A rationally designed monomeric peptide triagonist corrects obesity and diabetes in rodents

Brian Finan1–3,11, Bin Yang3,4,11, Nickki Ottaway5, David L Smiley3, Tao Ma3,6, Christoffer Clemmensen1,2, Joe Chabenne3,7, Lianshan Zhang4, Kirk M Habegger4, Katrin Fischer1,2, Jonathan E Campbell9, Darleen Sandoval3, Randy J Seeley5, Konrad Bleicher10, Sabine Uhles10, William Riboulet10, Jürgen Funk10, Cornelia Hertel10, Sara Belli10, Elena Sebokova10, Karin Conde-Knape10, Anish Konkar10, Daniel J Drucker9, Vasily Gelfanov3, Paul T Pfluger1,2, Timo D Müller1,2, Diego Perez-Tilve5, Richard D DiMarchi3 & Matthias H Tschöp1,2

We report the discovery of a new monomeric peptide that reduces body weight and diabetic complications in rodent models of obesity by acting as an agonist at three key metabolically-related peptide hormone receptors: glucagon-like peptide-1 (GLP-1), glucagon-dependent insulinotropic polypeptide (GIP) and glucagon receptors. This triple agonist demonstrates supraphysiological potency and equally aligned constituent activities at each receptor, all without cross-reactivity at other related receptors. Such balanced unimolecular triple agonism proved superior to any existing dual coagonists and best-in-class monoagonists to reduce body weight, enhance glycemic control and reverse hepatic steatosis in relevant rodent models. Various loss-of-function models, including genetic knockout, pharmacological blockade and selective chemical knockout, confirmed contributions of each constituent activity in vivo. We demonstrate that these individual constituent activities harmonize to govern the overall metabolic efficacy, which predominantly results from synergistic glucagon action to increase energy expenditure, GLP-1 action to reduce caloric intake and improve glucose control, and GIP action to potentiate the incretin effect and buffer against the diabetogenic effect of inherent glucagon activity. These preclinical studies suggest that, so far, this unimolecular, polypharmaceutical strategy has potential to be the most effective pharmacological approach to reversing obesity and related metabolic disorders.

Received 24 July; accepted 21 October; published online 8 December 2014; doi:10.1038/nm.3761

Prevention and treatment of obesity and type 2 diabetes (T2D) is urgently required because lifestyle modification has proven mostly ineffective. Despite this vast unmet need, potent and safe pharmacological options that effectively promote weight loss and improve metabolic health have largely remained elusive, partly because many drug interventions historically directed at single molecular targets have exhibited insufficient efficacy or unacceptable safety when used chronically. However, new multimolecular therapies have shown enhanced clinical weight loss and single-molecule peptides integrating the complementary actions of multiple endogenous metabolically-related hormones have emerged as one of the most promising clinical candidates for reversing obesity and type 2 diabetes, partly because many drug interventions historically directed at single molecular targets have exhibited insufficient efficacy or unacceptable safety when used chronically. However, new multimolecular therapies have shown enhanced clinical weight loss and single-molecule peptides integrating the complementary actions of multiple endogenous metabolically-related hormones have emerged as one of the more promising clinical candidates for reversing obesity and type 2 diabetes, partly because many drug interventions historically directed at single molecular targets have exhibited insufficient efficacy or unacceptable safety when used chronically.

We sought to explore the synergistic metabolic benefits of simultaneous modulation of glucagon, GLP-1 and GIP receptors through a single-molecule hybrid of the three hormones. Glucagon, GLP-1 and GIP are three distinct enteroinsular hormones with unique roles that complement, as well as oppose, each other in the regulation of energy and glucose homeostasis. Previously, we reported the ability to assemble balanced, high-potency coagonism for the GLP-1 and glucagon receptors (GLP-1R and GcgR, respectively) into a single peptide. This peptide exhibited a synergistic ability to lower body weight through coordinated thermogenic and anorectic actions, which can be attributed to the glucagon and GLP-1 components, respectively. Simultaneously, we discovered a high-potency, balanced coagonist for the GLP-1R and GIP receptors (GIPR) as a dual incretin coagonist displayed enhanced glycemic efficacy, diminished gastrointestinal toxicity and reduced body weight in preclinical studies, as well as the ability to lower hemoglobin A1C in humans with uncontrolled type 2 diabetes. Having established the unique efficacy of these two coagonists with glucagon and GIP independently complementing GLP-1 by different mechanisms, we hypothesized that, if chemically possible, simultaneous and aligned agonism at all three receptors through a single molecule would produce superior therapeutic outcomes. All three hormones are of comparable size and amino acid composition but sufficiently distinct to provide exquisite potency and specificity.

1Institute for Diabetes and Obesity, Helmholtz Zentrum München, German Research Center for Environmental Health, Neuherberg, Germany. 2Department of Medicine, Division of Metabolic Diseases, Technische Universität München, Munich, Germany. 3Department of Chemistry, Indiana University, Bloomington, Indiana, USA. 4Marcadia Biotech, Carmel, Indiana, USA. 5Metabolic Diseases Institute, Division of Endocrinology, Department of Internal Medicine, University of Cincinnati College of Medicine, Cincinnati, Ohio, USA. 6Research Center, Beijing Hanmi Pharm., Beijing, China. 7AIT Laboratories, Indianapolis, Indiana, USA. 8Comprehensive Diabetes Center, Department of Medicine, Division of Endocrinology, Diabetes and Metabolism, University of Alabama at Birmingham, Birmingham, Alabama, USA. 9Department of Medicine, Lunenfeld Tanenbaum Research Institute, Mt. Sinai Hospital, University of Toronto, Toronto, Ontario, Canada. 10Pharmaceutical Research and Early Development, Roche Innovation Center Basel, F. Hoffmann-La Roche Ltd., Basel, Switzerland. 11These authors contributed equally to this work. Correspondence should be addressed to B.F. (brian.finan@helmholtz-muenchen.de), R.D.D. (rdimarch@indiana.edu) or M.H.T. (tschoep@helmholtz-muenchen.de).
ARTICLES

for their individual receptors. These similarities render it hypotheti-
cally possible to chemically engineer a triple agonist (triagonist) with
potent and balanced promiscuity at these three receptors.

Through iterative chemical refinement, we have identified a high-
potency, balanced triagonist for GLP-1R, GIPR and GcgR. The tria-
gonist uses distinct amalgamated residues derived from each of the native
hormone sequences, which were selected to impart the desired activity
profile at each constituent receptor, as well as being optimized for the
necessary pharmacokinetics for suitable in vivo study. Ultimately, we
found that the final iteration of the various triagonists that we tested
delivered an apparent and heretofore unparalleled in vivo efficacy in
reversing diet-induced obesity and type 2 diabetes in rodent models
as compared to relevant monooagonists or coagonists.

RESULTS

Triple agonism generates synergistic metabolic benefits

We wished to determine whether adiposity and glycemia can be more
potently managed through simultaneous agonism at GLP-1R, GIPR and
GcgR. Thus, we compared the daily individual treatment of acylated
monooagonists at each receptor with the equimolar physical mixture of a
previously validated acylated GLP-1R and GIPR (GLP-1/GIP) coag-
onist8 with an appropriately matched, acylated GcgR agonist17 in diet-
induced obese (DIO) mice. The GIP analog decreased body weight by
6.4% (Fig. 1a) and modestly decreased food intake (Fig. 1b). The GLP-1
analog decreased body weight by 12.6% (Fig. 1a) and reduced cumula-
tive food intake by more than 50% (Fig. 1b). Both GIP and GLP-1 ana-
logs lowered ad libitum–fed blood glucose to a similar extent (Fig. 1c).
The glucagon analog decreased body weight by 11.1% (Fig. 1a) but did
not affect cumulative food intake (Fig. 1b) and increased blood glucose
throughout treatment (Fig. 1c). The GLP-1/GIP coagonist decreased body
weight by 15.4%, outperforming any of the monooagonists in this
regard (Fig. 1a). This body weight improvement by the coagonist was
associated with a lower cumulative food intake relative to that observed
with the GLP-1 analog (Fig. 1b). Additionally, ad libitum–fed blood
-glucose was decreased to a magnitude similar to that induced by either
incretin monooagonist (Fig. 1c). Co-administration of the GLP-1/GIP
coagonist with an equimolar dose of the glucagon analog decreased body
weight by 20.8% (Fig. 1a) without further suppression of food
intake than that observed with the coagonist alone (Fig. 1b). Moreover,
 simultaneous administration of the GLP-1/GIP coagonist and the GcgR
agonist was able to lower blood glucose levels to a point lower than
respective single treatments despite the hyperglycemic propensity of the
GcgR agonist alone (Fig. 1c). These observations served as the founda-
tion to pursue the discovery of unimolecular triagonists simultaneously
targeting GLP-1, GIP and glucagon receptors.

Discovery of a unimolecular, balanced, high-potency
GLP-1/GIP/glucagon selective triagonist

The structural and sequence similarities among the three hormones
(Fig. 1d), coupled with prior structure-function studies8,9, informed
the design of a sequence-hybridized peptide. The challenge was to
maintain the individual affinity of each ligand for its receptor and
eliminate the structural elements that convey selective preference for
each individual receptor, all while maintaining high potency in bal-
anced proportion to each other. Intermediary triagonist candidates
were progressively derived from a GLP-1/glucagon coagonist core
sequence8 in an iterative manner to introduce GIP agonism without
destroying GLP-1R and GcgR potency. We subsequently tested them for
in vitro activity at each constituent receptor (Supplementary Table 1).
All peptide sequences are displayed in Supplementary Figure 1, mass
spectrometry data are summarized in Supplementary Table 2, repre-
sentative HPLC and liquid chromatography-mass spectrometry data
are displayed in Supplementary Figure 2 and a detailed narrative of
triagonist evolution is in the Supplementary Results.

Through iterative refinement of the chemical structure, we ulti-
mately succeeded in engineering a highly modified peptide analog that
represents the first highly potent, balanced unimolecular triagonist
for GLP-1R, GIPR and GcgR. Aminoisobutyric acid was substituted
at position 2 to convey resistance to dipeptidyl peptidase IV–mediated
degradation and inactivation. We have previously observed that this
aminoisobutyric acid substitution also contributes to mixed agonism at
GLP-1R and GIPR but is detrimental to glucagon activity. Therefore,
we included Glu16, Arg17, Gln20, Leu27 and Asp28 to counteract
the negative impact on GcgR potency. These substitutions also serve
auxiliary functions to enhance solubility, secondary structure and
chemical stability15. To enhance time-action and in vivo utility, we site-
specifically lipidated Lys10 with palmitic acid (C16:0) through a
γ-carboxylate spacer. The acyl moiety promotes albumin binding while
also supporting mixed agonism19. Finally, the triagonist features
the C-terminal–extended residues from exendin-4, a reptilian-derived
GLP-1 paralog, which results in a single molecule of 39 residues that
shows superior solubility, potency and balance at each of the three
receptors (Fig. 1e–g and Supplementary Table 1). Although the tria-
onist was designed specifically to possess the requisite activity at human
receptors, it has a similar activity profile (based on cyclic AMP (cAMP)
induction) across all three constituent receptors originating from mice,
rats and cynomolgus monkeys (Supplementary Table 3). Therefore,
the triagonist is suitable for in vivo pharmacological and characteriza-
tion studies across different species in preclinical studies.

As we were able to introduce balanced agonism for three different
receptor targets within a single molecule, we surmised that the pep-
tide may bind to additional receptor targets. Therefore, we screened
the triagonist at over 70 different receptor targets in high–throughput
competitive binding assays. Using strict criteria for a positive result
(10% inhibition of native ligand binding), we show that the tria-
onist displayed no cross-reactive binding to any of the other screened
receptors (Supplementary Table 4). Notably, the triagonist did not
bind to the vasoactive intestinal peptide receptor or the pituitary ade-
nylate cyclase–activating polypeptide receptor, two members of the
glucagon/secretin class of peptide hormones. Therefore, we concluded
that the triagonist is highly specific for GLP-1R, GIPR and GcgR.

The unimolecular triagonist possesses in vitro and in vivo
activity attributed to each targeted receptor

To explore whether the triagonist possesses in vitro activity at each
constituent receptor, we investigated the effects on cAMP accumu-
lation in cell lines that represent conventional target tissues of GLP-1,
GIP and glucagon. In a mouse pancreatic beta cell line (MIN6) that
abundantly expresses GLP-1R, the GLP-1/GIP coagonist and the tria-
onist induced cAMP production with potency similar to that of native
GLP-1 (Supplementary Table 5), confirming full GLP-1R activity
at pancreatic beta cells. In differentiated 3T3-L1 mouse adipocytes,
which abundantly express GIPR, cAMP production was induced by
GIP, the GLP-1/GIP coagonist and the triagonist with comparable
potency (Supplementary Table 5), confirming full GIPR activity at
adipocytes. In rat hepatocytes, which endogenously express GcgR21,
cAMP production was induced by glucagon and the triagonist with
similar potency, whereas the GLP-1/GIP coagonist demonstrated
30-fold less potency (Supplementary Table 5), thus confirming full
GcgR activity at hepatocytes.
To investigate whether the triagonist possesses in vivo activity at each cognate receptor, we compared the acute glycemic effects using selective antagonists at each receptor in different rodent models. To confirm the presence of GLP-1 activity, we administered the triagonist to DIO mice pretreated with a validated GLP-1R antagonist. Pretreatment with the GLP-1R antagonist ameliorated the improved glucose tolerance observed with the triagonist alone (Fig. 1h), thus confirming in vivo GLP-1 activity. To confirm the presence of GIP activity, we administered the triagonist to DIO mice pretreated with a validated GIPR antagonist. Similarly, pretreatment with the GIPR antagonist blunted the improvement observed with the triagonist alone (Fig. 1i), thus demonstrating in vivo GIP activity.

To confirm the presence of GcgR activity in vivo, we administered the triagonist to streptozotocin-treated mice pretreated with a GcgR antagonist and assessed induced hyperglycemia brought on by the glucagon component of the peptide. Pretreatment with the GcgR antagonist inhibited the acute, transient hyperglycemic effect otherwise observed with the triagonist alone (Fig. 1j), which shows that the triagonist possesses classical in vivo GcgR activity.

**Metabolic benefits of the triagonist are superior to those of the respective dual agonists**

To confirm that the triagonist delivers enhanced metabolic improvement indicative of in vivo triple agonism, we compared chronic daily

---

**Figure 1** In vivo demonstration of GLP-1, GIP and glucagon triple agonism through coadministration and unimolecular peptides. (a–c) Effects on body weight change (a), cumulative food intake (b) and ad libitum fed blood glucose (c) of male DIO mice (age 9 months; n = 8 per group) treated with acylated analogs of GLP-1, GIP, dual incretin coagonist, glucagon or the equimolar coadministration of the dual incretin coagonist with the glucagon analog; all at a dose of 10 nmol per kg body weight (nmol kg⁻¹). (d) Sequences of native GLP-1, GIP and glucagon compared with the GLP-1/GIP/glucagon triagonist. Aminoisobutyric acid is denoted as X. Lysine with a \( \gamma \)C₁₆ acyl attached through the side chain amine is denoted as K. (e–g) Representative dose-response curves for activation of the native hormone and the triagonist at the human GLP-1R (e), GIPR (f) and GcgR (g). Data in (e–g) is the normalized response from three independent experiments. (h) Acute effects on intraperitoneal glucose tolerance in male DIO mice (age 12 months; n = 8 per group) treated with vehicle, the triagonist (1 nmol kg⁻¹) or a GLP-1R antagonist (1 µmol kg⁻¹), or pretreated with the GLP-1R antagonist before the triagonist. (i) Acute effects on intraperitoneal glucose tolerance in male GLP1r−/− DIO mice (age 12 months; n = 8 per group) treated with vehicle, the triagonist (2 nmol kg⁻¹) or a GIPR antagonist (2 µmol kg⁻¹), or pretreated with the GIPR antagonist before the triagonist. (j) Acute effects on glycemia in male STZ mice (age 13 months; n = 8 per group) treated with vehicle, the triagonist (1 nmol kg⁻¹) or a GcgR antagonist (1 µmol kg⁻¹) or pretreated with the GcgR antagonist before the triagonist. Data in a–j represent mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001, determined by regular one- or two-way analysis of variance (ANOVA) comparing vehicle with compound injections, and ###P < 0.001, as determined by ANOVA comparing the dual incretin coagonist with its coadministration with the glucagon analog. In both comparisons, ANOVA was followed by Tukey post hoc multiple comparison analysis to determine statistical significance.
treatment with the triagonist to treatment with the three structurally related and pharmacokinetically matched coagonists. As a first evaluation, we compared the triagonist with an equimolar dose of a GLP-1/glucagon coagonist and a GIP/glucagon coagonist in DIO mice, with liraglutide (a lipitated GLP-1 analog) included as a benchmark comparator. At this low dose, liraglutide and the GIP/glucagon coagonist did not improve body weight or glucose tolerance compared to vehicle in DIO mice, whereas the GLP-1/glucagon coagonist lowered body weight by 9.9% (Supplementary Fig. 3a). This body weight loss occurred without a concomitant improvement in intraperitoneal glucose tolerance (Supplementary Fig. 3b,c). Treatment with the triagonist lowered body weight by 15.1%, a substantial potentiation compared with liraglutide and both GIP/glucagon and GLP-1/glucagon coagonists. The triagonist was the only compound to markedly improve glucose tolerance (Supplementary Fig. 3b) and reduce food intake (Supplementary Fig. 3d) at this low dose. Notably, we were able to induce a similar effect on body weight loss with the triagonist as in our previous report of GLP-1/glucagon co-agonism\(^8\), but at nearly one-twentieth of the dose. We believe the glucagon component within the triagonist contributes substantial weight-lowering efficacy that can potentiate that of the dual incretin action. Thus, we extensively compared the metabolic efficacy of the triagonist with that of the validated GLP-1/GIP coagonist\(^8\) in subsequent studies.

We compared the triagonist with a GLP-1/GIP coagonist of matched potency at equivalent doses in DIO mice. The coagonist decreased body weight by 15.7%, whereas the triagonist decreased body weight by 26.6% after 20 d (Fig. 2a). The triagonist decreased body weight in a dose-dependent manner, such that a 33% lower dose of the triagonist decreased body weight by 14.7%, which is comparable to the coagonist at a threefold higher dose. Both mixed agonists caused a similar reduction in food intake (Fig. 2b) without influencing gastric emptying (Supplementary Fig. 4a). Such treatment generates the loss of fat mass without altering lean mass (Fig. 2c). The coagonist and the triagonist were equally effective in lowering ad \textit{lib}itum-fed blood glucose (Fig. 2d) and improving glucose tolerance (Fig. 2e) without inducing hypoglycemia (Supplementary Fig. 4b), demonstrating that chronic GcgR agonism does not diminish the sizable glycemic improvement achieved by simultaneous dual incretin receptor coagonism. However, the triagonist lowered circulating concentrations of insulin to a greater extent than that achieved with the coagonist (Fig. 2f), which is indicative of improved insulin sensitivity, reflected by an improved insulin tolerance and lower Homeostasis Model Assessment of insulin resistance score (Fig. 2g). The triagonist, but not the coagonist, increased plasma concentrations of fibroblast growth factor 21 (FGF21; Fig. 2h), which is a selective action of the glucagon component within the triagonist. Furthermore, the triagonist lowered the plasma concentration of cholesterol to a greater extent than the coagonist (Fig. 2i). Evaluations of other clinical chemical markers did not reveal any substantial differences between the treatments (Supplementary Fig. 4c–i). The triagonist had a pronounced effect on lowering hepatic lipid content and hepatocellular vacuolation (Fig. 2j). Collectively, these results demonstrate that greater metabolic efficacy can be gained by treatment with a unimolecular GLP-1R, GIPR and GcgR triagonist than with a dual incretin coagonist. A similar effect on body weight loss can be achieved with the triagonist as in our previous reports of co-agonism, but at a much lower dose of the triagonist.

We can exclude that prolonged duration of action is contributing to the enhanced efficacy of the triagonist compared with the GLP-1/GIP coagonist because the pharmacokinetic parameters across different
species are similar between the two peptides. The average half-life of the triagonist following a bolus subcutaneous dose is approximately 5 h in rodents (Supplementary Table 6), which is similar to the reported half-life of 4–8 h for liraglutide in rodents23.

As a point of reference, we compared the in vitro activity of this triagonist with two recently reported peptides with purported triagonism24,25. Both of these reported peptides (termed YAG-glucagon24 and [dA2]GLP-1/GcG (ref. 25)) demonstrate nearly one-thousandth the activity at least at one of the three receptors compared with the native hormones (Supplementary Table 1). Additionally, these peptides were massively imbalanced in activity, rendering them at best extremely low-potency coagonists and certainly not triagonists. In DIO mice, twice-daily administration of these compounds at a cumulative dose of 50 nmol kg$^{-1}$ d$^{-1}$, which is a dose 16 times higher than that of our triagonist, failed to reduce body weight, food intake or blood glucose or improve glucose tolerance compared with vehicle treatment, whereas exendin-4 improved all the aforementioned parameters (Supplementary Fig. 5a–d). These in vivo results confirm the imbalanced activity and reduced receptor potency of the purported triagonists and demonstrate that these peptides are substantially inferior in terms of metabolic efficacy, even compared with the classical GLP-1R monoagonist exendin-4. Accordingly, these peptides fail as potent mixed agonists; therefore, they do not qualify as triagonists and should be used with caution.

**Chronic treatment with the triagonist safely lowers body weight without hypoglycemic risk**

Although we did not observe hypoglycemia or a substantial loss of lean mass after chronic treatment in DIO mice, we were still concerned about the safety of the considerable glucose-lowering and weight-lowering efficacy. The triagonist induced a dose-dependent decrease in blood glucose following a single-bolus intraperitoneal injection in lean, euglycemic mice (Supplementary Fig. 6a). Notably, we did not observe hypoglycemia at any of the doses tested when monitored 24 h after injection. Additionally, the triagonist did not reduce body weight, lean mass, or food intake (Supplementary Fig. 6b–d) after chronic treatment at any of the doses tested in these lean mice.

To test whether the triagonist has sustained efficacy in a longer-term setting, we treated DIO rats for 11 weeks with two doses of the triagonist. By 11 weeks, the lower dose of the triagonist decreased body weight by 9.7% and the higher dose decreased body weight by 26.4% (Supplementary Fig. 6e), along with dose-dependent reductions in food intake (Supplementary Fig. 6f). This superior body weight loss induced by the triagonist may limit the dose escalation in translational and toxicological studies. However, 2-week follow-up analysis in DIO mice that received the triagonist for 3 weeks revealed that the mice regained body weight to near baseline levels, and mild hyperglycemia was recovered (Supplementary Fig. 6g,h), which indicates that the triagonist did not cause irreversible damage in these preclinical studies.
Improved metabolic and glycemic benefits of triple agonism depend on GLP-1R and GIPR signaling

We studied the contribution of the GLP-1 component of the triagonist by testing its metabolic efficacy in high-fat diet–fed (HFD) Glp1r−/− mice. The triagonist decreased body weight by 18.3% in wild-type HFD mice, yet by only 10.5% in HFD Glp1r−/− mice (Fig. 3a). This diminished efficacy was also reflected in fat mass change and cumulative food intake (Supplementary Fig. 7a,b). Without GLP-1 activity to enhance GIP activity in counteracting the hyperglycemic character of GcgR agonism, the triagonist increased blood glucose in HFD Glp1r−/− mice, a trait noticeably absent from wild-type HFD controls (Fig. 3b). Mirroring this, the triagonist improved glucose tolerance in wild-type mice but not in Glp1r−/− mice, in which it in fact worsened glucose tolerance (Fig. 3c). These results demonstrate the contribution of GLP-1 activity to the full efficacy of the triagonist and underscore the necessity of integrated GLP-1 activity to minimize the diabetogenic risk of chronic and maximal GcgR agonism.

We explored the contribution of the GIP component of the triagonist by testing its metabolic efficacy in HFD Gipr−/− mice. Unlike in HFD Glp1r−/− mice, the triagonist induced comparable reductions in body weight (Fig. 3d), fat mass and food intake (Supplementary Fig. 7c,d) in both HFD wild-type and HFD Gipr−/− mice. Similar to the case in HFD Glp1r−/− mice, the glucose-lowering effect of the triagonist was lost in HFD Gipr−/− mice (Fig. 3e), but the remaining GLP-1 activity was able to prevent the rise in blood glucose that we observed in HFD Glp1r−/− mice. This result shows that integrated GIP activity within the triagonist contributes to the glucose-lowering effect and helps buffer against the hyperglycemic effect of glucagon activity, albeit to a lesser degree than GLP-1. This was also evident in glucose tolerance, where the beneficial effect of triagonist was muted in HFD Gipr−/− mice (Fig. 3f). However, it cannot be entirely excluded that the loss of the glycemic benefits and lack of the weight-lowering effect of the triagonist in Gipr−/− mice was in part due to their inherent resistance to HFD-induced glucose intolerance26.

Improved energy metabolism benefits of tri-agonism depend on GcgR signaling

We studied the contribution of the glucagon component of the triagonist by testing its metabolic efficacy in GcgR−/− mice fed a HFD. The weight-lowering efficacy of the triagonist was lost in a low-dose treatment of these HFD GcgR−/− mice compared with HFD wild-type controls. The triagonist decreased body weight by 7.7% (Fig. 3g) and lowered fat mass in wild-type mice (Supplementary Fig. 7e), yet had no measurable effect on body weight loss in HFD GcgR−/− mice (Fig. 3g). Likewise, the triagonist lost its anorectic efficacy (Supplementary Fig. 7f) and lowering effect on fasted blood glucose (Fig. 3h), and failed to improve glucose tolerance in these HFD GcgR−/− (Fig. 3i), which may be partially attributed to the existing hypoglycemia and inherent protection from glucose intolerance of GcgR−/− mice27.

We have previously demonstrated that changes in energy expenditure are not a key contributor to the weight-lowering efficacy of the GLP-1/GIP coagonist16 but do contribute to the efficacy of the GLP-1/glucagon coagonist, which is attributed to the integrated glucagon pharmacology17. Supporting this, we did not observe any difference in food intake between wild-type mice treated with the dual incretin coagonist and those treated with the triagonist despite substantial differences in weight loss, thus supporting that energy expenditure mechanisms contribute to the overall efficacy. Therefore in DIO mice, we compared the metabolic efficacy of the triagonist to that of pair-fed controls. The triagonist caused a greater reduction in body weight (32.0%) compared to that observed in pair-fed controls (21.6%; Fig. 4a,b), which is the result of loss of fat mass, not lean mass (Fig. 4a,d). After body weight segregation, we observed significantly enhanced energy expenditure in triagonist-treated DIO mice compared with the pair-fed controls (Fig. 4e,f, P < 0.001). When
compared with both ad libitum–fed and pair-fed controls, triagonist-treated mice had a significantly lower respiratory quotient during times of feeding (Fig. 4g,h, P < 0.01) without any change in locomotor activity (Fig. 4i,j), suggesting altered nutrient partitioning to greater fat oxidation. Collectively, the data demonstrate that the weight-lowering efficacy of the triagonist is not simply a consequence of reduced caloric intake.

GcgR signaling component of the triagonist does not exacerbate preexisting hyperglycemia

We explored whether the enhanced metabolic efficacy of the triagonist observed in insulin-resistant obese mice would translate to rodent models of type 2 diabetes. We used db/db mice at 6 weeks of age, immediately before the development of hyperglycemia, and assessed the capacity of the triagonist to prevent the development of spontaneous diabetes compared with the dual incretin coagonist. The triagonist prevented the excessive weight gain observed in vehicle-treated mice and showed improvement over the effect observed with the coagonist (Fig. 5a). Despite these differences, cumulative food intake was not altered with either treatment (Fig. 5b). Furthermore, the triagonist protected db/db mice from fasting hyperglycemia at both doses tested, an effect comparable to that of the GLP-1/GIP coagonist (Fig. 5c). In fact, the triagonist was superior compared with the coagonist in lessening glucose intolerance (Fig. 5d) and preserving proper islet architecture, as assessed by reduced alpha cell infiltration within the core of pancreatic islets (Fig. 5e) after 4 weeks of treatment.

We next assessed the effects of the triagonist in Zucker diabetic fatty (ZDF) rats. In a dose-dependent manner, the triagonist improved body weight and fasting blood glucose with a rapid onset and sustained efficacy (Supplementary Fig. 8a,b), improved glucose tolerance and hemoglobin A1C, and preserved proper islet cytoarchitecture compared with vehicle control (Supplementary Fig. 8c–e). Notably, these glycemic improvements were maintained in the high-dose group 3 weeks after treatment cessation (Supplementary Fig. 8d,e), despite body weight regain back to a level comparable to that of vehicle-treated controls (Supplementary Fig. 8f), demonstrating an ability to ameliorate diabetes progression in rodent models of spontaneous diabetes.

Optimal metabolic benefits of triple agonism predominantly depend on fine-tuning the glucagon component

We explored the relative degree of glucagon activity necessary to maintain the maximal weight-lowering efficacy of a triagonist. We assembled a series of peptides selectively laddered in their relative GcgR character, and glutamic acid substitution (Glu3) resulted in an analog with 5% of initial glucagon character, and glutamic acid substitution (Glu3) resulted in negligible glucagon activity (Supplementary Table 1, peptides 21–27). Notably, substitution with methionine sulfoxide (Met(O)2) resulted in an analog with 5% of initial glucagon character, and glutamic acid substitution (Glu3) resulted in negligible glucagon activity (Supplementary Table 1, peptides 21–27). In DIO mice, the balanced triagonist decreased body weight by 22.8% and the sequence-matched coagonist reduced body weight by 14.9%, reemphasizing the relative importance of the integrated and balanced GcgR agonism to the weight-lowering efficacy. The Met(O)2 analog (imbalanced triagonist) decreased body weight by 19.3% (Fig. 6a), which is intermediate compared to the weight loss induced by the triagonist and GLP-1/GIP coagonist. However, this imbalanced triagonist had a more pronounced effect on lowering ad libitum–fed blood glucose than the balanced triagonist, resulting in enhanced glucose lowering analogous to that achieved with the GLP-1/GIP coagonist (Fig. 6b).

To more deeply determine the relative beneficial limits and risk associated with excessive glucagon pharmacology in DIO mice, we supplemented a constant low dose of the dual incretin coagonist9 with escalating doses of the acyl-glucagon agonist. The acylated glucagon analog alone displayed a dose-dependent and robust weight-lowering efficacy, which was appreciably enhanced when the dual-incretin coagonist was co-administered at a constant low dose (Fig. 6c).
The maximal dose of the glucagon analog alone increased blood glucose, but this rise was prevented when supplemented with the dual incretin coagonist, even at a relative ratio as little as one-third to one-tenth of the glucagon dose (Fig. 6d).

Finally, we challenged diabetic db/db mice with more extreme glucagon dosing to test the capacity of dual incretin coagonism to prevent the glucagon-mediated rise in blood glucose. We co-administered escalating doses of the acylated glucagon analog with a low dose of the GLP-1/GIP coagonist. This physical mixture resulted in a dose-escalating effect in blood glucose, depicted as equivalent molar ratio to the coagonist. Data in a–f represent mean ± s.e.m. **P < 0.01, ***P < 0.001, determined by ANOVA comparing vehicle with triagonist injections. #P < 0.05, ##P < 0.01, ###P < 0.001, determined by ANOVA comparing coagonist with triagonist injections. $P < 0.05, $$$P < 0.001 determined by ANOVA comparing triagonist with imbalanced triagonist injections. In all comparisons, ANOVA was followed by Tukey post hoc multiple comparison analysis to determine statistical significance.

DISCUSSION

Here we explored the chemical capability of combining agonism at the glucagon, GLP-1 and GIP receptors into a single molecule, as well as the synergistic efficacy of this concerted triple agonism to reverse perturbed metabolism in rodent models of obesity and diabetes. As a first-degree proof of principle, we simulated in vivo triple agonism by in situ co-administration of a glucagon analog as a second molecule with the validated GLP-1/GIP coagonist. This adjunctive triple agonism amplified the metabolic efficacy of the dual incretin in obese mice, thus establishing the pharmacological foundation to pursue the uncertain creation of a unimolecular triagonist with glucagon, GLP-1 and GIP activities.

The design of the triagonist was inspired by our previous observations in the discovery process of the mixed coagonists, as well as by the established sequence differences among the three endogenous hormones. The goal was to maintain high activity across all three receptors yet eliminate the inherent high selectivity resident in the native hormones. The core 29-residue GLP-1/glucagon coagonist peptide proved resistant to our best efforts to identify a change that would introduce GIP activity. The breakthrough was the addition of the exendin-4-based C-terminal extended sequence to a lipidated, dipeptidyl peptidase IV–protected intermediate analog. This elongation provided a triagonist of equal balance at all three receptors with superior potency relative to the native hormones. Notably, this single-peptide triagonist is a hybridized peptide, not simply a conjugate multimer of the native ligands, and it features a single receptor-binding face. This conveys concerted yet independent and promiscuous agonism at each constituent receptor without cross-reactivity at other related G protein–coupled receptors, essentially serving as a master key to unlock signaling at each individual receptor.

We demonstrate here that this unimolecular triagonist potently reverses diet-induced obesity and prevents diabetes progression in rodent models to a greater extent than reciprocal coagonism at the individual receptors. We were able to induce a comparable effect size with the triagonist, in terms of weight loss and glycemic control, as we independently reported with GLP-1/glucagon and GLP-1/GIP coagonists, but at substantially lower doses, which cannot be attributed to differences in pharmacokinetics. We demonstrated that concerted triple agonism exceeds dual incretin coagonism in terms of improving body weight and composition. This is partially attributed to the supramolecular nature of the glucagon-mediated rise in blood glucose, which is something that seemed more fragile when using just GLP-1 to buffer glucagon action.
high-potency, inherent mixed agonism within the triagonist advanta-
geously lessens the respective target receptor occupancy rates, which
more closely resembles a physiological response.

We confirmed constituent activity and demonstrated its contribu-
tion to the overall metabolic efficacy of the triagonist through indirect
and direct means. Indirectly, we showed this through the comparison of
in vivo metabolic profile of the triagonist to a matched set of the
three possible coagonist combinations. Directly, we confirmed con-
stituent activity through in vitro assays in target cell populations and
in vivo studies using acute antagonist challenges and chronic studies
in individual receptor–knockout mice. These results demonstrated
that the triagonist possesses selected and independent pharmaco-
logical virtues indicative of each hormone, such that these otherwise
independent virtues synergize to achieve superior metabolic efficacy.
The thermogenic properties of glucagon supplements the body weight
lowering of dual incretin action, and the additional glycemic efficacy
of GIP supports GLP-1 to further buffer against the inherent
hyperglycemic risk of integrated glucagon action. Through a series of
imbalanced triagonists with a step-wise selective blunting of glucagon
activity, we also demonstrated that glucagon activity that is aligned
with GLP-1 and GIP activity is necessary for the maximal weight
loss induced by the triagonist, but dampening the glucagon activity
can promote better glycemic outcomes, albeit with less weight loss.
However, in no instance did we witness elevation of glucose when
using the balanced triagonist alone or in combination with the dual
incretin coagonist with supplemental glucagon agonism, even at levels
where the glucagon was much in excess. This stands in contrast to
our prior observations in which the specific ratio of glucagon activity
relative to GLP-1 monoagonism was of central importance to body
weight lowering and the propensity to elevate glucose28.

Through studies using individual receptor–knockout mice, we were
able to clearly demonstrate the presence of each constituent activ-
ity within the triagonist, and we were also able to ascertain some of
the contribution of each constituent to the overall metabolic ben-
efits induced by the triagonist. In both Glp1r−/− and Gcgr−/− mice,
we observed that loss of GLP-1 and glucagon functionality results
in reduced weight-lowering efficacy. Additionally, in Glp1r−/− and
Gipr−/− mice, we observed that a loss of individual incretin functional-
y results in differing degrees of buffering against a glucagon-medi-
ated negative impact on blood glucose. At minimum, the studies in the
individual receptor–knockout mice clearly demonstrated the presence
of each constituent activity within the triagonist, but gaining insight
into the contribution of each to the overall metabolic benefits elic-
ited by the triagonist is less clear. This is mostly owing to the altered
physiology that is the result of compensatory mechanisms arising
from germline knockout. As just one example, Glp1r−/− (ref. 29),
Gipr−/− (ref. 26) and Gcgr−/− (ref. 27) mice are all protected from HFD-
induced adiposity and have differential glucose and insulin sensitivi-
ties. Together, the body weight phenotypes of all three knockout
models would suggest that all three receptors should be targets of inhibition
to gain beneficial effects on body composition, which is certainly not
the case and should give warning to investigators that pharmacologi-
cal outcomes of a particular target are often difficult to predict from
targeted mutation models. Furthermore, the altered endoenocrine
responses in these three knockout lines contribute to the observed
phenotypes, which here confound interpretations of data to deter-
mine the overall contributions of each component of the triagonist.
Ultimately, in terms of demonstrating in vivo constituent activity and
understanding how activity contributes to the overall effect of polyag-
onists, we believe there is no equivalent to a set of chemically matched
analogs in which a single component activity has been deleted and
subsequently tested in wild-type animals. Together with the arduous
use of selective antagonists in wild-type conditions, albeit with its
own limitations, these sets of reagents can circumvent the liabilities
associated with the use of germline knockout models.

The magnitude of weight loss in a clinical setting and the speed with
which it is achieved needs to be thoroughly established in subsequent
development work. Intensive toxicological analyses are warranted,
including a thorough assessment of effects on the cardiovascular sys-
tem, as recent reports suggest direct chronotropic action of GLP-1R30
and GcgR31 agonism. In addition to cardiovascular outcomes, a thor-
ough assessment of long-term outcomes on diabetic ketoacidosis is
warranted because of the observed high degree of weight loss and
reduction of circulating insulin induced in these preclinical studies.
The therapeutic index of these peptides for chronic human use can-
not be accurately determined from these preclinical studies; however,
in long-term efficacy studies and follow-up analysis after treatment
cessation, we witnessed sustained efficacy with no apparent adverse
pharmacology. If it proves in subsequent testing that less aggressive
glucagon agonism is preferable for long-term dosing, it is possible
that the series of imbalanced triagonists with a selective titration of
glucagon activity might ultimately prove to be more suitable therapeu-
tics. Notably, the glucagon activity can be selectively fine-tuned with
minimal structural or chemical change (a single amino acid change
at position 3), providing the opportunity for a more personalized
medicinal approach to obesity therapy that reflects the heterogeneous
nature of the human condition. In entering the translational stage, the
ability to choose among several options that differ in their inherent
molecular pharmacology increases the likelihood of ultimate success,
as well as the opportunity to explore unconventional uses, such as for
Prader-Willi syndrome, neurodegenerative diseases or nondiabetes
liver diseases associated with excessive fat deposition.

Our results call into question previous reports of purported uni-
molecular triagonists where the body weight lowering was paltry rela-
tive to what we have observed and unsupportive of concerted triple
agonism at GLP-1R, GIPR and GcgR24,25,32. In the most recent report25,
the total reported weight loss after three weeks of twice-daily adminis-
tration was no more than 3% relative to vehicle control at a total dose
of 50 nmol kg−1 d−1. Our synthesis of these purported triagonists and
subsequent in vitro analysis revealed these peptides to be dramatically
reduced in potency relative to the native hormones (nearly 100-fold)
and to our triagonist (nearly 1,000-fold), and also failed to achieve
aligned activity balance across the three receptors. These in vitro results
suggest a basis for their limited body weight lowering relative to our
more extensive results presented herein. Here, we confirmed that these
compounds provide negligible metabolic benefits in DIO mice and
thus do not biochemically qualify to be characterized as triagonists.
Consequently, we believe that the peptides presented here represent
the first discovery of balanced triagonists and that YAG–glucagon24 and

We report the discovery of a peptide with balanced superpotency at
tree different metabolically relevant receptor targets: GcgR, GLP-1R
and GIPR. To our knowledge, this unmolecular triagonist represents
the only pharmacotherapy to date that can achieve such preclinical
efficacy at such low doses to rectify obesity and its metabolic compi-
lcations in rodents. It is becoming increasingly evident that adjusted
tenendoendocrine responses contribute to the massive and rapid meta-
abolic improvements achieved by bariatric surgeries33, which suggests
that the simultaneous and encompassing modulation of these molecu-
lar pathways may offer a pharmacological opportunity to replicate the
altered physiology seen with bariatric surgery. Accordingly, we believe the bundled multitagonism within this single molecule embodies such a physiological-mimicking polypharmacy. The metabolic improvements elicited by the triagonist rival those induced by bariatric surgery, at least when compared to the published reports in rodent models, albeit without the consequential surgical risks and invasiveness of bariatric surgeries. Ultimately, the triagonist represents a sizable step forward beyond previous attempts of coagamism and reflects the growing notion that single-molecule polytherapies are emerging as the gold standard for obesity and diabetes medicines.  

It is conceivable that this newly discovered triagonist could be used to deepen and broaden the efficacy in targeting of nuclear hormones or find application in combination with other protein-based therapeutics as we search for that pharmacology that more closely replicates physiology in the pursuit of medicinal alternatives to bariatric surgery.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**ACKNOWLEDGMENTS**

We thank J. Levy for technical and chemical support of peptide synthesis. We thank J. Ford for cell culture maintenance. We thank J. Patterson, J. Day, B. Ward and C. Ouyang for discussions on chemical structure-activity relationships and seminal work in mixed agonist peptides. We thank J. Holland, J. Hembree, C. Raver, S. Amburg, J. Pressler, J. Sorrell, D. Küchler and L. Seherr for assistance during in vivo pharmacological studies. At F. Hoffmann-La Roche Ltd., we thank A. Roccek, A. Vandjour and E. Hainaut for assistance during in vivo pharmacological studies; M. Brecheisen, C. Richardson, G. Brandlee and V. Ott for necropsy and immunohistological procedures; C. Apfel, C. Wohlgesinger and V. Griesser for bioanalytics; and M. Kapps, C. Flament, P. Schrag, C. Rapp, M.S. Gruyer, V. Dall’Aven, F. Schuler and M. Otteneder for assistance in pharmacokinetic studies. We thank M. Charroin (Albert Einstein College of Medicine) for providing Gpr105−/− mice and Y. Seino (Kansai Electric Power Hospital) for providing Gpr105−/− mice. Partial research funding was provided by Marcadia Biotech, which has been acquired by F. Hoffmann-La Roche Ltd., and by grants from the Deutsche Forschungsgesellschaft (DFG; TS226/1-1), Deutsches Zentrum für Diabetesforschung (DZD), EuroCHIP (FP-7-HEALTH-2009-241592), Helmholtz Alliance Icemed—Imaging and Curing Environmental Metabolic Diseases (through the Initiative and Networking Fund of the Helmholtz Association) and the Canadian Institutes of Health Research (93749).

**AUTHOR CONTRIBUTIONS**

B.F. designed and performed in vitro, in vivo, and ex vivo rodent experiments, synthesized and characterized compounds, analyzed and interpreted data, and co-wrote the manuscript. B.Y. designed, synthesized and characterized compounds, performed in vitro experiments, and analyzed and interpreted data. N.O. designed and led in vivo pharmacology and metabolism rodent studies and interpreted data. D.P.T., P.T.P., K.M.H., J.E.C., D.S., R.J.S., C.C., D.J.D., E.S., A.K. and T.D.M. designed, supervised and performed in vivo experiments and interpreted data. L.Z. designed in vivo experiments and interpreted data. K.F. performed in vivo experiments. J.C. and D.L.S. designed, synthesized and characterized compounds. K.R. designed and synthesized compounds. S.U., W.R., C.H., E.S., K.K.-K. and A.K. designed and performed in vivo and ex vivo analyses in ZDF rats and interpreted data. J.F. performed liver histology and interpreted data. S.U. performed pancreas histology and interpreted data. C.H., A.K. and V.G. designed and performed in vitro experiments and interpreted data. S.B. led pharmacokinetic studies and interpreted data. R.D.D. and M.H.T. conceptualized, designed and interpreted all studies and wrote the manuscript together with B.F.

**COMPETING FINANCIAL INTERESTS**

The authors declare competing financial interests: details are available in the online version of the paper.

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.
ONLINE METHODS

Peptide synthesis. Peptides were synthesized by solid-phase peptide synthesis methods using in situ neutralization for both Boc-based and Fmoc-based chemistries. For Boc-based neutralization peptide synthesis, 0.2 mmol 4-methylbenzhydrylamine (MBHA) resin (Midwest Biotech) was used on a highly modified Applied Biosystems 430A peptide synthesizer by standard Boc methods using DEPBT/DIEA for coupling and TFA for deprotection of amino-terminal amines. Peptidyl resins were treated with hydrofluoric (HF) acid/p-cresol (10:0.5 vol/vol) at 0 °C for 1 h with agitation. HF was removed in vacuo and precipitated the cleaved and deprotected peptide in diethyl ether. For Fmoc-based neutralization peptide synthesis, 0.1 mmol Rink MBHA resin (Novabiochem) was used on an Applied Biosystems 433A peptide synthesizer by standard Fmoc methods using DIC/CH3 HOBT for coupling and 20% piperidine/dimethylformamide (DMF) for deprotection of N-terminal amines. Completed peptidyl resins were treated with TFA/TIS/anisole (9:0:5.0:5.0 vol/vol/v) for 2 h with agitation. After removal of the ether, the crude peptide was dissolved in aqueous buffer containing at least 20% acetonitrile (ACN) and 1% acetic acid (AcOH) before lyophilization. Peptide molecular weights were confirmed by electrospray ionization (ESI) or matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (summarized in Supplementary Table 1) and confirmed by analytical reversed-phase HPLC in 0.1% TFA with an ACN gradient on a Zorbax C8 or C18 column (0.46 × 5 cm).

Peptide purification. Following cleavage from the resin, crude extracts were purified by semi-preparative reversed-phase HPLC in 0.1% TFA with an ACN gradient on a Vydac C8 column (2.2 × 25 cm). Preparative fractions were analyzed for purity by analytical reversed-phase HPLC using the conditions listed above. Peptide molecular weights were confirmed by ESI or MALDI-TOF mass spectrometry. Purified peptides were lyophilized, aliquoted, and stored at 4 °C.

Cell lines. All cell lines used were confirmed to be mycoplasma free and tested on a monthly basis. Parent HEK-293 cells used for in vitro receptor activation were obtained from the ATCC. MIN6, 3T3-L1, and rat hepatocytes were obtained from internal stock at F. Hoffmann-La Roche. None of the cell lines were authenticated in house by short tandem repeat (STR) DNA profiling.

Human GLP-1, GIP, and glucagon receptor activation. Each peptide was individually tested for its ability to activate the GLP-1, GIP, or glucagon receptor through a cell-based luciferase reporter gene assay that indirectly measures cAMP induction. Human embryonic kidney (HEK293) cells were co-transfected with each individual receptor cDNA (zeocin-selection) and a luciferase reporter gene construct fused to a cAMP response element (CRE) (hygromycin B-selection). Cells were seeded at a density of 22,000 cells per well and serum deprived for 16 h in DMEM (HyClone) supplemented with 0.25% (vol/vol) bovine growth serum (BGS) (HyClone). Serial dilutions of the peptides were added to 96-well cell-culture treated plates (BD Biosciences) containing the serum-deprived, co-transfected HEK293 cells, and incubated for 5 h at 37 °C and 5% CO2 in a humidified environment. To stop the incubation, an equivalent volume of Steady Lite HTS luminescence substrate reagent (PerkinElmer) was added to the cells to induce lysis and expose the lysates to luciferin. The cells were agitated for 5 min and stored for 10 min in the dark. Luminescence was measured on a MicroBeta-1450 liquid scintillation counter (PerkinElmer). Luminescence data were graphed against concentration of peptide and EC50 values were calculated using Origin software (OriginLab).

Mouse, rat, and cynomolgus monkey receptor activation. CHO-K1 cells were transiently transfected with expression plasmids constructed from pcDNA3.1 plasmids (Life Technologies) containing the native sequence for each individual receptor originating from mouse, rat, and cynomolgus monkey, which were based on reference database sequences, as well as an artificial Kozak sequence (GCCGCCATCATG) to further aid expression. CHO-K1 cells were cultured in Ham’s F-12 media (GIBCO) supplemented with 10% FBS, 2 mM glutathione, 500 μg/ml Geneticin, 100 μg/ml penicillin and 100 μg/ml streptomycin. cAMP production was assessed following the method below.

CAMP assay. Mouse insulinoma MIN6 cells were cultured in RPMI 1640 medium supplemented with 10% FBS (PAA Laboratories), 1 mM HEPES, 1 mM sodium pyruvate, 50 μM 2-β-mercaptoethanol, 100 μg/ml penicillin and 100 μg/ml streptomycin and were grown at 37 °C and 5% CO2 in a humidified incubator. Rat hepatocytes were thawed from cryopreserved stock immediately before the experiment. Mouse 3T3-L1 cells were grown in DMEM/10% FBS and 1% penicillin/streptomycin in a humidified incubator with 5% CO2 at 37 °C. Confluent 3T3-L1 cells were initiated to differentiate with DMEM containing 20% FBS, 0.5 mM IBMX, 0.4 μg/ml dexamethasone and 5 μg/ml insulin. After 5 days, media was replaced with DMEM containing 20% FBS, 4 μg/ml insulin and 10 μM rosiglitazone. After 3 days, media was replaced with DMEM containing 10% FBS. Cells were used 10 days after initiation of differentiation.

For CAMP production assessment, the medium was removed and the cells were washed with PBS. For MIN6 cells, cell monolayers were incubated for 5 min at 37 °C with 5 μl Cell Dissociation solution (Gibco) to dislodge the cells. For suspended MIN6 cells and fresh rat hepatocytes, cells were transferred to a 96-well plate at a seeding density of 5000 cells/well. For 3T3-L1 cells, cells were seeded directly into 96-well plates and differentiated. The cell suspension (MIN6 and hepatocytes) or the cell monolayer (3T3-L1) was then incubated for 30 min at room temperature with the peptides. The reaction was stopped by addition of lysis buffer and cAMP generated was determined using the cAMP Dynamic 2 Kit (Cisbio) following the manufacturer’s instructions. The time-resolved fluorescence signal was determined using an EnVision (PerkinElmer), and cAMP production was calculated based on standard curve run in parallel for every experiment. Data were analyzed using GraphPad Prism.

CEREP assay for non-selective receptor binding. The cross-reactive binding of triagonist was profiled in a high-throughput custom-made competitive binding screen at 73 targets, including GLP-1R and GcgR as positive controls (Cerep). The exact specifications of each individual assay are detailed in Supplementary Table 3, which includes receptor source, labeled and unlabeled ligands, and incubation conditions. The specific ligand binding to the respective receptors is defined as the difference between the total binding and the non-specific binding determined in the presence of an excess of a respective native, unlabeled ligand. Results are expressed as percentage specific binding, calculated by [(triagonist specific binding / control specific binding) × 100], and also as percentage inhibition of control, calculated by [100 – (triagonist specific binding / control specific binding) × 100]. In general, results showing a percentage inhibition of control that is greater than 25% is considered a positive result of specific receptor binding, and values less than 25% are considered a negative result of specific receptor binding.

General experimental approaches for in vivo pharmacology experiments. For in vivo pharmacological studies, a group size of eight was preferentially used, which was determined from previous experiments where we determined this to be optimal for in vivo evaluation. Smaller group sizes were used in the studies utilizing genetically modified animals because there were not sufficient numbers available to reach a group size of eight. For studies using lean and obese mice, mice were randomized into the treatment groups based upon body weight and body fat/lean mass and levels of blood glucose. Rodents were single- or group-housed on a 12-h/12-h light-dark cycle at 22 °C with free access to food and water. Group size estimations were based upon a power calculation to minimally yield an 80% chance to detect a significant difference in body weight of P < 0.05 between the treatment groups. For studies using diabetic rodents, rodents were randomized based on body weight, body fat/lean mass, and levels of blood glucose. The investigators were not blinded during group allocation or during in vivo profiling of compounds. No samples or animals were excluded from the longitudinal in vivo pharmacological profiling, challenge tests, or port mortem analysis. The age and strain of the mice are indicated below and in the figure legends.

DIO mice. With all DIO mice, male C57BL/6 mice (Jackson Laboratories) were fed a diabetogenic diet (Research Diets), which is a high-sucrose diet with 58% kcal from fat, 25.5% kcal from carbohydrates, and 16.4% kcal from protein, beginning at 8 weeks or 2 months of age. DIO mice were single- or group-housed on a 12-h/12-h light-dark cycle at 22 °C with free access to food and water.
Mice were maintained under these conditions for a minimum of 16 weeks before initiation of pharmacological studies and were between the ages of 6 months and 18 months old. All injections and tests were performed during the light cycle. Mice were randomized and evenly distributed to test groups according to body weight and body composition. No animals were excluded due to illness or outlier results; therefore, no exclusion determination was required. If ex vivo molecular biology/histology/biochemistry analyses were performed, the entire group of mice for each treatment was analyzed.

**Glp1r−/− mice.** Male Glp1r−/− mice and wild-type littermates (C57BL/6 background) were bred in house and fed the aforementioned diabetogenic diet for 12 weeks before initiation of injections. All tests were performed on mice aged 8 months and injections were administered during the light cycle. Mice were single- or group-housed on a 12-h/12-h light-dark cycle at 22 °C with free access to food and water.

**Gcgr−/− mice.** Male Gcgr−/− mice and wild-type littermates (C57BL/6 background) were bred in house and, starting at 12 weeks of age, were fed the aforementioned diabetogenic diet for 6 weeks before initiation of injections. All tests were performed on mice aged 5 months and injections were administered during the light cycle. Mice were single- or group-housed on a 12-h/12-h light-dark cycle at 22 °C with free access to food and water.

**Gipr−/− mice.** Male Gipr−/− mice and wild-type littermates (C57BL/6 background) were bred in house and, starting at 12 weeks of age, were fed the aforementioned diabetogenic diet for 6 weeks before initiation of injections. All tests were performed on mice aged 5 months and injections were administered during the light cycle. Mice were single- or group-housed on a 12-h/12-h light-dark cycle at 22 °C with free access to food and water.

**db/db mice.** Six-week-old male db/db mice (Jackson Laboratories; C57BL/6 background) were housed 4 per cage and provided access to standard chow diet and water ad libitum. Mice were 9 weeks old when used for the indicated studies. Mice were randomized by ad libitum–fed blood glucose and body weight, and were double-housed for the study. All injections and tests were performed during the light cycle.

**STZ mice.** Thirteen-month-old male C57BL/6 mice (Jackson Laboratories) were housed 4 per cage and provided access to standard chow diet and water ad libitum. Streptozotocin was administered at a dose of 150 mg per kg body weight via a single intraperitoneal injection. After 3 d, mice with a blood glucose level exceeding 200 mg/dl were considered sufficiently diabetic and were treated with long-acting insulin (made in house) via daily subcutaneous injections for 4 weeks to maintain euglycemia. Mice were used for the glucagon challenge test after a 72-h washout period of the insulin and were randomized based on ad libitum–fed blood glucose and body weight. For the challenge test, mice were subjected to 6 h of fasting at the onset of the light cycle and injected intraperitoneally with GgcR antagonist 15 min before administration of the triagonist. Tail blood glucose concentrations were measured using a handheld glucometer (TheraSense FreeStyle) before antagonist administration (~15 min), before triagonist administration (0 min), and at 15, 30, 60, and 120 min after injection of the triagonist. All injections and tests were performed during the light cycle.

**DIO rats.** Twelve-week-old male Long Evens rats were fed a diabetogenic diet (Research Diets), which is a high-sucrose diet with 58% kcal from fat, for 40 weeks before initiating the long-term study. The DIO rats were single-housed on a 12:12-h light-dark cycle at 22 °C with free access to food and water. Rats were randomized and distributed to test groups according to body weight and body composition. All injections and tests were performed during the light cycle.

**ZDF rats.** Nine-week-old male ZDF rats (Charles River Laboratories, USA) were fed a special diet (Purina PMI 5008) and housed 1 per cage at room temperature (−21 °C) and relative humidity 55–65%. A 12-h light-dark cycle was maintained in the rooms with all tests being performed during the light phase. Access to food and water was ad libitum. After 2 weeks of acclimatization, measurement of fasting blood glucose concentrations for randomization was performed by tail puncture in conscious animals. ZDF rats were distributed into groups (n = 8/group) according to body weight and fasting glucose concentrations. Immediately after treatment cessation, n = 4 rats per group were blindly selected for ex vivo analysis. The remaining n = 4 rats were monitored for 21 additional days after treatment termination and were subsequently killed for ex vivo analysis.

**Ethical approval.** All rodent studies were approved by and performed according to the guidelines of the Institutional Animal Care and Use Committee of the Helmholtz Center Munich, University of Cincinnati, and under an animal research protocol (ARP) authorization to F. Hoffmann-La Roche by the Swiss Cantonal Veterinary Office Basel-Stadt and in accordance with guidelines of the Association for the Assessment and Accreditation of Laboratory and Animal Care (AAALAC unit no. 001057) and appropriate federal, state and local guidelines.

**Rodent pharmacological and metabolism studies.** Compounds were administered by repeated subcutaneous injections in the middle of the light phase at the indicated doses with the indicated durations. Co-administration of compounds was administered by single formulated injections. Body weights and food intake were measured every day or every other day after the first injection. All studies in wild-type mice were performed with a group size of n = 8 per group using mice on a C57BL6 background. Fasted blood glucose was measured upon study initiation and termination following 6 h of fasting. For assessment of glucose and insulin tolerance during chronic treatment, the challenge tests were performed at least 24 h after the last administration of compounds. The investigators were not blinded to group allocation during the in vivo experiments or to the assessment of experimental end points.

**Body composition measurements.** Whole-body composition (fat and lean mass) was measured using magnetic resonance technology (EchoMRI).

**Energy balance physiology measurements.** Energy intake, energy expenditure, and home-cage activity were assessed using a combined indirect calorimetry system (TSE Systems). O₂ consumption and CO₂ production were measured every 10 min for a total of 120 h (including 48 h of adaptation) to determine the respiratory quotient and energy expenditure after an initial treatment regimen for 2 weeks. Food intake was determined continuously for 120 h at the same time as the indirect calorimetry assessments by integration of scales into the sealed cage environment. Home-cage locomotor activity was determined using a multidimensional infrared light beam system with beams scanning the bottom and top levels of the cage, and activity being expressed as beam breaks.

**Blood parameters.** Blood was collected after a 6-h fast from tail veins or after euthanasia using EDTA-coated microvette tubes (Sarstedt), immediately chilled on ice, centrifuged at 5,000g and 4 °C, and plasma stored at −80 °C. Plasma insulin was quantified by a radioimmunoassay from Linco (Sensitive Rat Insulin RIA; Linco Research) or by an ELISA assay (Alpco). Plasma FGF21, adiponectin, GIP, GLP-1, and glucagon were quantified by an ELISA assay (Millipore). Plasma cholesterol, triglycerides, ALT, and AST were measured using enzymatic assay kits (Thermo Fisher). Plasma free fatty acids and ketones were measured using enzymatic assay kits (Wako). All assays were performed according to the manufacturers’ instructions.

**Acute glucose tolerance test with GLP-1R antagonist.** For the determination of the effects of the triagonist on intraperitoneal glucose tolerance with concomitant antagonism of the GLP-1R, 6-h fasted male C57BL/6/DIO mice (n = 8 per group; age 9 months) were pretreated with vehicle or the GLP-1R antagonist (1 μmol per kg body weight) via an intraperitoneal injection 30 min before the intraperitoneal glucose challenge (2 g of glucose per kg body weight). Vehicle or the triagonist (1 mmol per kg body weight) was administered 15 min before the glucose challenge via intraperitoneal injections. Tail blood glucose concentrations were measured using a handheld glucometer (TheraSense FreeStyle) before subsequent injections at ~30, −15, 0, 15, 30, 60, and 120 min after the glucose administration.

---

NATURE MEDICINE
doi:10.1038/nm.3761
Acute glucose tolerance test with GIPR antagonist. For the determination of the effects of the triagonist on intraperitoneal glucose tolerance with concomitant antagonism of the GIPR, 6-h fasted male Glp1r−/− DIO mice (n = 8 per group; age 12 months) were pretreated with vehicle or the GIPR antagonist (2 µmol per kg body weight) via an intraperitoneal injection 30 min before the intraperitoneal glucose challenge (1.5 g of glucose per kg body weight). Vehicle or the triagonist (2 nmol per kg body weight) was administered 15 min before the glucose challenge via intraperitoneal injections. Tail blood glucose concentrations were measured by using a handheld glucometer (TheraSense FreeStyle) before subsequent injections at −30, −15, 0, 15, 30, 60, and 120 min after the glucose administration.

Acute glucose tolerance test with GcgR antagonist. For the determination of the effects of the triagonist on glycemia with concomitant antagonism of the GcgR, 6-h fasted male STZ C57BL/6 lean mice (n = 8 per group; age 13 months) were pretreated with vehicle or the GcgR antagonist (1 µmol per kg body weight) via an intraperitoneal injection 15 min before the intraperitoneal injection with vehicle or the triagonist (1 nmol per kg body weight). Tail blood glucose concentrations were measured by using a handheld glucometer (TheraSense FreeStyle) before subsequent injections at −15, 0, 15, 30, 60, and 120 min after the glucose administration.

Glucose tolerance test. For the determination of glucose tolerance, mice were subjected to 6 h of fasting at the onset of the light cycle and injected intraperitoneally with 1.5 g glucose per kg body weight for DIO mice or 2 g glucose per kg body weight for lean mice (20% w/v d-glucose (Sigma) in 0.9% w/v saline). For acute glucose tolerance tests using antagonists, the antagonists, at a 1000-fold excess dose of the triagonist, were administered 15 min before agonist administration and 30 min before the glucose challenge. For db/db mice, mice were subjected to 6 h of fasting at the onset of the light cycle and injected intraperitoneally with 1 g glucose per kg body weight. Tail blood glucose concentrations were measured using a handheld glucometer (TheraSense FreeStyle) before (0 min) and at 15, 30, 60, and 120 min after injection. For the acute effects of the triagonist with or without respective antagonists, blood glucose concentrations were measured before administration of the antagonist (−30 min) and the triagonist (−15 min) in addition to the measurement times listed above. For ZDF rats, oral glucose tolerance was assessed in 8 rats per group after an overnight fasting period (16 h) and blood glucose was measured before oral glucose challenge (2 g kg−1), and subsequently at 15, 30, 60 and 120 min post-glucose challenge. Blood glucose was monitored using the AccuCheck glucometer system.

Insulin tolerance test. For the determination of insulin tolerance, mice were subjected to 6 h of fasting at the onset of the light cycle and injected intraperitoneally with 0.75 units of insulin per kg body weight. Tail blood glucose concentrations were measured by using a handheld glucometer (TheraSense FreeStyle) before (0 min) and at 15, 30, 60, and 120 min after injection.

Histopathology and immunohistochemistry. The methodology has been described. In brief, tissue samples were fixed in 10% neutral-buffered formalin for 24 h, dehydrated and subsequently embedded into paraffin. Standard hematoxylin and eosin staining was performed to assess liver histology. The following immunofluorescence stainings on tissue sections of 4 µm were carried out to assess islet morphology: anti-insulin (1:50; Dako; A0564), anti-glucagon (1:50; Dako; A0565) and 4′, 6-diamidino-2-phenylindole (DAPI; 1:1,000; Roche; 10236276001), followed by respective secondary fluorescent antibodies (Alexa Fluor). Digital imaging fluorescence microscopy of the pancreas was performed using a scanning platform (MetaSystems) with a Zeiss Imager Z.2 microscope (Carl Zeiss MicroImaging, Inc.). Quantitative image analysis of islet morphology was performed using Definiens Architect XD (Definiens AG). Investigators were not blinded during analysis.

Triagonist pharmacokinetic studies. All pharmacokinetic studies were conducted with the approval of the local veterinary authority in strict adherence to the Swiss federal regulations on animal protection and to the rules of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Studies with rodents and nonhuman primates were conducted according to Roche animal permissions for performing PK studies at Roche Basel. Adult male C57BL/6 mice weighing approximately 30 g each (Harlan Laboratories), adult male DIO mice weighing approximately 60 g each (Charles River), and adult male Wistar rats weighing approximately 250 g each (Harlan Laboratories) were housed in a controlled environment (temperature, humidity, and 12 h light/dark cycle) with free access to food and water. Six adult male cynomolgus monkeys weighing approximately 10 kg each (Roche monkey PK colony) were routinely employed for pharmacokinetic studies. Nonrandomized rodents were administered with the compound as subcutaneous injection in the neck (45 µg kg−1) and nonrandomized monkeys in the flank (15 µg kg−1). Blood from mice and rats was collected under anesthesia (5% isoflurane inhalation in pure oxygen), while no anesthesia was needed for the monkeys. Blood samples from mice (n = 2 samples per time point), rats (n = 3 samples per time point), and monkeys (n = 3 samples per time point) were collected up to 24 h postdose by heart puncture, sublingually, or from the brachial vein, respectively, and placed on ice into EDTA-coated polypropylene tubes. Plasma was prepared from blood within 30 min by centrifugation at 3000g for 5 min at 4 °C and frozen immediately. All samples were stored at −20 °C. Concentration data in plasma were determined by LC-MS/MS and respective pharmacokinetic parameters were determined by Non-Compartmental Analysis (NCA) with the industry standard software Phoenix WinNonlin (Build 6.2.0.495, Pharsight). Researchers were not blinded during the investigation.

GLP-1/GIP coagonist pharmacokinetic studies. All procedures in this protocol are in compliance with the US Department of Agriculture’s (USDA) Animal Welfare Act (9 CFR Parts 1, 2, and 3); the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Academy Press, Washington, D.C., 1996; and the National Institutes of Health, Office of Laboratory Animal Welfare. Studies with rodents and nonhuman primates were conducted according to Roche animal permissions for performing PK studies at Roche Basel. Whenever possible, procedures in this study are designed to avoid or minimize discomfort, distress, and pain to animals. Three adult cynomolgus monkeys were arbitrarily chosen from an in house colony and individually housed in a controlled environment on a 12-h light/dark cycle. Whole blood was collected via the femoral vein/artery at 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h, 48 h, 72 h, and 96 h after compound administration via a subcutaneous injection in the flank. Unaudited plasma and tissue pharmacokinetic analysis data were analyzed using WinNonlin, version 5.2.1 software (Pharsight, Inc.) and Microsoft Office Excel 2003. Use of Excel was limited to receipt of bioanalytical data and transfer into WinNonlin for pharmacokinetic analysis. Researchers were not blinded during the investigation.

Statistical analyses. Statistical analyses were performed on data distributed in a normal pattern using a regular one-way or two-way analysis of variance (ANOVA) with Tukey post hoc multiple comparison analysis to determine statistical significance between treatment groups. Differences with P values less than 0.05 were considered significant. Group size estimations were based upon a power calculation to minimally yield an 80% chance to detect a significant difference in body weight of P < 0.05 between the treatment groups.