p < 0.0001$) for vehicle vs. CRB-913 (2.5, 5, and 10 mg/kg) and rimonabant; $p < 0.05$; ***$p < 0.001$, for CRB-913 (2.5 mg/kg) vs. CRB-913 (10 mg/kg). All other interactions (including lean mass) were not significant. All data are mean ± SEM from six mice/group. For all box and whisker plots, the lower and upper bounds of the rectangles represent the first and third quartiles, the horizontal line represents the median, the whiskers extend to the highest and lowest values within 1.5 × the interquartile range. DIO, diet-induced obesity [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Changes in body weight at Day 18 for CRB-913 monotherapy or combination therapy with tirzepatide,semaglutide, or liraglutide

		Tirzepatide, SC, every 3 days[a]		Semaglutide, SC, every 3 days[a]		Liraglutide, SC, twice daily[b]
CRB-913, oral dose, twice daily		5 nmol/kg	10 nmol/kg	10 nmol/kg	30 nmol/kg	13 nmol/kg
0 mg/kg		−11.9%	−21.7%	−7.8%	−14.3%	−7.9%
2.5 mg/kg	−10.2%[a]/−8.5%[b]	−24.7%	−28.9%	−19.0%	−24.2%	−16.8%
5 mg/kg	−18.5%[a]/−10.0%[b]	−28.1%	−32.6%	−24.3%	−26.7%	−19.5%

Abbreviation: SC, subcutaneous.
[a]Values obtained from tirzepatide/semaglutide study.
[b]Values obtained from liraglutide study.

stabilized (Day 14) was CRB-913 again combined with incretin analogue treatment. As such, the −32.6% and −26.7% decreases in body weight on Day 18 for CRB-913 in combination with tirzepatide or semaglutide may not have realized the full combination effect.

CRB-913 monotherapy also induced additive weight loss in combination with liraglutide (Table 1 and Supporting Information Figure S1A). CRB-913 (2.5 mg/kg) and liraglutide monotherapy (−7.9% and −8.5%, respectively) improved to −16.8% body weight loss for the combination. Although CRB-913 (5 mg/kg) monotherapy weight loss in the liraglutide study was lower (−10.0%) than two other studies, the weight loss in mice in combination with liraglutide was nevertheless additive (−19.5%).

Food intake mirrored weight loss with an initial marked reduction in Days 1 through 7 gradually stabilizing at a lower final daily

intake than vehicle (Figure 4E–H) in all CRB-913 monotherapy and combination treatments. Food intake was further reduced in the combination cohorts compared with the individual monotherapy groups. Oscillations in daily food intake observed in the incretin analogue dose groups were consistent with the PK of these agents and the every 3 days dosing schedule.

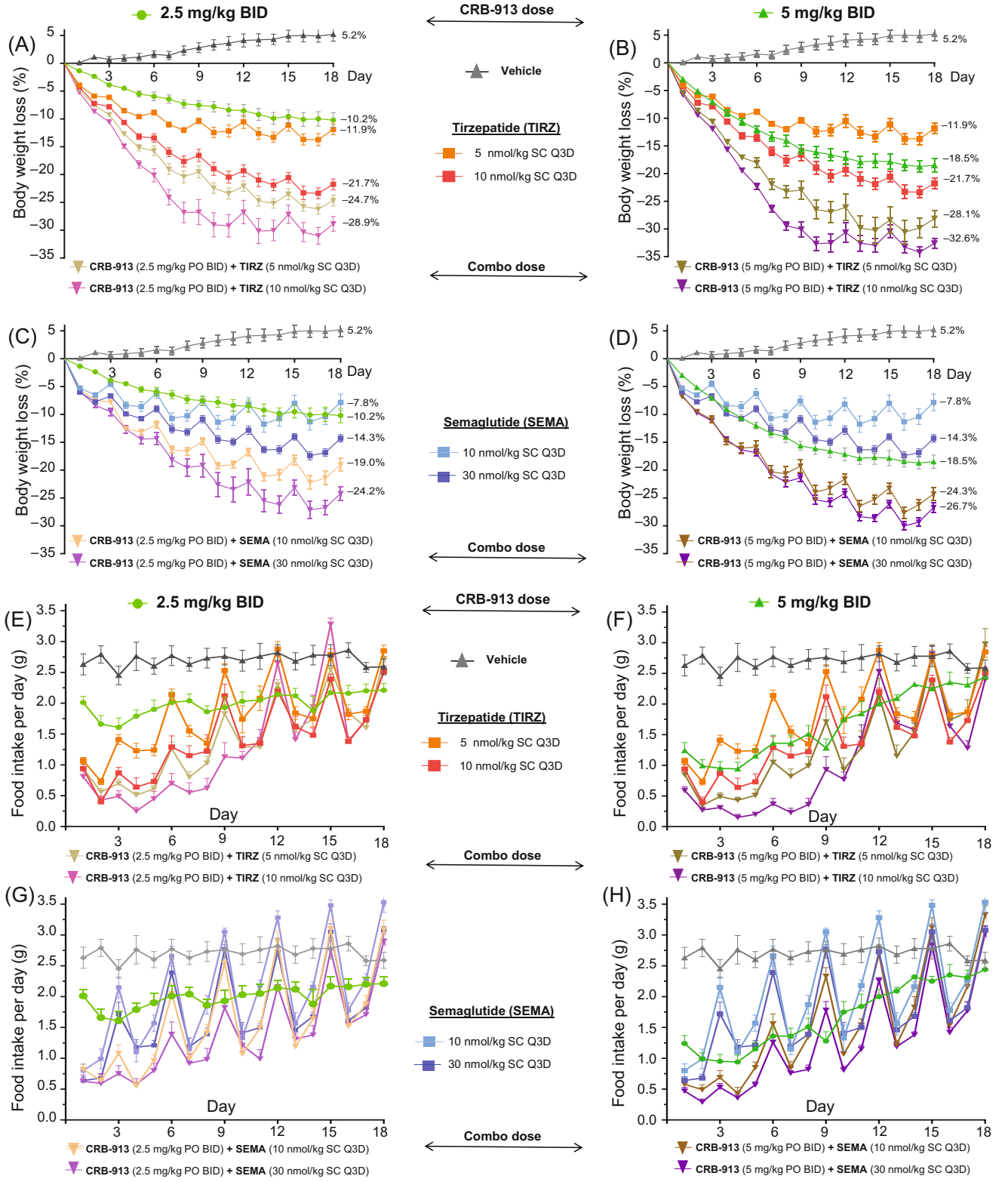

FIGURE 4 Legend on next page.

Combined CRB-913 and incretins improve leptinemia and insulin resistance

In DIO mice, CRB-913 (2.5 mg/kg) reduced leptin levels at the end of the study from 71.8 to 30.2 ng/mL, comparable to the effect observed with 5 nmol/kg tirzepatide (28.4 ng/mL) and 10 nmol/kg semaglutide (39.0 ng/mL) (Figure 5A). CRB-913 (2.5 mg/kg) in combination with incretin analogue therapy further decreased leptin levels in all combination arms. At the higher 5 mg/kg CRB-913 dose, leptin levels were further reduced (17.4 ng/mL), paralleling reductions observed at 10 nmol/kg tirzepatide (17.6 ng/mL) and 30 nmol/kg semaglutide (22.9 ng/mL) (Figure 5B). Further decreases were again observed in the high-dose incretin analogue combination arms (9.9–11.9 ng/mL). In general, reductions due to the combinations were not statistically significant at the higher CRB-913 levels compared with the monotherapy values. In the liraglutide study, leptin levels reached similar values in the CRB-913 (2.5 and 5 mg/kg) combination groups (11.9 and 13.8 ng/mL; Supporting Information Figure S1B).

To determine whether weight loss was accompanied by improved glycemic control, we examined blood glucose and insulin levels by oGTT in both our rimonabant comparison (Supporting Information Figure S2A,B) and incretin combination DIO studies (Supporting Information Figure S2D–G). Fasting blood glucose and peak and area under the curve ($AUC_{0\text{-}120min}$) blood glucose following glucose challenge decreased following CRB-913 dosing, comparable to rimonabant at ≥5 mg/kg CRB-913. Peak and $AUC_{0\text{-}120min}$ blood glucose were further reduced in the tirzepatide, semaglutide, and liraglutide monotherapy and combination groups; differences between these groups were not statistically significant (Supporting Information Figures S1D and S2D–G). In contrast, CRB-913 monotherapy decreased peak plasma insulin levels measured during the first 30 minutes after the oral glucose challenge to levels lower than semaglutide and similar to tirzepatide (Figure 5C–F). The CRB-913 and incretin analogue combinations further decreased peak insulin. Together, these results indicate that CRB-913 exerts potent antidiabetic effects in a dose-dependent manner as a monotherapy that are further increased when combined with all three incretin analogues evaluated.

CRB-913 and its combination with incretins reduce liver fat deposits and improve liver lipid profile

The effect of several CB1R inverse agonists and incretin analogues on liver fat deposits has been described previously [22, 28, 29]. Monotherapies and incretin analogue combination therapy with CRB-913 reduced liver fat content as determined by histology (Figure 6 and Supporting Information Figure S1K). In all cases, combination therapy showed superior fat reduction by Oil Red O staining over CRB-913 or incretin analogue monotherapies, with particularly significant reduction in combination with tirzepatide. Consistent with decreased liver fat, liver triglyceride levels (Figure 7A,B) decreased concomitantly with increased nonesterified fatty acid levels (Figure 7C,D). Reflective of the beneficial effects on liver fat burden, alanine aminotransferase measurement at the end of the study generally showed improvement over vehicle control across the monotherapy and combination therapy cohorts (Supporting Information Figures S1E and S3A,B). Aspartate aminotransferase levels were also slightly reduced, though not statistically different from vehicle (Supporting Information Figures S1F and

FIGURE 4 Weight loss efficacy and food intake of CRB-913 (2.5 and 5 mg/kg, oral dose, twice daily) in combination with incretins in the DIO mouse model. DIO mice were treated daily with vehicle CRB-913 (2.5 mg/kg, oral dose, twice daily), TIRZ (5 or 10 nmol/kg, SC, every 3 days), SEMA (10 or 30 nmol/kg, SC, every 3 days), or the combination of the two agents for 28 days ($n = 8$ mice/group). Food intake for each animal was recorded before dosing in the morning. As oral glucose tolerance testing was done on Day 19, body weight (panels A–D) and food intake (panels E–H) are reported up to fasting on the evening of Day 18. All data are mean ± SEM from eight mice/group. Body weight and food intake changes were analyzed by two-way ANOVA repeated measurements with the Tukey multiple comparison test for individual time points. (A) Treatment effect: F (130, 1134) = 9.557; $p < 0.0001$; difference by day: F (26, 1701) = 219.6; $p < 0.0001$. Posttest results were statistically significant ($p < 0.0001$) for: vehicle vs. TIRZ (5 and 10 nmol/kg) and vehicle vs. CRB-913 (2.5 mg/kg) + TIRZ (5 or 10 nmol/kg) from Day 2; vehicle vs. CRB-913 (2.5 mg/kg) from Day 6. (B) Treatment effect: F (130, 1134) = 11.07; $p < 0.0001$; difference by day: F (26, 1134) = 131.8; $p < 0.0001$. Posttest results were statistically significant ($p < 0.0001$) for: vehicle vs. CRB-913 (5 mg/kg) + TIRZ (5 or 10 nmol/kg) from Day 2; vehicle vs. CRB-913 (5 mg/kg) from Day 3. (C) Treatment effect: F (130, 1134) = 6.719; $p < 0.0001$; difference by day: F (26, 1134) = 62.95; $p < 0.0001$. Posttest results were statistically significant ($p < 0.0001$) for: vehicle vs. CRB-913 (2.5 mg/kg) + SEMA (10 or 30 nmol/kg) from Day 2; vehicle vs. CRB-913 (2.5 mg/kg) from Day 6. (D) Treatment effect: F (130, 1134) = 10.53; $p < 0.0001$; difference by day: F (26, 1134) = 102.8; $p < 0.0001$. Posttest results were statistically significant ($p < 0.0001$) for: vehicle vs. CRB-913 (5 mg/kg) + SEMA (10 or 30 nmol/kg) from Day 1; vehicle vs. CRB-913 (5 mg/kg) from Day 3. (E) Treatment effect: F (125, 1092) = 7.952; $p < 0.0001$; difference by day: F (25, 1092) = 84.39; $p < 0.0001$. Posttest results were statistically significant ($p < 0.0001$) for vehicle vs. TIRZ (5 and 10 mg/kg) and vehicle vs. CRB-913 (2.5 mg/kg) + TIRZ (5 or 10 mg/kg) from Day 1; $p < 0.1$, vehicle vs. CRB-913 (2.5 mg/kg) from Day 1. (F) Treatment effect: F (125, 1092) = 8.969; $p < 0.0001$; difference by day: F (25, 1092) = 111.1; $p < 0.0001$. Posttest results were statistically significant ($p < 0.0001$) for vehicle vs. CRB-913 (5 mg/kg), vehicle vs. TIRZ (5 and 10 mg/kg), and vehicle vs. CRB-913 (5 mg/kg) + TIRZ (5 or 10 mg/kg), from Day 1. (G) Treatment effect: F (125, 1092) = 8.246; $p < 0.0001$; difference by day: F (25, 1092) = 91.66; $p < 0.0001$. Posttest results were statistically significant ($p < 0.0001$) for vehicle vs. all cohorts except $^*p < 0.1$, vehicle vs. CRB-913 (2.5 mg/kg) from Day 1. (H) Treatment effect: F (125, 1092) = 11.65; $p < 0.0001$; difference by day: F (25, 1092) = 147.5; $p < 0.0001$. Posttest results were statistically significant ($p < 0.0001$) for vehicle vs. all cohorts from Day 1. DIO, diet-induced obesity; SEMA, semaglutide; SC, subcutaneous; TIRZ, tirzepatide [Color figure can be viewed at wileyonlinelibrary.com]

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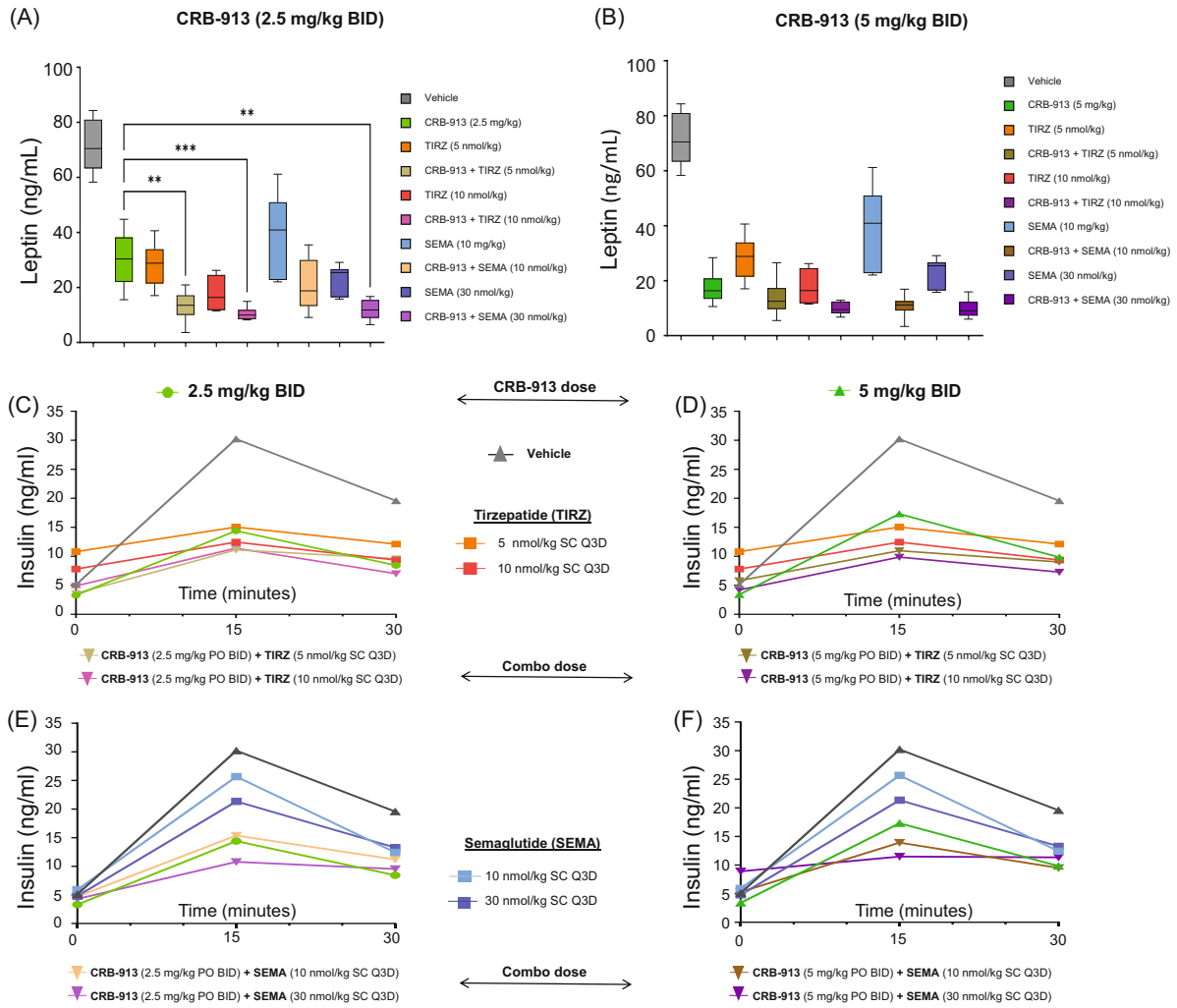

FIGURE 5 Leptin levels and insulin response to CRB-913 (2.5 mg/kg, oral dose, twice daily) monotherapy and in combination with incretins in the DIO mouse model. Data were analyzed by two-way ANOVA repeated measurements with the Tukey multiple comparison test for individual time points. (A,B) Leptin level changes were measured at end of study for DIO mice treated daily with vehicle, CRB-913 (2.5 or 5 mg/kg, oral dose, twice daily), TIRZ (5 or 10 nmol/kg, SC, every 3 days), SEMA (10 or 30 nmol/kg, SC, every 3 days), or the combination of CRB-913 + TIRZ for 28 days ($n = 8$ mice/group). All cohorts were $p < 0.0001$ vs. vehicle, $F(9, 69) = 35.39$; $p < 0.0001$ and $F(9, 62) = 46.47$; $p < 0.0001$. The following comparision against CRB-913 monotherapy was significant for CRB-913 + TIRZ (5 mmol/kg), $^{**}p < 0.01$, CRB-913+ TIRZ (10 nmol/kg), $^{***}p<0.001$, and CRB-913 + SEMA (30 nmol/kg), $^{**}p<0.01$. (C,D) Effects on insulin from oGTT in DIO mice on Day 19 after overnight fasting ($n = 8$ mice/group). CRB-913 was administered 60 min before glucose administration on Day 19; TIRZ was administered 12 h prior to glucose administration. Mice were treated with vehicle, CRB-913 (2.5 or 5 mg/kg, oral dose, twice daily), TIRZ (5 and 10 nmol/kg, SC, every 3 days), or the combination of the two agents. Treatment effect: $F(10, 120) = 5.328$; $p < 0.0001$, time effect: $F(2, 120) = 45.34$; $p < 0.0001$. Posttest results were $p < 0.0001$, for vehicle vs. all cohorts for peak insulin at 15 min, and $p < 0.0001$, for vehicle vs. CRB-913 (2.5 mg/kg) + TIRZ (10 nmol/kg SC), and $p < 0.001$, for vehicle vs. CRB-913 (2.5 mg/kg) or TIRZ (10 nmol/kg) at 30 min. (D) Treatment effect: $F(10, 126) = 5.357$; $p < 0.0001$, time effect: $F(2, 126) = 43.39$; $p < 0.0001$. Posttest results were $p < 0.0001$, for vehicle vs. all cohorts for peak insulin at 15 min, and $p < 0.001$, for vehicle vs. CRB-913 (5 mg/kg) + TIRZ (10 nmol/kg, SC) at 30 min. Effects on insulin from oGTT in DIO mice on Day 19 after overnight fasting ($n = 8$ mice/group). CRB-913 was administered 60 min before glucose administration on Day 19; SEMA was administered 12 h prior to glucose administration. Mice were treated with vehicle, CRB-913 (2.5 or 5 mg/kg, oral dose, twice daily), SEMA (10 and 30 nmol/kg, SC, every 3 days), or the combination of the two agents. (E) Treatment effect: $F(10, 120) = 3.408$; $p = 0.0006$, time effect: $F(2, 120) = 84.34$; $p < 0.0001$. Posttest results were $p < 0.0001$, for vehicle vs. CRB-913 (2.5 mg/kg), CRB-913 (2.5 mg/kg) + SEMA (10 or 30 nmol/kg), and not significant for vehicle vs. SEMA (10 nmol/kg), and $p < 0.1$, for vehicle vs. SEMA (30 nmol/kg) for peak insulin at 15 minutes. At 30 min, $p < 0.1$ for vehicle vs. CRB-913 (2.5 mg/kg) or vehicle vs. CRB-913 (2.5 mg/kg) + SEMA (30 nmol/kg SC). (F) Treatment effect: $F(10, 126) = 3.942$; $p = 0.0001$, time effect: $F(2, 126) = 71.70$; $p < 0.0001$. Posttest results were $p < 0.0001$, for vehicle vs. CRB-913 (5 mg/kg) + SEMA (10 or 30 nmol/kg SC), and $p < 0.01$, for vehicle vs. CRB-913 (5 mg/kg) at 15 min. At 30 min, $p < 0.1$, for vehicle vs. CRB-913 (5 mg/kg) or vehicle vs. CRB-913 (5 mg/kg) + SEMA (10 nmol/kg SC). DIO, diet-induced obesity; oGTT, oral glucose tolerance test; SC, subcutaneous; SEMA, semaglutide; TIRZ, tirzepatide [Color figure can be viewed at wileyonlinelibrary.com]

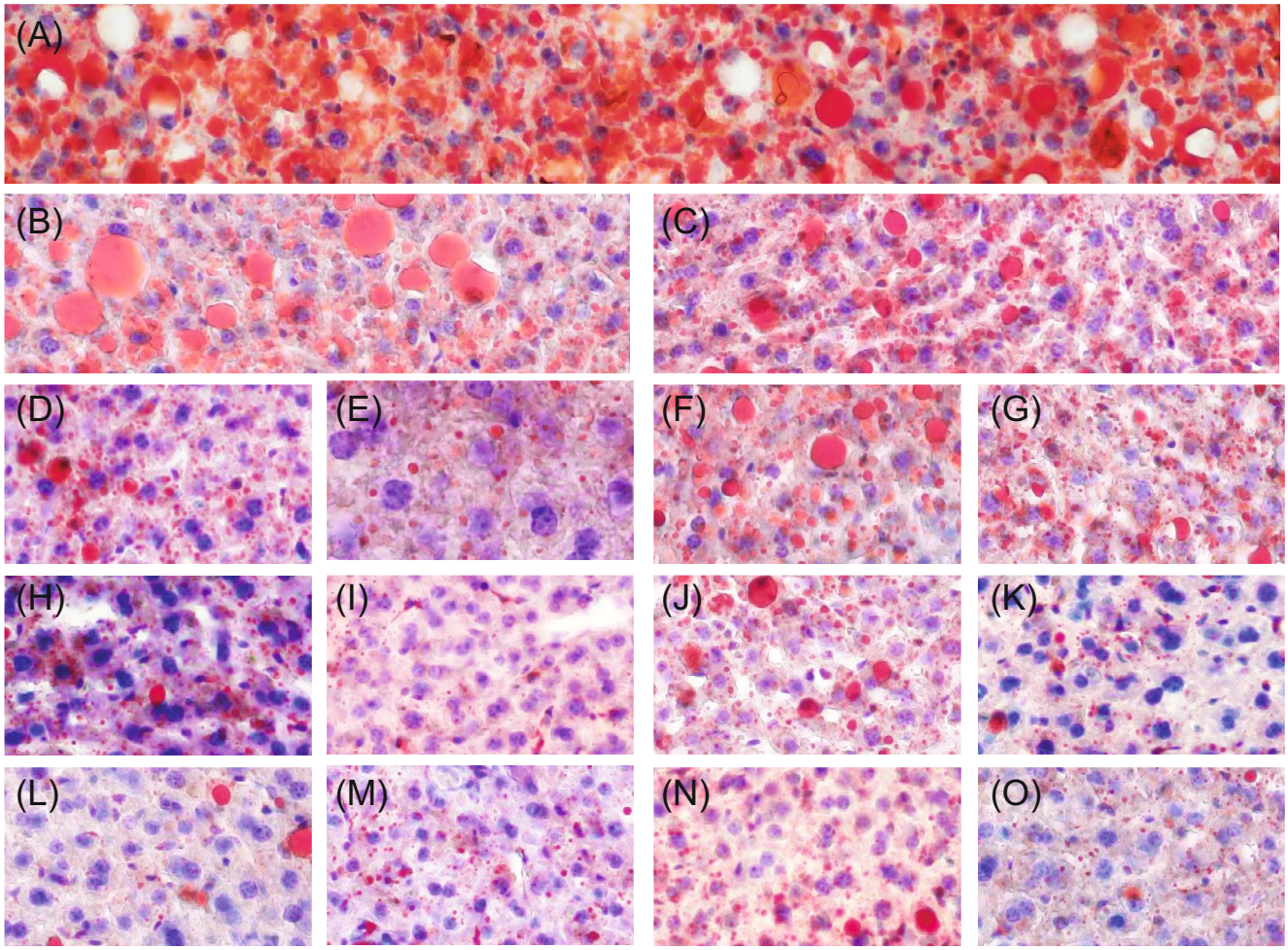

FIGURE 6 Reduction of liver fat deposits in the DIO mouse model by CRB-913 (2.5 mg/kg, oral dose, twice daily) alone and in combination with tirzepatide and semaglutide. Staining of hepatic lipids with Oil Red O on frozen sections of fed DIO male mice treated with (A) vehicle, (B) CRB-913 (2.5 mg/kg), (C) CRB-913 (5 mg/kg), (D) TIRZ (5 nmol/kg), (E) TIRZ (10 nmol/kg), (F) SEMA (10 nmol/kg), (G) SEMA (30 nmol/kg), or the combination of the incretins with CRB-913 ([H] CRB-913 [2.5 mg/kg] + TIRZ [5 nmol/kg], [I] CRB-913 [2.5 mg/kg] + TIRZ [10 nmol/kg], [J] CRB-913 [2.5 mg/kg] + SEMA [10 nmol/kg], [K] CRB-913 [2.5 mg/kg] + SEMA [30 nmol/kg], [L] CRB-913 [5 mg/kg] + TIRZ [5 nmol/kg], [M] CRB-913 [5 mg/kg] + TIRZ [10 nmol/kg], [N] CRB-913 [5 mg/kg] + SEMA [10 nmol/kg], [O] CRB-913 [5 mg/kg] + SEMA [30 nmol/kg]) on Day 28. Stained areas from randomly selected liver slices at 20× magnification with scale at 100 μm. DIO, diet-induced obesity; SEMA, semaglutide; TIRZ, tirzepatide [Color figure can be viewed at wileyonlinelibrary.com]

S3C,D). Additional serum measurements (low-density lipoprotein, high-density lipoprotein, and cholesterol) did not show meaningful differences (Supporting Information Figures S1G–I and S3E–J).

DISCUSSION

Both CB1R and incretin receptors are validated clinical targets in treating obesity [5, 30]. Although several incretin analogues are available to patients, no approved CB1R-targeting therapeutics are currently available. The only such drug previously approved, the brain-penetrant CB1R inverse agonist rimonabant, was withdrawn worldwide following the FDA rejection of its marketing authorization in 2007 [13]. The rejection cited the increased risk of suicidal ideation associated with rimonabant usage [31]. To eliminate such potential central nervous system-related effects, subsequent research has focused on minimizing brain penetration with a new generation of CB1R inverse agonists, of which CRB-4001 is a well-studied example. We have further improved this compound, resulting in CRB-913, a novel CB1R inverse agonist that maintains high CB1R affinity and selectivity but with markedly lower brain exposure. Compared with rimonabant, CRB-913 brain concentration exhibited ~20-fold lower C_{max} and 9.5-fold lower AUC_{last} while eliciting equivalent weight loss effects in the DIO mouse model at the same total daily dose. Together, these attributes could translate to an improved safety-benefit profile for CRB-913.

Previous reports have shown that CB1R inverse agonists such as CRB-4001 can amplify the weight loss and anorectic effects of a glucagon-like peptide 1 (GLP-1) receptor agonist and point to the use of combination treatments with orthogonal mechanisms of action to further improve the efficacy of these emerging antiobesity therapies [25, 32]. Treatment of DIO mice with CRB-4001 in combination with

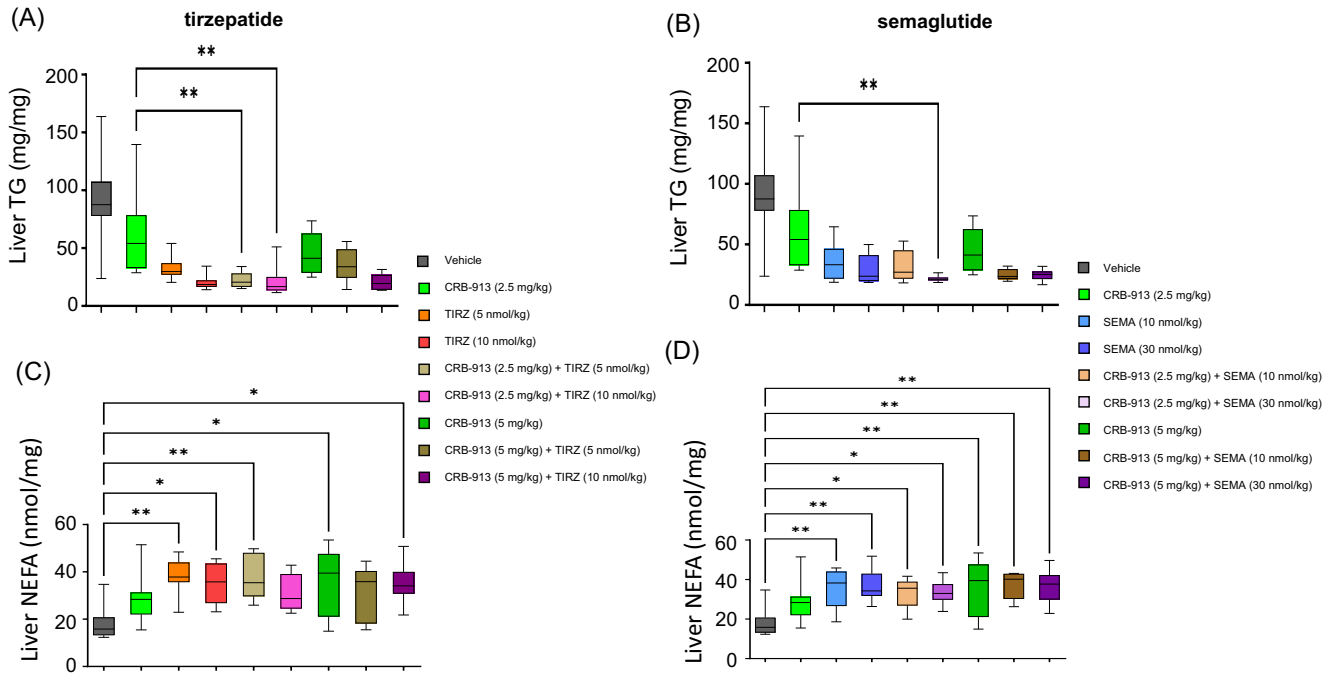

FIGURE 7 TG and NEFA response to CRB-913 (2.5 mg/kg, oral dose, twice daily) monotherapy and in combination with incretins in the DIO mouse model. Terminal fasting TG and NEFA levels in DIO mice on Day 28 were measured after fasting for 5 and 1 h after CRB-913 dosing. (A,B) Liver TG levels. Comparisons between treatments vs. vehicle were analyzed by one-way ANOVA repeated measurements with the Dunnett test (TIRZ treatment effect F (8, 63) = 11.08; $p < 0.0001$; SEMA treatment effect: F (8, 63) = 9.906; $p < 0.0001$). Posttest results were statistically significant when compared with vehicle for all cohorts except for CRB-913 (2.5 mg/kg) monotherapy cohort. (C,D) Liver NEFA levels. Comparisons between treatments vs. vehicle were analyzed by one-way ANOVA repeated measurements with the Dunnett test (TIRZ treatment effect F (8, 63) = 3.615; $p = 0.0002$; SEMA treatment effect: F [3, 28] = 5.967; $p = 0.0028$). *P* values listed vs. vehicle. $^{**}p < 0.01$ and $^{*}p < 0.1$. DIO, diet-induced obesity; NEFA, nonesterified fatty acid; SEMA, semaglutide; TG, triglycerides; TIRZ, tirzepatide [Color figure can be viewed at wileyonlinelibrary.com]

semaglutide yielded superior results in weight loss, leptinemia, triglyceride levels, and liver fat compared with monotherapy treatments [25]. We extend these findings in our studies with CRB-913 and demonstrate additive effects on weight loss with three approved incretin analogues: liraglutide, semaglutide, and tirzepatide. Weight loss induced by CRB-913, characterized by reduced fat mass with minimal loss of muscle mass, was driven by decreased food intake and resolution of leptinemia. Furthermore, CRB-913 improved glycemic control and liver function via reductions in liver lipid storage and triglycerides, in accordance with the known role of CB1R in modulating lipogenesis in liver and adipose tissues [28, 33, 34]. These benefits, also observed with the three GLP-1 agonists, were further improved under the combination treatments, which produced additive effects on body weight loss.

CB1R and incretin receptor pathways interact across multiple tissues and a recent noteworthy review by Aseer and Egan [35] provides an overview of the proposed molecular mechanism by which this cross talk occurs based on cellular and animal models. Although much is still unclear, both modulate intracellular cAMP levels to control pancreatic insulin secretion while regulating food intake and satiety via central neural circuits. Reflecting the oppositional functions of these receptor pathways, González-Mariscal et al. [36] found that CB1R activation reduced GLP-1 receptor-mediated insulin secretion in murine insulinoma and human islet cells. This latter effect was prevented by the blockade of the CB1R receptor by an inverse agonist (AM-251 or CRB-4001). Like incretin receptor agonism, CB1 inverse agonism (including by CRB-4001) has been extensively shown to reduce leptin levels and improve hyperleptinemia [22, 25, 37], restoring the sensitivity of normal adiposity signaling in murine models. In humans, increased endocannabinoid levels in individuals with obesity modulate incretin and insulin secretion [38, 39], although the research is hampered by a lack of a clinically approved CB1R inverse agonist.

The respective contribution of peripheral versus central CB1R inhibition in driving weight loss remains a matter of intense debate [40–44]. Reduction in food intake suggests a centrally mediated mechanism whereas mobilization of liver fat, changes in insulin sensitivity, and an improvement in adipocyte and other peripheral organ leptin resistance indicate peripheral mechanisms driving weight loss. Despite the extremely low central nervous system levels of CRB-913, both peripheral and central mechanisms plausibly contribute to the observed weight loss.

We hypothesize that the convergent physiological benefits of these two distinct mechanisms of action could translate to improved obesity management. CRB-913 may boost the efficacy of current incretin analogue therapeutics and provide a daily oral treatment that

could improve patient compliance. Alternatively, it may help address incretin analogue tolerability limitations by allowing a reduction in the dose of the incretin analogue which could be compensated by weight loss generated from CB1R inverse agonism. We plan on advancing CRB-913 into clinical trials exploring its effect both as monotherapy and in combination with incretin therapy in patients.O

AUTHOR CONTRIBUTIONS

Marshall Morningstar, Andrew Kolodziej, Tracy Blumen, and Yuval Cohen wrote the manuscript and analyzed the data. Andrew Kolodziej designed the diet-induced obesity studies. Suzie Ferreira reviewed/edited the manuscript and contributed to pharmacokinetics studies. Rachael Brake contributed to the discussion and reviewed/edited the manuscript. Marshall Morningstar, Andrew Kolodziej, and Yuval Cohen are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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DATA AVAILABILITY STATEMENT

All data and resources are available from the corresponding author upon request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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