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GLP-1/GCGR dual agonism reverses obesity in mice

Short-running title: Dual GLP-1/GCGR agonist for the treatment of obesity

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Objective— Oxyntomodulin (OXM) is a GLP-1 receptor (GLP1R)/glucagon receptor (GCGR) dual agonist peptide that reduces body weight in obese subjects through increased energy expenditure and decreased energy intake. The metabolic effects of OXM have been attributed primarily to GLP1R agonism. We examined whether a long acting GLP1R/GCGR dual agonist peptide exerts metabolic effects in diet-induced obese mice that are distinct from that obtained with a GLP1R-selective agonist.

Research design and methods— We developed a protease-resistant dual GLP1R/GCGR agonist, DualAG, and a corresponding GLP1R-selective agonist, GLPAG, matched for GLP1R agonist potency and pharmacokinetics. The metabolic effects of these two peptides with respect to weight loss, caloric reduction, glucose control, and lipid lowering, were compared upon chronic dosing in DIO mice. Acute studies in DIO mice revealed metabolic pathways that were modulated independent of weight loss. Studies in *Glp1r*$^{-/-}$ and *Gcgr*$^{-/-}$ mice enabled delineation of the contribution of GLP1R versus GCGR activation to the pharmacology of DualAG.

Results— Peptide DualAG exhibits superior weight loss, lipid lowering activity, and antihyperglycemic efficacy comparable to GLPAG. Improvements in plasma metabolic parameters including insulin, leptin, and adiponectin were more pronounced upon chronic treatment with DualAG than with GLPAG. Dual receptor agonism also increased fatty acid oxidation and reduced hepatic steatosis in DIO mice. The anti-obesity effects of DualAG require activation of both GLP1R and GCGR.

Conclusions— Sustained GLP1R/GCGR dual agonism reverses obesity in DIO mice and is a novel therapeutic approach to the treatment of obesity.

Obesity is an important risk factor for T2DM and approximately 90% of patients with T2DM are overweight or obese (1). Among new therapies for T2DM, peptidyl mimetics of the gut-derived incretin hormone glucagon-like peptide 1 (GLP-1) stimulate insulin biosynthesis and secretion in a glucose-dependent manner (2; 3) and cause modest weight loss in T2DM patients. The glucose lowering and anti-obesity effects of incretin-based therapies for T2DM has prompted evaluation of the therapeutic potential of other glucagon-family peptides, in particular oxyntomodulin (OXM). The OXM peptide is generated by post-translational processing of preproglucagon (PPG) in the gut and is secreted post-prandially from L-cells of the jejuno-ileum together with other PPG-derived peptides including GLP-1 (4; 5). In rodents, OXM reduces food intake and body weight, increases energy expenditure, and improves glucose metabolism (6-8). A 4-week clinical study in obese subjects demonstrated that repeated subcutaneous administration of OXM was well tolerated and caused significant weight loss with a concomitant reduction in food intake (9). An increase in activity-related energy expenditure was also noted in a separate study involving short term treatment with the peptide (10).

OXM activates both, the GLP-1 receptor (GLP1R) and glucagon receptor (GCGR) *in vitro,* albeit with 10-100-fold reduced potency compared to the cognate ligands GLP-1 and glucagon, respectively (11-13). It has been proposed that OXM modulates glucose and energy homeostasis solely by GLP1R agonism, since its acute metabolic effects in rodents are abolished by co-administration of the GLP1R antagonist exendin(9-39) and are not observed in *Glp1r*$^{-/-}$ mice (7; 8; 14; 15). Other aspects of OXM pharmacology, however, such as protective effects on murine islets and inhibition of gastric acid secretion appear to be independent of GLP1R signaling (14). In addition, pharmacological activation of GCGR by glucagon, a master regulator of fasting metabolism (16), decreases food intake in rodents and humans (17-19), suggesting a potential role for GCGR signaling in the pharmacology of OXM. Since both OXM and GLP-1 are labile *in vivo* ($T_{1/2}$ ~12 min and 2-3 min, respectively (20; 21), and are substrates for the cell surface protease dipeptidyl peptidase 4 (DPP-4) (22), we developed two long acting DPP-4-resistant OXM analogs as pharmacological agents to better investigate the differential pharmacology and therapeutic potential of dual GLP1R/GCGR agonism versus GLP1R-selective agonism. Peptide DualAG exhibits *in vitro* GLP1R and GCGR agonist potency comparable to that of native OXM and is conjugated to cholesterol via a Cys sidechain at the C-terminus for improved pharmacokinetics. Peptide GLPAG differs from DualAG by only one residue ($Gln^3 \rightarrow Glu$) and is an equipotent GLP1R agonist, but has no significant GCGR agonist or antagonist activity *in vitro*. The objective of this study was to leverage the matched GLP-1R agonist potencies and pharmacokinetics of peptides DualAG and GLPAG in comparing the metabolic effects and therapeutic potential of a dual GLP1R/GCGR agonist versus a GLP1R-selective agonist in a mouse model of obesity.

RESEARCH DESIGN AND METHODS

Animals. Experiments were performed in lean and diet-induced obese (DIO) C57BL/6 mice and in weight- and sex-matched *Gcgr*$^{-/-}$ (23), *Glp1r*$^{-/-}$ (24), or lean C57BL/6 control mice. All mice were obtained from Taconic Farms (Germantown, NY, USA) and were maintained on either standard chow (Teklad 7012; Harlan Teklad)

or high fat diet (D12492: 60% kcal from fat; Research Diets) in a 12 h light/12 h dark cycle (light: 3:00 AM -3:00 PM). Animal protocols used in these studies were approved by the Merck Research Laboratories Institutional Animal Care and Use Committee in Rahway, NJ.

Peptides. The free thiol-containing peptide precursors of DualAG and GLPAG were synthesized by standard solid-phase peptide synthesis using Fmoc/t-Bu chemistry. Peptides were synthesized by reverse-phase HPLC using water/acetonitrile (0.1% trifluoroacetic acid) gradients. Cholesterol-peptide conjugates were synthesized by reaction of the thiol-containing peptide precursors with cholest-5-en-3-yl 1-bromo-2-oxo-6,9,12,15-tetraoxa-3-azaoctadecan-18-oate, which was previously assembled by standard solution chemistry. The peptide conjugates were purified by reverse-phase chromatography using water/acetonitrile (0.2% acetic acid) gradients. Purified peptides were characterized by electrospray MS on a Micromass LCZ platform spectrometer.

Determination of murine GLP1R and GCGR agonist potency. *In vitro* agonist potency of peptides was determined in CHO cells stably expressing murine GLP1R or murine GCGR using the Cisbio CAMP Dynamic 2 assay. Peptides were diluted in assay buffer and incubated with cells in the presence of 20% mouse plasma. The assay was terminated with the addition of the Cisbio detection reagents as per the manufacturer's instructions. cAMP was detected by a decrease in time-resolved fluorescence energy transfer (TR-FRET) using an EnVision platereader (PerkinElmer).

Ex-vivo liver glycogenolysis assay. The ability of DualAG and GLPAG to stimulate glycogen breakdown was evaluated *ex vivo* in perfused livers harvested from C57BL/6 mice using a ^{13}C NMR-based assay as previously described (25).

Measurement of plasma peptide exposures at the end of the chronic study. Diet-induced obese (DIO) mice, 23-weeks old and maintained for 16-weeks on a high fat diet, were anesthetized with isofluorane and blood was collected by cardiocentesis into EDTA-coated microtainer tubes containing DPP-4 inhibitor and aprotinin. The *in vitro* cell-based cAMP bio-assay for determining GLP1R agonist potency was used with CHO cells stably transfected with human GLP1R to determine peptide concentrations by comparing the degree of cAMP accumulation in plasma samples from treated animals against a cAMP standard curve generated by spiking peptide standards into mouse plasma.

Single dose studies in DIO mice. Weight- (~45 g) and age-matched DIO mice (23-weeks old) were s.c. injected at 9:00 AM with vehicle, DualAG, or GLPAG at a dose of 191 nmol/kg. Food was removed and 6 hours later (3:00 PM, ~T_{max} for GLPAG and DualAG) the animals were sacrificed for collection of plasma and tissue samples, which were immediately stored at -80 °C.

RNA preparation and quantitative RT-PCR. RNA was isolated from tissues using UltraspecTM Total RNA Isolation Reagent (Biotex Laboratories, Inc.). The resulting total RNA was subjected to DNAse treatment using RNAse-freeDNAse (Qiagen). Following reverse transcription of the RNA to generate cDNA, quantitative RT-PCR was performed with TaqMan PCR Reagent using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). All Taqman probes were purchased from Applied Biosystems: *ChREBP* (Mm00498811_m1), *Cpt1a* (Mm00550438_m1), *Fgf21* (Mm00840165_g1), *Ldlr* (Mm00440169_m1), *Pck1* (Mm00440636_m1), *Pgc-1α* (Mm00447183_m1). Expression was normalized to the copy number for β–actin (*Actb*).

Quantitation of malonyl-CoA and acetyl-CoA. Mouse liver samples (~100 mg) were homogenized in 1 mL of 10% sulfosalicylic acid, 10 mM DTT. The samples were spun at 15000x g and the supernatant analyzed by LC/MS after 10-fold dilution using an Agilent 1100 series capillary pump interfaced to an LTQ ion trap mass spectrometer (Thermo Fisher Scientific). The assay was adapted from Minkler *et al.* (26).

Chronic dosing of DualAG and GLPAG in diet-induced obese DIO mice. Male DIO mice (23-weeks old, n=8/dosing group, maintained for 16 weeks on a high fat diet), were acclimated to non-specific stress for 10 days prior to the onset of the chronic dosing study. DualAG (1.9 μmol/kg), GLPAG (1.9 μmol/kg), or vehicle (water) was injected subcutaneously (s.c.) every other day (QOD) for 2 weeks. Body weight and food intake were measured daily. An intraperitoneal glucose tolerance test (IPGTT, 1.5 g/kg dextrose challenge) was performed on day 13 of the chronic study at 10:00 AM. Whole body composition analysis of conscious mice was conducted prior to (Day 0) and at the conclusion of the study by EchoMRI (Echo Medical Systems). Plasma samples for measurement of terminal plasma concentration of active GLPAG and DualAG were obtained 18 h after the last injection by cardiocentesis.

Food intake and body weight studies in Glp1r$^{-/-}$ and Gcgr$^{-/-}$ mice. Single-caged weight-matched (~30 g) wild type (n=24), *Glp1r*$^{-/-}$ (n=24) and *Gcgr*$^{-/-}$ (n=21) mice were injected daily (s.c.) with vehicle, DualAG (1.9 μmol/kg), or GLPAG (1.9 μmol/kg), 30 min before the onset of the dark cycle for 5 days. Food intake and body weight were recorded daily for the duration of the study .

Histology. Liver histology was performed as described elsewhere (27).

Biochemical analyses. Insulin and leptin levels in plasma were measured by ELISA (Linco/Millipore). Plasma free fatty acids and ketone bodies were measured using commercially available enzyme-coupled spectrophotometric assays (Wako Chemicals). Plasma triglyceride and total cholesterol were determined using an Olympus AU400e Bioanalyzer. Adiponectin was measured using a mouse adiponectin RIA kit (Linco/Millipore). Blood glucose levels were measured using a OneTouch glucometer (Ultra LifeScan).

Statistics. All data are presented as mean ± SEM. Comparisons among groups were made using ANOVA or unpaired Student's t-test, as appropriate. $p<0.05$ was regarded as statistically significant.

RESULTS

Development of long-acting GLP1R/GCGR dual agonist (DualAG) and GLP1R-selective agonist (GLPAG) peptides. As summarized in Figure 1A, Glucagon and OXM, the only members of the glucagon superfamily that activate GCGR, incorporate a neutral polar residue at position 3 (glutamine, Q). In contrast, GLP-1 and the GLP-1 mimetic exendin-4 exhibit no significant GCGR agonist activity and incorporate an acidic residue at this position (glutamate, E), which decreases binding affinity to GCGR (28; 29). Peptide DualAG is a long acting analog of OXM with a Ser^2→DSer substitution for resistance to DPP-4 cleavage. A Gln^3→Glu substitution was introduced in DualAG to generate analog GLPAG. As illustrated in Figure 1A, a cholesterol moiety was conjugated to the thiol side chain of a Cys residue appended to the C-terminus of each OXM analog to improve pharmacokinetics and enhance metabolic stability via plasma lipid and protein binding. The insertion of a short PEG spacer between the cholesterol moiety and the peptides ameliorated a decrease in potency of the conjugates in the presence of plasma that occurs due to protein/lipid binding. A more complete description of the discovery and

development of these peptides will be provided elsewhere (Bianchi E *et al.*, manuscript in preparation).

Consistent with a previous report of DSer^2-glucagon being resistant to DPP-4 cleavage (30), peptides DualAG and GLPAG exhibited no loss of in vitro potency following overnight incubation at 37° C with human recombinant soluble DPP-4 (31). Protease resistance of the peptides was further supported by pharmacokinetics data. The T_{max} (time corresponding to peak plasma concentration post-dose) for DualAG administered s.c. in lean C57Bl/6 mice at a dose of 3 mg/kg was 5 h, as determined using a bioassay for GLP1R agonism. The corresponding *in vivo* half life ($T_{1/2}$) for circulating active peptide was 1.7 h (compared to $T_{1/2}$ ~8-12 min for native OXM (32). Peptide GLP1AG exhibited comparable *in vivo* stability to DualAG as reflected by comparable plasma concentrations of bioactive peptides measured at 24 h post-dose in mice (Figure 2B, inset).

As summarized in Figure 1B, DualAG is a full agonist of both, mGLP1R ($EC_{50,\ cAMP}$ = 3.4 nM) and mGCGR ($EC_{50,\ cAMP}$ = 1.5 nM) and is comparable in potency to OXM *in vitro*, using a cell-based assay that measures cAMP accumulation in CHO cells stably transfected with the respective recombinant murine receptor. GLPAG is an equipotent mGLP1R agonist ($EC_{50,\ cAMP}$ =1.7 nM), with at least 100-fold reduced mGCGR agonist potency compared to DualAG. The GCGR receptor selectivity of these long acting peptides was further confirmed using an *ex vivo* assay that monitors glycogenesis and glycogenolysis in a perfused mouse liver (25). Briefly, a ^{13}C NMR-visible pool of glycogen was created by perfusion of the gluconeogenic substrate [2-^{13}C] pyruvate and liver glycogen was monitored in real time via the C1 resonance of the glucosyl units in the glycogen chain. DualAG or GLPAG was subsequently infused and loss of label from glycogen (glycogenolysis) was monitored by ^{13}C NMR to assess GCGR activation (Supplemental Figure 1, A and B in the online appendix available at http://diabetes.diabetesjournals.org). Peptide DualAG induced full glycogenolysis at a perfusate concentration of ~1 nM ($EC_{50,\ glyco}$ ~0.5 nM), which compares favorably with its *in vitro* potency against mGCGR. In contrast, a much higher peptide perfusate concentration (~300 nM) was required for maximal stimulation of glycogenolysis by GLPAG ($EC_{50,\ glyco}$ ~208 nM), consistent with >100-fold reduced agonist potency against mGCGR. Peptides DualAG and GLPAG showed no antagonist activity at GCGR and were inactive at other receptors of the glucagon-secretin family (PAC1, VPAC1, VPAC2, and GIPR, $EC_{50,\ cAMP}$ >10 μM).

DualAG reverses obesity and improves glucose metabolism in mice. DIO mice were injected s.c. with DualAG or GLPAG (1.9 μmol/kg) in a 14-day chronic study (Figure 2A). Although peak plasma peptide concentrations (C_{max}) were achieved ~5 h following s.c. injection and the plasma $T_{1/2}$ suggests daily dosing as optimal, both peptides were injected every other day to minimize the stress caused by frequent injections. DualAG exhibited superior weight loss efficacy compared to GLPAG at day 14 (25% and 12% decrease in body weight, respectively, relative to vehicle-treated mice, Figure 2B). As noted earlier, plasma peptide exposures measured at 24 h following the last injection were similar for DualAG and GLPAG (Figure 2B, inset), confirming the matched pharmacokinetics of the two peptides. As depicted in Figure 2C, cumulative food intake was reduced to a greater extent with the DualAG than with GLPAG (30% and 12% reduction, respectively, relative to vehicle). Body composition analysis confirmed that the decrease in body weight was primarily accounted for by a proportional decrease in

fat mass (Figure 2D). An IPGTT performed on day 13 (20 h after the last injection) revealed that glucose tolerance was significantly and comparably improved in both treatment groups (Figure 2E). Furthermore, basal blood glucose levels were normalized by chronic treatment with either peptide (Figure 2E, t = 0 measurement pre-dextrose challenge in the IPGTT). Several other metabolic parameters in plasma were also improved by chronic treatment with the peptides (Table 1). Increases in adiponectin and decreases in leptin and insulin levels correlated with the decreased adiposity observed at the end of the study in each treatment group. Reduced cholesterol and triglyceride levels, increased ketone bodies, and decreased hepatic steatosis (Figure 3) relative to vehicle treatment were also noted, especially for animals treated with DualAG.

To identify metabolic pathways acutely altered by DualAG and GLPAG prior to any significant weight loss, age- and weight-matched DIO mice were injected with vehicle, DualAG, or GLPAG (191 nmol/kg s.c. each peptide) and sacrificed 6 h later to collect plasma for metabolites and liver tissue samples for gene expression analysis. Despite the acute treatment, decreased plasma triglycerides and increased ketone bodies were detected albeit only in animals treated with the dual agonist (Suppl. Fig. 2). Increased expression of the gluconeogenic genes *Pck1, Pgc1α,* and *Pdha1* was observed with DualAG treatment, but not with GLPAG or vehicle (Figure 4, A-C). Liver pools of acetyl-CoA, the main product of pyruvate decarboxylation, and malonyl-CoA were decreased by DualAG (Figure 4, D-E). In addition, DualAG caused a significant upregulation of genes that induce fatty acid oxidation (FAO) in the liver, including *Fgf21* and *Cpt1a* (Figure 4, F-G), and the downregulation of lipogenic genes such as *ChREBP* (Figure 4H). A robust (~15 fold) upregulation of *Ldlr* by DualAG (Figure 4I) was also noted in the context of reduced cholesterol levels previously observed with chronic treatment (Table 1).

Metabolic effects of DualAG and GLPAG in Glp1r$^{-/-}$ and Gcgr$^{-/-}$ mice. To provide mechanistic insight into the relative contributions of each target receptor to the metabolic effects of the long-acting peptides, we compared the effect of repeat administrations of DualAG and GLPAG (1.9 μmol/kg s.c.) on body weight and food intake in wild type mice versus animals lacking either GLP1R (*Glp1r$^{-/-}$)* or GCGR (*Gcgr$^{-/-}$)*. Since both receptor knockout mouse lines are resistant to diet induced obesity, weight-matched lean mice were used in the study. Both peptides significantly reduced cumulative food intake and body weight in wild type mice. As observed previously, DualAG treatment effected superior body weight loss in wild type mice compared to animals treated with GLPAG (Figure 5). In mice lacking either GLP1R or GCGR, however, the efficacy of DualAG was sustained but partially attenuated compared to the weight loss achieved in wild type animals. These results implicate both GLP1R and GCGR activation in the mechanism of action of DualAG. The weight loss efficacy of GLPAG in *Gcgr$^{-/-}$* mice was comparable to the attenuated body weight effects of DualAG in these animals and was completely abolished in *Glp1r$^{-/-}$* mice, confirming the GLP1R selectivity of this peptide.

DISCUSSION

Herein, we compare the anti-obesity effects of a long-acting dual GLP-1/glucagon agonist (DualAG) to that of a long-acting GLP1R selective agonist (GLPAG) in a mouse model of obesity. To avoid confounding effects in these studies, a specific effort was made to match the pharmacokinetics, GLP1R agonist potencies, and plasma exposures of the two peptides during chronic dosing studies. Long acting

peptides DualAG and GLPAG lowered blood glucose, reduced food intake, and decreased body weight in DIO mice. We report for the first time that chronic treatment with a dual GLP1R/GCGR agonist causes superior weight loss and lipid lowering in DIO mice compared to a GLP1R selective agonist, without causing hyperglycemia. Instead, ambient glucose levels were normalized by both peptides, and glucose tolerance was comparably improved in both groups as measured in an IPGTT conducted at the end of the study. Of note, improvements in metabolic parameters such as plasma insulin, leptin, and adiponectin were typically more pronounced upon chronic treatment with DualAG than with GLPAG, consistent with the increased weight loss efficacy of the dual agonist.

To evaluate the contributions of GLP1R versus GCGR agonism to the observed pharmacology of the long-acting peptides, we evaluated the metabolic effects of repeat dosing with DualAG and GLPAG in *Glp1r*$^{-/-}$ and *Gcgr*$^{-/-}$ mice. Our studies clearly establish the importance of dual GLP1R/GCGR agonism in the increased weight loss efficacy of DualAG. Specifically, weight loss was observed with DualAG treatment in both *Glp1r*$^{-/-}$ and *Gcgr*$^{-/-}$ mice, although efficacy was reduced compared to body weight effects observed in weight-matched wild type animals. In contrast, the metabolic effects of GLPAG were completely ablated in *Glp1r*$^{-/-}$ mice, confirming its GLP1R selective effects.

A unified hypothesis for the mechanism of action of peptide DualAG is illustrated in Figure 5G. Under conditions of fasting metabolism, GCGR signaling pharmacologically accentuates the catabolic aspects of metabolism that favor weight loss. DualAG activates GCGR in the liver, rapidly upregulating key gluconeogenic genes. However, increased GLP1R signaling in the post-prandial state is sufficient to improve glucose tolerance with a dual agonist peptide, as has been reported with native OXM in mice (6; 14). Our studies also confirm the contribution of GLP1R agonism to the chronic anti-obesity effect of DualAG, which is driven primarily by a reduction in adiposity. As illustrated in Figure 5G, DualAG modulates metabolic pathways that decrease acetyl-CoA and malonyl-CoA pools in the liver, increase ketogenesis, and decrease plasma lipids. Hormone sensitive lipase (HSL) mRNA, which is downregulated in animal models of obesity (33) and in obese humans (34; 35), is significantly upregulated in adipose tissue obtained from DIO mice treated with DualAG (data not shown). While the role of glucagon in fat cell metabolism in humans is unclear, pharmacological activation of GCGR in adipose tissue may activate HSL, resulting in an increased free fatty acid pool available for β-oxidation (36). Our data suggest that fatty acid oxidation is acutely upregulated in rodents by GLP1R/GCGR dual agonism prior to any weight loss. The observed upregulation of liver *Fgf21* by DualAG may also contribute to the action of a dual GLP1R/GCGR agonist since pharmacological levels of FGF21 stimulate hepatic fatty acid oxidation and increase energy expenditure (37; 38).

The increased weight loss efficacy of DualAG compared to GLPAG is consistent with previous research on the pharmacology of glucagon. The hormone has been reported to decrease total cholesterol in rats, and to cause a greater reduction in body weight compared to a pair-fed group of animals due to both, reduced food intake and increased energy expenditure (19). Conversely, Langhans *et al.* showed that intraperitoneal (i.p.) injections of anti-glucagon antibodies in food-deprived rats increased meal size and duration (39). Furthermore, chronic administration of glucagon in humans has been reported to increase satiety and decrease hunger scores (17; 18). Of note, however,

GCGR-selective agonism is typically associated with the risk of hyperglycemia, since elevation of endogenous glucagon levels and concomitant reduction in insulin levels/action are accepted as key players in the pathogenesis of diabetic hyperglycemia (40). According to this bihormonal hypothesis for diabetes, hyperglucagonemia results in excessive hepatic glucose production, which is not balanced by glucose utilization under conditions of hypoinsulinemia and insulin resistance. Indeed, the development of GCGR antagonists for the treatment of T2D is being actively pursued (41-44) since these agents act towards restoring normal GCGR tone. Distinct from the demonstrated imbalance in endogenous hormonal action that characterizes the pathology of T2D, however, we now report for the first time that *concomitant* activation of GLP1R in rodents mitigates the metabolic risks associated with GCGR activation while leveraging the beneficial pharmacological effects of activating each receptor, including enhanced weight loss efficacy, antihyperglycemic activity, and lipid lowering effects. Our hypothesis is that pharmacological GLP1R agonism results in enhanced glucose-dependent insulin secretion, which enhances glucose disposal and provides sufficient anabolic tone to balance the glucoregulatory and catabolic effects of concomitant GCGR agonism. Hence, the GLP1R/GCGR dual agonist peptide DualAG mediates safe and effective weight loss and improves glucose tolerance in rodents without causing hyperglycemia or cachexia. Whether the observed rodent pharmacology is predictive of clinical effects with a long acting dual agonist peptide remains to be determined, although the weight loss obtained with native OXM in overweight subjects is encouraging (9), given the rapid clearance of this peptide.

In conclusion, we propose that long-acting GLP1R/GCGR dual agonists like peptide DualAG represent novel pharmaceutical agents for the treatment of obesity.

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FIGURE LEGENDS

Figure 1. Sequence alignment and receptor agonist potencies of OXM and related peptides (A) Sequence alignment of OXM, exendin-4, and related glucagon superfamily peptides including the long acting OXM analogs DualAG and GLPAG. Conserved residues are highlighted. Gln^3 is important for GCGR agonist activity. DualAG and GLPAG incorporate a $DSer^2$ (S) substitution that confers resistance to DPP-4. The cholesterol moiety (chol) at the C-terminus of these peptides enhances metabolic stability and receptor affinity, and the intervening PEG spacer minimizes the loss in agonist potency due to plasma protein/lipid binding. (**B**) In vitro receptor agonist potencies (cAMP release) against mGLP1R and mGCGR, and ED_{50} (nM) in the *ex vivo* mouse liver glycogenolysis assay (mGlyco). The $Gln^3 \rightarrow$ Glu substitution in GLPAG reduces GCGR agonist activity ~120-400-fold compared to DualAG. mGLP1R: murine GLP-1R, mGCGR: murine GCGR.

Figure 2. Superior efficacy of a long-acting dual GLP1/GCGR agonist in reducing body weight in DIO mice. (A) Study protocol for chronic dosing of animals with vehicle or peptides DualAG and GLPAG (1.9 μmol/kg s.c., QOD). (**B)** Cumulative changes in body weight. Plasma exposures of each peptide measured at the end of the study were comparable (inset). (**C**) Cumulative food intake and **(D)** body composition changes for each treatment group. (**E)** IPGTT conducted on day 13 of the study. *p<0.05, DualAG and GLPAG vs vehicle; ^p<0.05, DualAG vs GLPAG.

Figure 3. Histological analysis (photomicrographs) of lipid accumulation in liver obtained from DIO mice
treated chronically with DualAG and GLPAG. H&E stain of hepatic histological sections obtained from animals treated with **(A)** vehicle, (**B**) DualAG, and **(C)** GLPAG; magnification: x300.

Figure 4. Gene expression changes measured in liver tissue obtained from DIO mice treated with Vehicle, DualAG or GLPAG. *(***A***) Pck1*, (**B**) *Pgc-1α* and (**C**) *Pdha1* mRNA. (**D**) acetyl-CoA and (**E**) malonyl-CoA levels. (**F**) *Fgf21*, (**G**) *Cpt1a*, (**H**) *ChREBP* and (**I**) *Ldlr* mRNA. *p<0.05 DualAG and GLPAG vs. vehicle; ^p<0.05 DualAG vs. GLPAG.

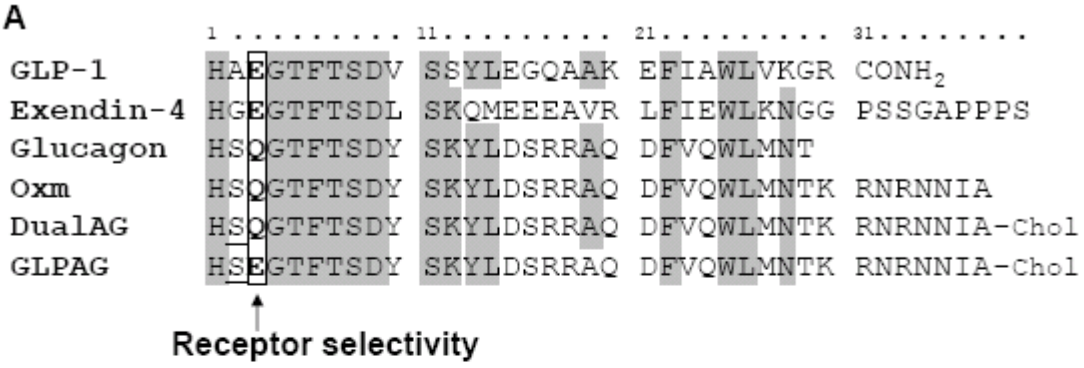
Figure 5. DualAG lowers body weight and food intake via activation of GLP1R and GCGR. Effect of repeated injections of DualAG or GLPAG on cumulative food intake and body weight in wild type (**A, B**), *Glp1r*$^{-/-}$ (**C, D**) and *Gcgr*$^{-/-}$ (**E, F**) mice. The anti-obesity effects of DualAG are attenuated but not ablated in either receptor-knockout mouse. **(G)** Proposed mechanism of action of DualAG. In addition to the known effects associated with GLP1R activation, hepatic GCGR activation increases liver glucose production and stimulates fatty acid oxidation. The acetyl-CoA generated by β-oxidation challenges the processing capacity of the TCA cycle and is used in the biosynthesis of ketone bodies. Consistent with the decrease in plasma cholesterol, animals treated with DualAG showed a robust up-regulation of liver LDLr expression. In the adipose tissue, pharmacological activation of GCGR and GLP1R may stimulate hydrolysis of triglycerides. Upregulation of liver *Fgf21* in animals treated with DualAG may contribute to stimulation of fatty acid oxidation and ketogenesis.

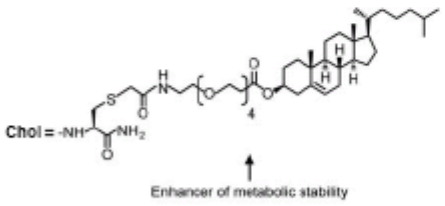
Table 1. Chronic treatment of DIO mice with DualAG and GLPAG: plasma parameters measured at the end of the 14 day study

	vehicle	**DualAG** **1.9 μ M/kg/day**	**GLPAG** **1.9 μ M/kg/day**
N	6	7	7
Insulin (ng/mL)	13.2±0.7	4.0±0.2*†	7.8±1.1*
Leptin (ng/mL)	32±4	14±1*†	19±1*
Adiponectin (μg/mL)	15±1	28±2*†	20±1*
FFA (mM)	0.2±0.0	0.4±0.1	0.3±0.0
Cholesterol	153±6	76±7*†	107±5*
TG (mg/dL)	68±8	44±5*	47±6*
BHBA (mg/dL)	4.1±0.3	9.3±0.9*	7.2±0.4*

Data are means ± SEM. FFA, free fatty acids; TG, triglycerides; BHBA, β-hydroxybutyrate. *p<0.05 vs vehicle, p<0.05 †DualAG vs GLPAG

A

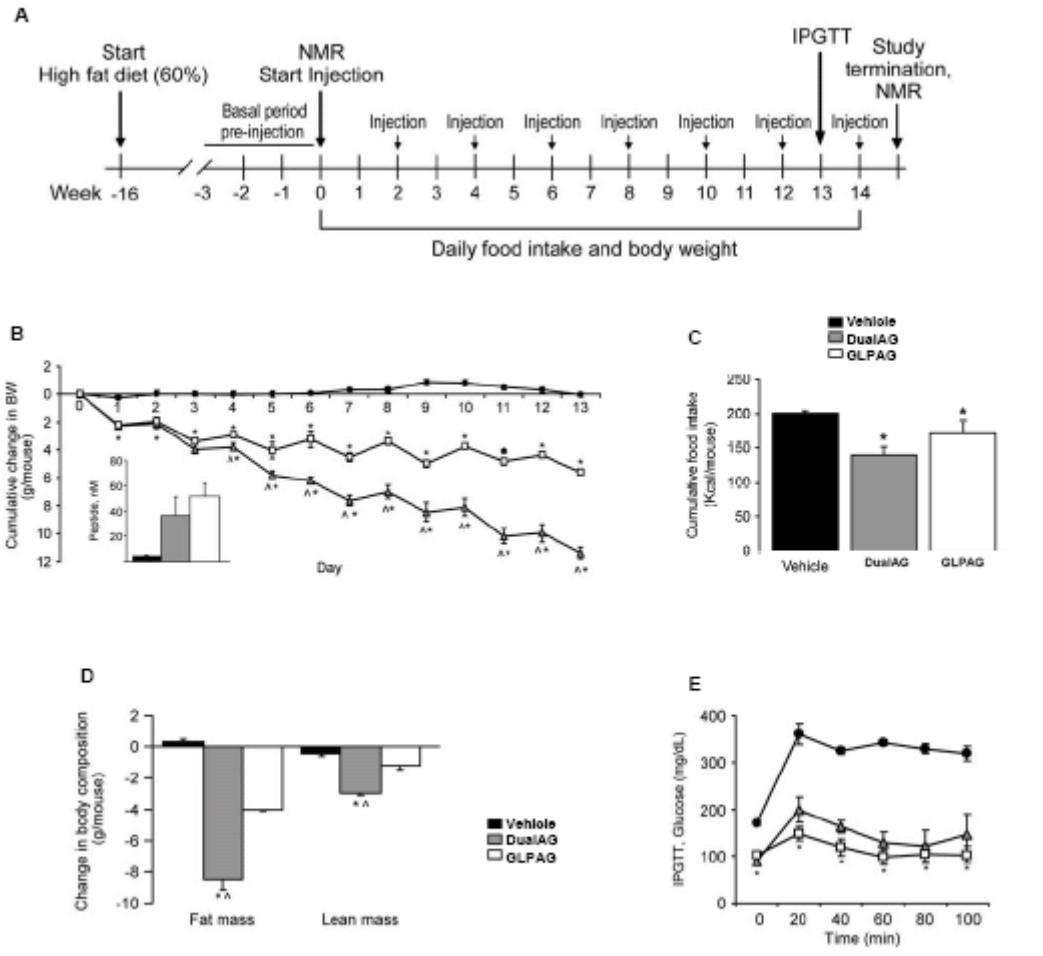




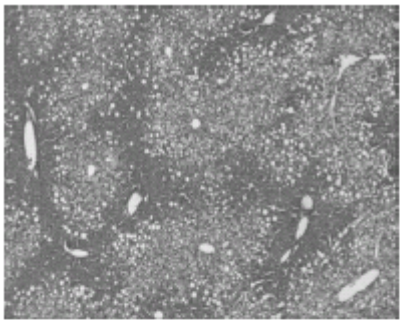
B

$EC_{50,\ cAMP,}$ nM	mGLP 1R	mGCGR	mGlyco
GLP-1	0.09	>1000	>1000
Glucagon	41	0.5	0.02
Oxm	2.5	6.2	0.5
DualAG	3.4	1.5	0.5
GLPAG	1.7	180	208

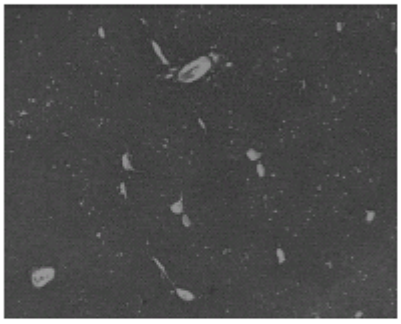
Pocai/Fig.1


A
Start
High fat diet (60%)
NMR
Start Injection
IPGTT
Study
termination,
NMR
Basal period
pre-injection
Injection
Week -16
Daily food intake and body weight
B
Cumulative change in BW
(g/mouse)
Day
Plasma, nM
C
Vehicle
DualAG
GLPAG
Cumulative food intake
(Kcal/mouse)
D
Change in body composition
(g/mouse)
Fat mass
Lean mass
E
IPGTT, Glucose (mg/dL)
Time (min)

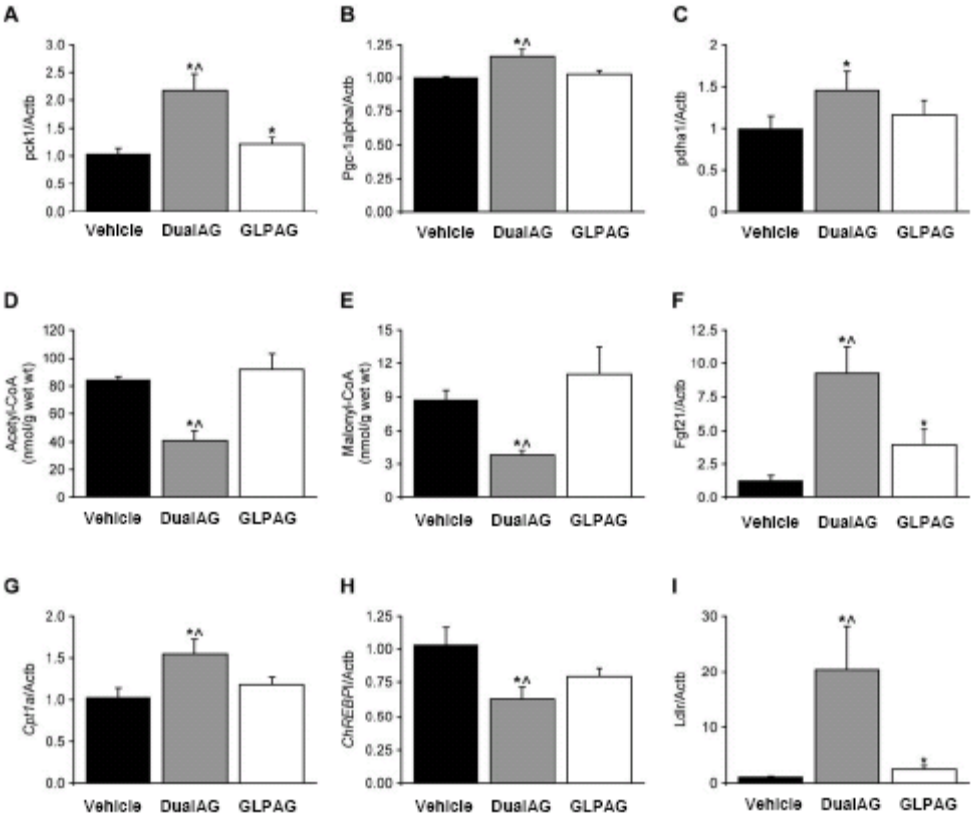
Pocai/Fig.2



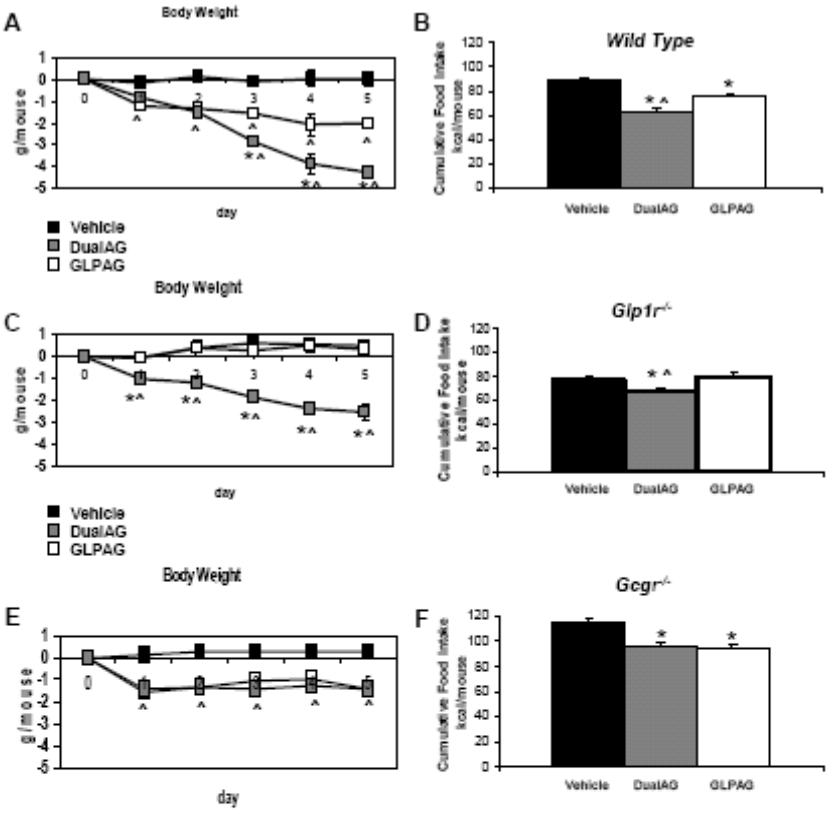
Vehicle

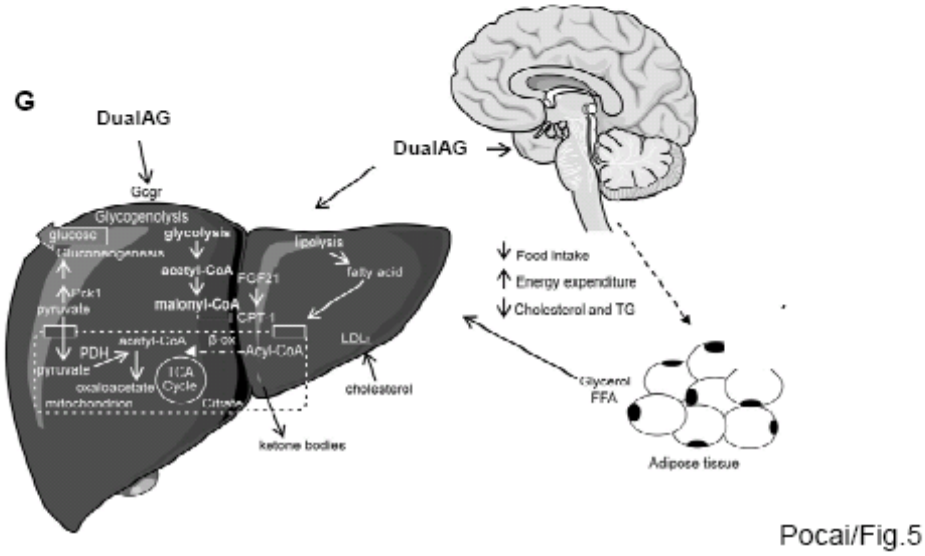


DualAG



Pocai/Fig.4


A
Body Weight
g/mouse
day
Vehicle
DualAG
GLPAG
B
Wild Type
Cumulative Food intake kcal/mouse
Vehicle
DualAG
GLPAG
C
Body Weight
g/mouse
day
Vehicle
DualAG
GLPAG
D
Glp1r-/-
Cumulative Food intake kcal/mouse
Vehicle
DualAG
GLPAG
E
Body Weight
g/mouse
day
F
Gcgr-/-
Cumulative Food intake kcal/mouse
Vehicle
DualAG
GLPAG


G
DualAG
Gcgr
DualAG
Glycogenolysis
glucose
glycolysis
lipolysis
acetyl-CoA
FGF21
Tatty acid
PFK1
pyruvate
malonyl-CoA
CPT-1
PDH
acetyl-CoA
β-OX
Acyl-CoA
LDL
pyruvate
oxaloacetate
TCA Cycle
cholesterol
mitochondrion
Citrate
ketone bodies
Food intake
Energy expenditure
Cholesterol and TG
Glycerol
FFA
Adipose tissue

Pocai/Fig.5