Glucagon receptor antagonist and GIP agonist combination for diet-induced obese mice

L M McShane¹, N Irwin¹, D O’Flynn², Z J Franklin¹, C M Hewage² and F P M O’Harte¹

¹SAAD Centre for Pharmacy and Diabetes, University of Ulster, Coleraine, Northern Ireland, UK
²Conway Institute of Biomolecular and Biomedical Research, UCD, Belfield, Dublin 4, Ireland
(L M McShane is now at School of Life Sciences, Faculty of Health and Life Sciences, University of Liverpool, Liverpool, UK)
(Z J Franklin is now at Diabetes Research Group, Division of Diabetes and Nutritional Sciences, King’s College London, London, UK)

Abstract

Ablation of glucagon receptor signaling represents a potential treatment option for type 2 diabetes (T2DM). Additionally, activation of glucose-dependent insulino-tropic polypeptide (GIP) receptor signaling also holds therapeutic promise for T2DM. Therefore, this study examined both independent and combined metabolic actions of desHis¹Pro⁴Glu⁹(Lys¹²PAL)-glucagon (glucagon receptor antagonist) and d-Ala²GIP (GIP receptor agonist) in diet-induced obese mice. Glucagon receptor binding has been linked to alpha-helical structure and desHis¹Pro⁴Glu⁹(Lys¹²PAL)-glucagon displayed enhanced alpha-helical content compared with native glucagon. In clonal pancreatic BRIN-BD11 beta-cells, desHis¹Pro⁴Glu⁹(Lys¹²PAL)-glucagon was devoid of any insulinotropic or cAMP-generating actions, and did not impede d-Ala²GIP-mediated (P<0.01 to P<0.001) effects on insulin and cAMP production. Twice-daily injection of desHis¹Pro⁴Glu⁹(Lys¹²PAL)-glucagon or d-Ala²GIP alone, and in combination, in high-fat-fed obese mice failed to affect body weight or energy intake. Circulating blood glucose levels were significantly (P<0.05 to P<0.01) decreased by all treatments regimens, with plasma and pancreatic insulin elevated (P<0.05 to P<0.001) in all mice receiving d-Ala²GIP. Interestingly, plasma glucagon concentrations were decreased (P<0.05) by sustained glucagon inhibition (day 28), but increased (P<0.05) by d-Ala²GIP therapy, with a combined treatment resulting in glucagon concentration similar to saline controls. All treatments improved (P<0.01) intraperitoneal and oral glucose tolerance, and peripheral insulin sensitivity. d-Ala²GIP-treated mice showed increased glucose-induced insulin secretion in response to intraperitoneal and oral glucose. Metabolic rate and ambulatory locomotor activity were increased (P<0.05 to P<0.001) in all desHis¹Pro⁴Glu⁹(Lys¹²PAL)-glucagon-treated mice. These studies highlight the potential of glucagon receptor inhibition alone, and in combination with GIP receptor activation, for T2DM treatment.

Key Words
- glucagon
- glucose-dependent insulino-tropic polypeptide (GIP)
- glucose homeostasis
- insulin secretion
- insulin sensitivity
- glucagon receptor antagonist
- GIP receptor agonist
- diet-induced obese diabetic mice
Introduction

Through advances in our understanding of the pathways involved in glucose homeostasis, and an appreciation that type 2 diabetes (T2DM) is a bihormonal disorder, it is clear that abnormalities of insulin secretion and action in T2DM are present in the setting of glucagon excess (Unger & Cherrington 2012). Thus, improved control of glucagon signaling represents a rational therapeutic target for T2DM. In agreement with this, early proof-of-concept studies using the orally available glucagon receptor antagonist, Bay 27-9955, have shown initial promise in humans (Petersen & Sullivan 2001). Additionally, more recent clinical trials with similar orally available glucagon receptor inhibitors, MK-0893 and LY-2409021, reveal further potential for the treatment of T2DM (Xiong et al. 2012, Kelly et al. 2015). A separate, but somewhat comparable approach, to reduce glucagon receptor expression through the use of antisense oligonucleotides, has also reached Phase 2 clinical trials (Sehgal et al. 2013). However, the ultimate approval of these types of low-molecular-weight therapies will depend upon specificity and off-target effects, toxicity, and potential for immune responses (Peng et al. 2014, Kelly et al. 2015, Lefèbvre et al. 2015).

Therefore, we have recently characterized the novel peptide-based glucagon receptor antagonist, desHis1Pro4Glu9(Lys12PAL)-glucagon (O’Harte et al. 2014), that represent a more specific approach to inhibit glucagon receptor action. Indeed, this peptide analog induced significant improvements in metabolic control following a chronic dosing regimen in diet-induced obese (DIO) mice as well as ob/ob diabetic mice (O’Harte et al. 2014). Importantly, we did not observe any evidence of adverse effects, and further studies in normal mice indicate that this peptide-based glucagon receptor antagonist represents a safe and effective treatment option for T2DM (Franklin et al. 2014). Interestingly, Mu and coworkers reported that coadministration of the glucagon antagonist, Cpd-A, with a dipeptidylpeptidase-4 (DPP-4) inhibitor in diabetic mice resulted in additional improvements in glycemic control when compared with either treatment alone (Mu et al. 2011). It follows that a combined therapy of a glucagon receptor antagonist with an incretin-based drug could offer an advantageous approach for the treatment of T2DM.

The incretin hormones, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), are recognized to account for approximately 50–70% of insulin secretion following a meal (Nauck et al. 1986). However, this incretin contribution to postprandial insulin release falls to less than 20% in T2DM (Nauck et al. 1986). The reduction is attributable to decreased GLP-1 release (Vilsbøll et al. 2001) and resistance to the insulinotropic actions of GIP in T2DM (Nauck et al. 1993). Accordingly, enzymatically stable GLP-1 mimetics that enhance circulating physiological levels of GLP-1 have gained notable success in the T2DM clinic (Gupta 2013, Chaplin & Joseph 2014), whereas GIP mimetics are yet to reach the clinic due to insensitivity in T2DM patients (Nauck et al. 1993). More encouragingly, GIP resistance in T2DM appears to be reversible in both animals and human through tight glycemic control or combinational drug therapy (Meneilly et al. 2003, Piteau et al. 2007, Højberg et al. 2009). In addition to this, there is a suggestion that GIP, unlike GLP-1, can promote glucagon release (Meier & Nauck 2004, 2015), which would further detract from its therapeutic efficacy in T2DM. Thus, coadministration of a specific glucagon receptor antagonist (O’Harte et al. 2014), with a stable long-acting GIP mimetic, such as d-Ala2GIP (Hinke et al. 2002, Gault et al. 2003), should offer a meaningful therapeutic advantage.

To evaluate the potential of combined glucagon receptor inhibition and GIP receptor activation in T2DM, we have investigated the effects of subchronic treatment with the peptide-based glucagon receptor antagonist, desHis1Pro4Glu9(Lys12PAL)-glucagon, and d-Ala2GIP in DIO mice fed a high-fat diet. The results provide experimental evidence that GIP mimetics may prove to be surprisingly useful for the treatment of T2DM when combined with a glucagon receptor antagonist.

Materials and methods

Peptide synthesis

Glucagon, d-Ala2GIP, and desHis1Pro4Glu9(Lys12PAL)-glucagon were produced (> 95% purity) by Fmoc solid-phase peptide synthesis and purchased from GL Biochem Ltd (Shanghai, China). All peptides were further characterized in-house using matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry, as described previously (O’Harte et al. 2013).

Circular dichroism (CD)

CD spectra for glucagon and desHis1Pro4Glu9(Lys12PAL)-glucagon were acquired at the far-UV region (190–250 nm) using a JASCO J-810 spectropolarimeter.
Peptide samples were prepared by dissolving the analogs in water or 20 mmol/L phosphate buffer at pH 7.0 to a final concentration of 30 μM, and the concentrations of trifluoroethanol (TFE) used was 15, 30, 50, and 70% for each peptide. Parameters used for CD experiments were response time of 2 s, bandwidth of 1 nm, scanning speed of 50 nm/min, and a data pitch of 0.2 nm. All spectra were acquired at 25°C by accumulation of 15 scans in a 1 mm quartz cell, and the baseline corrected. Calculation of alpha-helical and beta-sheet content was carried out by the K2D3 program using the DICHROWEB web interface (Louis-Jeune et al. 2012).

Acute in vitro insulin release and cAMP measurements

BRIN-BD11 cells were cultured in RPMI-1640 culture medium containing 10% v/v FBS and 11.1 mmol/L glucose, and were seeded at a density of 1 × 10^5 cells/well in 24-well plates for insulin release studies or 8 × 10^4 cells/well in 96-well plates for cAMP studies. Cells were allowed to attach overnight at 37°C in a LEEC incubator (Laboratory Technical Engineering, Nottingham, UK) in an atmosphere of 5% CO₂ and 95% air. Before insulin and cAMP studies, the tissue culture medium was removed and cells were preincubated with 1 mL KRB buffer (pH 7.4) supplemented with bovine serum albumin (0.5% w/v), containing 1.1 mmol/L glucose, at 37°C for 40 min. Test incubations were conducted at 5.6 mmol/L glucose over a 20 min incubation period, using individual and combined peptide treatments as shown in the figures. For insulin release, supernatants were removed and frozen at −20°C before measurement of insulin by radioimmunoassay (Flatt & Bailey 1981). For cAMP measurements, cells were lysed and total cAMP content was determined using a commercially available chemiluminescent cAMP immunoassay kit (R&D Systems).

Animals

NIH Swiss male mice (Harlan Ltd, Oxon, UK) were used at 18 weeks of age. The animals were housed individually in an air-conditioned room at 22 ± 2°C with a 12 h light:12 h darkness cycle (lights off between 20:00 and 08:00 h). All animals had free access to drinking water and a high-fat diet (45% fat, 35% carbohydrate, and 20% protein, Dietex International Ltd, Witham, Essex, UK) for 100 days before the commencement of studies. Obesity and glycemic dysregulation were clearly manifested compared with age-matched mice maintained on normal laboratory chow (10% fat, 30% protein, and 60% carbohydrate, Trouw Nutrition, Cheshire, UK) as verified by body weight and blood glucose analyses. All experiments were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986, under project licences approved by the local Ethical Committee.

Study design

Mice received twice-daily intraperitoneal (i.p.) injections of saline (0.9% NaCl w/v) at 10:00 and 16:30 h over a 6-day acclimatization period. Following this, mice received twice-daily i.p. administration (10:00 and 16:30 h) of saline vehicle (0.9% NaCl w/v) or desHis^1Pro^4Glu^9(Lys^12PAL)-glucagon alone, d-Ala^2GIP alone, or a combination of both peptides (all treatments at 25 nmol/kg body weight) over a 28-day period. Doses were chosen based on our previous extensive in vivo assessments with glucagon antagonist and GIP agonist peptides (Martin et al. 2013, O’Harte et al. 2014). Food intake was monitored daily, whereas body weight, circulating blood glucose, and plasma insulin concentrations were assessed at 3–4-day intervals in non-fasted mice at 09:00 h before the normal morning 10:00 h peptide administration. At the end of the treatment period, oral and i.p. (18 mmol/kg bw) glucose tolerance tests were performed in overnight-fasted mice. In addition, an insulin sensitivity (10 U/kg bw) test was also performed in non-fasted mice. At termination, pancreatic tissue was excised and insulin content was measured following extraction with 5 mL/g of ice-cold acid ethanol (75% ethanol, 23.5% water, 1.5% concentrated HCl).

Measurement of metabolic rate and locomotor activity

Metabolic rate and locomotor activity were measured using an Oxymax Complex Laboratory Animal Monitoring System or CLAMS (Columbus Instruments, OH, USA) on day 28. Mice were acclimatized to the air-tight metabolic chambers for 18 h before the commencement of observations. Oxygen consumption and carbon dioxide production were monitored for 30 s at 15 min intervals over a period of 24 h, and respiratory exchange ratios (RER’s) were produced to calculate energy expenditure using the following equation: (3.815 + 1.232 × RER) × VO₂. Ambulatory locomotor activity
was assessed using the optical beams (Opto M3, Columbus Instruments, OH, USA). Consecutive photo-beam breaks were scored as an ambulatory movement. Activity counts in X- and Z-axes were recorded each minute for 24 h.

Biochemical analyses

Blood samples were collected from the cut tip on the tail vein of conscious mice into chilled fluoride/heparin glucose microcentrifuge tubes (Sarstedt, Numbrecht, Germany) at the time points indicated in the figures. Blood glucose was measured directly using a handheld Ascencia Contour blood glucose meter (Bayer). For plasma insulin analysis, blood samples were immediately centrifuged using a Beckman microcentrifuge (Beckman Instruments, Galway, Ireland) for 1 min at 13,000 g and stored at −20°C. Plasma and pancreatic insulin was assayed by a modified dextran-coated charcoal RIA (Flatt & Bailey 1981). In addition, a terminal blood sample was also collected for the measurement of plasma glucagon via a sandwich immunoassay using a commercially available kit (Meso Scale Discovery, Gaithersburg, MD, USA).

Statistical analyses

Results are expressed as mean ± s.e.m. and data compared using ANOVA, followed by a Student–Newman–Keuls post hoc test. Incremental areas under plasma glucose and insulin curves (AUC) were calculated using a computer-generated program (Prism 5, San Diego, CA, USA).

Figure 1

Circular dichrosim spectra of (A) glucagon and (B) desHis1Pro4Glu9(Lys12PAL)-glucagon. Spectra were observed in the far-UV region under different concentrations of TFE, as indicated in the figure.

Figure 2

Insulinotropic- and cAMP-generating effects of desHis1Pro4Glu9(Lys12PAL)-glucagon and ω-AlaωGIP. (A) BRIN-BD11 cells were exposed to varying concentrations (10−12 to 10−6 M) of desHis1Pro4Glu9(Lys12PAL)-glucagon, ω-AlaωGIP, and ω-AlaωGIP in the presence of 10−7 M desHis1Pro4Glu9(Lys12PAL)-glucagon for 20 min at 5.6 mmol glucose.

Figure 3

Consecutive photo-beam breaks were scored as an ambulatory movement.
Glucagon antagonism and GIP receptor activation

Effects of desHis1Pro4Glu9(Lys12PAL)-glucagon and d-Ala2GIP on acute insulin secretion and cAMP production in BRIN-BD11 cells

The acylated glucagon receptor antagonist, desHis1Pro4Glu9(Lys12PAL)-glucagon, had no significant stimulatory effect on either insulin secretion (Fig. 2A) or cAMP production (Fig. 2B) in BRIN-BD11 cells. However, the stable GIP agonist, d-Ala2GIP, induced a significant (P < 0.01 to P < 0.001) concentration-dependant (10–8 to 10 –6 M) increase in insulin secretion when compared with a 5.6 mmol/L glucose control (Fig. 2A). Coincubation of desHis1Pro4Glu9(Lys12PAL)-glucagon with d-Ala2GIP had no effect on d-Ala2GIP-mediated insulin release (Fig. 2A). Furthermore, desHis1Pro4Glu9(Lys12PAL)-glucagon (10 –7 M) had no inhibitory effect on d-Ala2GIP-induced (P < 0.01) cAMP production (Fig. 2B).

Circular dichroism analysis of peptides

In aqueous conditions, all peptides had an overall random structure. Upon addition of TFE, two negative dichroic bands at 208 and 222 nm were observed indicating the formation of alpha-helical conformations within the peptide analogs. The alpha-helical content of glucagon was calculated at 26–31% at high TFE concentrations (Fig. 1A). As the concentration of TFE increased, desHis1Pro4Glu9(Lys12PAL)-glucagon revealed an overall trend of increasing alpha-helical concentration with decreased beta-sheet content (Fig. 1B). In comparison to native glucagon (Fig. 1A), desHis1Pro4Glu9(Lys12PAL)-glucagon had increased alpha-helical content at 15–70% TFE concentrations (Fig. 1B).

Results

Circular dichroism analysis of peptides

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Employing the trapezoidal rule with baseline subtraction. P<0.05 was considered to be significantly different.
Effects of 28-days administration of desHis1ProGlu(Lys12PAL)-glucagon and d-Ala2GIP on metabolic parameters in high-fat-diet-induced obese mice

Twice-daily treatment with desHis1ProGlu(Lys12PAL)-glucagon or d-Ala2GIP alone, and in combination, for 28 days had no significant effect on body weight (Fig. 3A) or food intake (Fig. 3C). However, total body fat mass was significantly (P<0.01 to P<0.001) reduced in all treatment groups (Fig. 3B), specifically saline control-, desHis1ProGlu(Lys12PAL)-glucagon-, and d-Ala2GIP-treated high-fat mice had body fat masses of 40.3±0.6%, 33.8±0.8%, and 37.7±0.5%, respectively, compared with a body fat mass of 37.8±0.5% in lean control mice. In addition, d-Ala2GIP-treated mice and those given the combination of both peptides had increased (P<0.01) body fat percentage compared with desHis1ProGlu(Lys12PAL)-glucagon treatment alone (Fig. 3B). Furthermore, a significant (P<0.05 to P<0.001) decrease in circulating blood glucose was observed in all three treatment groups from day 10 onward when compared with saline controls (Fig. 3D). In addition, d-Ala2GIP induced a highly significant (P<0.05 to P<0.001) increase in circulating insulin on day 28 compared with all other groups (Fig. 3E), whereas desHis1ProGlu(Lys12PAL)-glucagon monotherapy had no effect on plasma insulin levels (Fig. 3E). d-Ala2GIP-mediated elevations in plasma insulin were partially restrained by a combined desHis1ProGlu(Lys12PAL)-glucagon therapy, although values still remained significantly (P<0.05) higher compared with desHis1ProGlu(Lys12PAL)-glucagon alone from days 17 through 28 (Fig. 3E). Circulating plasma glucagon concentrations were significantly (P<0.01) elevated in d-Ala2GIP mice compared with saline- and desHis1ProGlu(Lys12PAL)-glucagon-treated mice at the end of the study, whereas desHis1ProGlu(Lys12PAL)-glucagon treatment alone resulted in a significant (P<0.05) reduction in glucagon concentrations (Fig. 3F). A combined administration of both peptides resulted in no significant change in plasma glucagon concentrations compared with high-fat control mice (Fig. 3F). Thus, desHis1ProGlu(Lys12PAL)-glucagon prevented the significant (P<0.05) augmentation of circulating glucagon induced by d-Ala2GIP treatment alone. Interestingly, the insulin:glucagon molar ratios on day 28 were 23:1, 71:1, and 44:1 in desHis1ProGlu(Lys12PAL)-glucagon, d-Ala2GIP, and the combined treatment groups, respectively, compared with 17:1 in saline-treated controls.

Effects of 28-days administration of desHis1ProGlu(Lys12PAL)-glucagon and d-Ala2GIP on glucose tolerance and insulin sensitivity in high-fat-diet-induced obese mice

All treatment groups had significantly (P<0.05 to P<0.01) reduced blood glucose excursions during an i.p. glucose tolerance test when compared with saline controls (Fig. 4A and B). In addition, d-Ala2GIP treatment was associated with a significantly (P<0.01) enhanced overall...
insulinotropic response in comparison with control mice (Fig. 4C and D). Similarly, during an oral glucose challenge, blood glucose levels were significantly ($P < 0.01$) reduced 30 and 60 min post administration in all treatment groups (Fig. 5A). In harmony with observations following an i.p. glucose load, $\delta$-Ala$^2$GIP treatment significantly enhanced the individual ($P < 0.05$ to $P < 0.001$) and overall ($P < 0.05$) insulin secretory response following oral glucose delivery when compared with all other groups of mice (Fig. 5C and D). Likewise, a combined treatment of $\delta$-Ala$^2$GIP with desHis$^1$Pro$^4$Glu$^6$(Lys$^{12}$PAL)-glucagon also enhanced ($P < 0.05$) the overall insulin secretory response (Fig. 5C and D). As shown in Fig. 6, treatment with $\delta$-Ala$^2$GIP alone, or in combination with desHis$^1$Pro$^4$Glu$^6$(Lys$^{12}$PAL)-glucagon, significantly ($P < 0.01$) improved the glucose-lowering action of exogenous insulin at 30 and 60 min post insulin injection when compared with saline controls (Fig. 6A). Treatment with desHis$^1$Pro$^4$Glu$^6$(Lys$^{12}$PAL)-glucagon alone also resulted in a significant ($P < 0.01$) reduction in blood glucose levels at 60 min post insulin injection (Fig. 6A). Moreover, the overall glucose-lowering effect of insulin was significantly ($P < 0.05$ to $P < 0.01$) enhanced in all treatment groups (Fig. 6B). Interestingly, pancreatic insulin content was significantly ($P < 0.05$ to $P < 0.01$) higher in mice treated with $\delta$-Ala$^2$GIP alone, or in combination with desHis$^1$Pro$^4$Glu$^6$(Lys$^{12}$PAL)-glucagon, when compared with saline controls or desHis$^1$Pro$^4$Glu$^6$(Lys$^{12}$PAL)-glucagon treatment alone (Fig. 6C).

Discussion

Notwithstanding encouraging preclinical data (Bagger et al. 2011, Trujillo & Nuffer 2014), the progression
Glucagon antagonism and GIP receptor activation

L M McShane and others

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of monotherapy glucagon antagonist- or GIP agonist-based therapies to the clinic is lacking. This is despite knowledge that a potential major beneficial effect of the most widely used antidiabetic drug, metformin, is mediated through inhibition of glucagon action (Pernicova & Korbonits 2014). Furthermore, recent studies have shown that targeting multiple regulatory hormone receptors may be a viable treatment option for T2DM (Patel et al. 2013, Trevaskis et al. 2013, Skarbaliene et al. 2015). As such, the dual activation of incretin-related pathways coupled with glucagon receptor blockade significantly improves metabolic control in diabetes (Claus et al. 2007, Mu et al. 2011).

Given that a documented therapeutic drawback of GIP mimetics relates to elevation of glucagon levels (Meier & Nauck 2004, 2015), a combined therapy with a specific glucagon antagonist would seem logical. Here, we assessed the benefits of combining the glucagon receptor antagonist, desHis1Pro4Glu9 (Lys12PAL)-glucagon (O’Harte et al. 2014), with the well characterized DPP-4-resistant GIP analog, d-Ala2GIP (Hinke et al. 2002, Widenmaier et al. 2010). We aimed to prove the concept that desHis1Pro4Glu9 (Lys12PAL)-glucagon could counter GIP-related elevations of blood glucagon levels.

Structure function studies with native glucagon have shown that the C-terminal portion of peptide, which exhibits an alpha-helical conformation, is involved in receptor recognition, with the N-terminal more important for receptor signaling (Sturm et al. 1998). In this study, desHis1Pro4Glu9 (Lys12PAL)-glucagon had an increased α-helical content when compared with native glucagon, a trait that is strongly associated with increased receptor-binding potency (Krstenansky et al. 1988). As previously shown by the leading synthetic peptide groups of Hruby and Merrifield, and later independently confirmed in our laboratory, His1, Gly4, and Asp9 are essential for normal agonist activity of glucagon at the level of the receptor (Hruby 1982, Unson et al. 1991, 1993, Ahn et al. 2001, O’Harte et al. 2013, Franklin et al. 2014, McShane et al. 2014). In complete harmony with this structural data, desHis1Pro4Glu9 (Lys12PAL)-glucagon is known to inhibit glucagon-induced elevations of cAMP generation and insulin secretion (O’Harte et al. 2013). Both the glucagon and GIP receptors belong to the same family of G-protein-coupled receptors (Brubaker & Drucker 2002) and share considerable structural homology (Kogire et al. 1992). However, desHis1Pro4Glu9 (Lys12PAL)-glucagon did not adversely hinder the insulinotropic and cAMP potentiating effect of d-Ala2GIP (Martin et al. 2013), further confirming specificity.

Figure 6
Effects of twice-daily treatment with desHis1Pro4Glu9 (Lys12PAL)-glucagon and d-Ala2GIP alone, or in combination, on peripheral insulin sensitivity and pancreatic insulin content in high-fat mice. Tests were conducted after 28 days twice-daily treatment with saline vehicle (0.9% w/v NaCl), desHis1Pro4Glu9 (Lys12PAL)-glucagon, d-Ala2GIP, or desHis1Pro4Glu9 (Lys12PAL)-glucagon in combination with d-Ala2GIP (all at 25 nmol/kg bw). (A) Insulin (10 U/kg bw) was administered by i.p. injection at t = 0 in nonfasted mice. (B) Blood glucose AAC values for 0–60 min post injection are also shown, where the baseline is 100%. (C) Pancreatic insulin content was assessed on day 28 following acid-ethanol extraction and measurement of insulin concentrations by RIA. Values are mean ± s.e.m. for eight mice. *P < 0.05 and **P < 0.01 compared with saline-treated control group. + P < 0.05 compared with desHis1Pro4Glu9 (Lys12PAL)-glucagon treatment alone. - P < 0.05 compared with the combined treatment group.
As would be expected, twice-daily treatment with either desHis1Pro4Glu9(Lys12PAL)-glucagon or d-Ala2GIP in high-fat-fed mice reproduced many of the beneficial effects previously noted with sustained GIP receptor activation (Kerr et al. 2009, Porter et al. 2011) or glucagon receptor blockade (Lotty et al. 2014, McShane et al. 2014, O’Harte et al. 2014). This included significant reductions in circulating blood glucose levels and improvements in peripheral glucose disposal. Beneficial effects of both treatment regimens were independent of alterations in body weight or energy intake. Previous studies have indicated that glucagon can decrease food intake (Habegger et al. 2010, Kosinski et al. 2012); however, our studies with peptide-based glucagon antagonists suggest that contrasting elevations of energy intake do not occur with glucagon receptor inhibition (Franklin et al. 2014, McShane et al. 2014, O’Harte et al. 2014). This probably reflects the complex neural pathways and plasticity involved in the regulation of feeding and energy balance (Dockray & Burdyga 2011). A combined therapy with desHis1Pro4Glu9(Lys12PAL)-glucagon and d-Ala2GIP did not result in discernible benefits on blood glucose or glucose disposal when compared with either treatment alone. This likely reflects the good efficacy of each treatment alone and the relatively high doses employed, which could preclude additive action. Indeed, circulating blood glucose levels were around 5–6 mmol/L in each treatment group by the end of the study. As has been proposed, circulating glucagon levels were significantly elevated by d-Ala2GIP treatment (Meier & Nauck 2004, 2015), but this detrimental effect was completely annulled by concurrent administration of desHis1Pro4Glu9(Lys12PAL)-glucagon. Moreover, circulating insulin concentrations were reduced in mice treated with a combination of desHis1Pro4Glu9(Lys12PAL)-glucagon and d-Ala2GIP, when compared with d-Ala2GIP alone, implying improved insulin action in these mice, since ambient glucose levels were essentially similar. Indeed, pancreatic insulin stores and the insulin:glucagon ratio were substantially augmented by dual therapy on day 28 when compared with desHis1Pro4Glu9(Lys12PAL)-glucagon treatment alone, which also points toward decreased insulin demand in the combined treatment group. Plasma glucagon levels were actually reduced by desHis1Pro4Glu9(Lys12PAL)-glucagon monotherapy, which is somewhat unexpected given previous observations (Bagger et al. 2011), and, therefore, does require further investigation.

Interestingly, total body fat mass was lowered in all treatment groups without change in overall body weight, suggesting a possible shift toward the use of stored fat as an energy source. Somewhat surprisingly, although in agreement with increased fat utilization, energy expenditure was increased during the dark phase in all mice receiving desHis1Pro4Glu9(Lys12PAL)-glucagon treatment. Thus, glucagon receptor activation is generally associated with enhanced energy expenditure (Campbell & Drucker 2015) implying that counter-regulatory mechanisms may be important for the benefits of sustained glucagon receptor inhibition in this study. However, respiratory exchange ratio was unaltered between groups with values of approximately 0.85, indicating a similar combination of fat and carbohydrates utilization. Interestingly, mice with genetic knock out of synaptotagmin-7, a regulator of glucagon and insulin secretion, present with reduced

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**Figure 7**
Effects of twice-daily treatment with desHis1Pro4Glu9(Lys12PAL)-glucagon and d-Ala2GIP alone, or in combination, on metabolic rate and locomotor activity in high-fat mice. Parameters were measured after 28 days twice-daily treatment with saline vehicle (0.9% w/v NaCl), desHis1Pro4Glu9(Lys12PAL)-glucagon, d-Ala2GIP, or desHis1Pro4Glu9(Lys12PAL)-glucagon in combination with d-Ala2GIP (all at 25 nmol/kg bw). (A) Energy expenditure, (B) respiratory exchange ratio, (C) ambulatory X counts, and (D) total Z counts were assessed by CLAMS. Values are mean ± s.e.m. for six mice. *P < 0.05 and **P < 0.001 compared with saline group. ΔP < 0.05 and ΔΔΔP < 0.001 compared with desHis1Pro4Glu9(Lys12PAL)-glucagon treatment alone. ηP < 0.05, ηηP < 0.01, and ηηηP < 0.001 compared with the combined treatment group.
circulating glucagon levels and increased energy expenditure (Lou et al. 2011), in harmony with the current findings. Ambulatory locomotion was also elevated only in mice where glucagon receptor action was inhibited. The overall significance of these centrally mediated effects requires further detailed elucidation. Moreover, the passage of both desHis-Pro-Glu(Lys32-PAL)-glucagon and d-Ala2GIP through the blood-brain barrier also requires investigation.

Improvements in glucose tolerance and glucose-stimulated insulin release are a previously reported feature of d-Ala2GIP treatment in high-fat-fed mice (Gault et al. 2011). Indeed, studies suggest that high-fat feeding increases islet GIP receptor expression (Harada et al., Moffett et al. 2015). Thus, the GIP analog may be able to independently overcome any potential GIP resistance (Nauck et al. 1993) in this mouse model of T2DM. Similarly, improvements in response to both oral and intraperitoneal glucose challenge were observed in desHis-Pro-Glu(Lys32-PAL)-glucagon mice, consistent with previous studies (O’Harte et al. 2013). This was despite any obvious increase in glucose-stimulated insulin secretion in desHis-Pro-Glu(Lys32-PAL)-glucagon treated mice. In agreement with this, peripheral insulin sensitivity was dramatically improved by desHis-Pro-Glu(Lys32-PAL)-glucagon; however, this was also the case for all treatment paradigms. Thus, improved insulin action might simply be a reflection of decreased glucose toxicity in each treatment group, due to lower circulating blood glucose concentrations. This might also be a reason for the lack of benefit of the combined treatment regimen. More interestingly, d-Ala2GIP-induced elevations of insulin secretion appeared to be blunted by the coadministration of desHis-Pro-Glu(Lys32-PAL)-glucagon following intraperitoneal glucose, but much less so following oral glucose administration. This would suggest d-Ala2GIP treatment alone, and in combination with desHis-Pro-Glu(Lys32-PAL)-glucagon, enhances the incretin axis in high-fat-fed mice (Moffett et al. 2015). Indeed, this could be linked to augmented secretion and/or action of GLP-1 following an oral glucose challenge in these mice, as suggested previously (Parker et al. 2002, Gelling et al. 2003).

In conclusion, this study indicates that twice-daily injection of either d-Ala2GIP or desHis-Pro-Glu(Lys32-PAL)-glucagon is an effective means of improving diabetic control in obese-diabetic high-fat-fed mice. There was some limited evidence for benefits following combined treatment, but this requires further detailed study to assess the relative importance. As such, studies utilizing various concentration and ratios of individual peptides could be interesting and might reveal further benefits. Importantly, however, a combined therapy of desHis-Pro-Glu(Lys32-PAL)-glucagon with d-Ala2GIP did completely annul GIP-induced elevations of circulating glucagon levels and augments pancreatic insulin stores, confirming proof of concept. Furthermore, it may be interesting to examine the metabolic benefits of sustained glucagon inhibition in combination with GLP-1 receptor activation, or in other animal models of diabetes. Taken together, the data presented here provide evidence for the usefulness of peptide-based GIP receptor agonist and glucagon receptor antagonist therapies for the treatment of T2DM.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
L McS, D O F, and Z J F were involved in performing this research for cell work, in vivo work in mice, and CD analysis. CMH was involved in CD work and supervision. L McS, N I, and FOH were involved in the preparation of data, writing up this work, and proofreading the document.

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