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

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**Author contribution:**

LMcS, DOF, ZJF were involved in performing this research for cell work, \textit{in vivo} work in mice and CD analysis. CMH was involved in CD work and supervision. LMcS, NI and FOH were involved in preparation of data, writing up this work and proof reading the document.

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ABSTRACT

Ablation of glucagon receptor signalling represents a potential treatment option for type 2 diabetes (T2DM). Additionally, activation of glucose-dependent insulinoetric polypeptide (GIP) receptor signalling also holds therapeutic promise for T2DM. Therefore, the present study examined both independent and combined metabolic actions of desHis$^{1}$Pro$^{4}$Glu$^{9}$Lys$^{12}$PAL$^{v}$glucagon (glucagon receptor antagonist) and D-Ala$^{2}$GIP (GIP receptor agonist), in diet induced obese mice. Glucagon receptor binding has been linked to alpha-helical structure and desHis$^{1}$Pro$^{4}$Glu$^{9}$Lys$^{12}$PAL$^{v}$glucagon displayed enhanced alpha-helical content compared to native glucagon. In clonal pancreatic BRIN-BD11 beta-cells, desHis$^{1}$Pro$^{4}$Glu$^{9}$Lys$^{12}$PAL$^{v}$glucagon was devoid of any insulinoetric or cAMP generating actions, and did not impede D-Ala$^{2}$GIP-mediated (p<0.01 to p<0.001) effects on insulin and cAMP production. Twice daily injection of desHis$^{1}$Pro$^{4}$Glu$^{9}$Lys$^{12}$PAL$^{v}$glucagon or D-Ala$^{2}$GIP alone, and in combination, in high fat fed 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-Ala$^{2}$GIP. Interestingly, plasma glucagon concentrations were decreased (p<0.05) by sustained glucagon inhibition (day 28), but increased (p<0.05) by D-Ala$^{2}$GIP therapy, with 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-Ala$^{2}$GIP 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
desHis$^1$Pro$^4$Glu$^9$(Lys$^{12}$PAL)-glucagon treated mice. These studies highlight the potential of glucagon receptor inhibition alone, and in combination with GIP receptor activation, for T2DM treatment.

INTRODUCTION

Through advances in our understanding of the pathways involved glucose homeostasis, and an appreciation that type 2 diabetes (T2DM) is a bi-hormonal 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 signalling 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-240921, 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 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; Lefebvre et al. 2015; Kelly et al. 2015).

Therefore, we have recently characterised the novel peptide-based glucagon receptor antagonist, desHis$^1$Pro$^4$Glu$^9$(Lys$^{12}$PAL)-glucagon (O’Harte et al. 2014), that should represent a more specific approach to inhibit glucagon receptor action. Indeed, this peptide analogue induced significant improvements in metabolic control following a chronic dosing regimen in diet induced obese (DIO) as well as in 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 colleagues reported
that co-administration of the glucagon antagonist, Cpd-A, with a dipeptidylpeptidase-4 (DPP-
4) inhibitor in diabetic mice resulted in additional improvements in glycaemic control when
compared to either treatment alone (Mu et al. 2011). It follows that 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 recognised 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 (Vilsboll 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
man through tight glycaemic 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, co-administration of a specific
glucagon receptor antagonist (O’Harte et al. 2014), with a stable long-acting GIP mimic,
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 sub-chronic treatment with the peptide-based glucagon receptor antagonist, desHis\(^1\)Pro\(^4\)Glu\(^9\)(Lys\(^{12}\)PAL)-glucagon, and D-Ala\(^2\)GIP 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-Ala\(^2\)GIP and desHis\(^1\)Pro\(^4\)Glu\(^9\)(Lys\(^{12}\)PAL)-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 previously described (O’Harte et al. 2013).

Circular dichroism (CD)

CD spectra for glucagon and desHis\(^1\)Pro\(^4\)Glu\(^9\)(Lys\(^{12}\)PAL)-glucagon were acquired at the far-UV region (190-250 nm) using a JASCO J-810 spectropolarimeter. Peptide samples were prepared by dissolving the analogues in water or in 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\(^{-1}\) glucose, and were seeded at a density of 1x10\(^5\) cells/well in 24 well plates for insulin release studies or 8x10\(^4\) 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% CO\(_2\) and 95% air. Prior to insulin and cAMP studies, the tissue culture medium was removed and cells were pre-incubated 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\(^{-1}\) 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 prior to 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 Europe Ltd., Abingdon, UK).

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 dark cycle (lights off between 20:00 and 08:00 h). All animals had free access to drinking water and a high fat (45% fat, 35% carbohydrate and 20% protein, Dietex International Ltd. Witham, Essex, UK) diet for 100 days prior to commencement of studies. Obesity and glycaemic dysregulation were clearly manifested compared to 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 acclimatisation 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\(^1\)Pro\(^4\)Glu\(^9\)(Lys\(^{12}\)PAL)-glucagon alone, D-Ala\(^2\)GIP alone or a combination of both peptides (all treatments at 25 nmol kg\(^{-1}\) 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 prior to the normal morning 10:00 h peptide administration. At the end of the treatment period, oral and i.p. (18 mmol kg\(^{-1}\) bw) glucose tolerance tests were performed in overnight fasted mice. In addition, an insulin sensitivity (10 U kg\(^{-1}\) bw) test was also performed in non-fasted mice. At termination, pancreatic tissue was excised and insulin content measured following extraction with 5 ml g\(^{-1}\) 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 acclimatised to the air tight metabolic chambers for 18 h prior to commencement of observations. Oxygen consumption and carbon dioxide production were monitored for 30 sec 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 x RER) x 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- axis 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 micro-centrifuge tubes (Sarstedt, Numbrecht, Germany) at the time points indicated in the Figures. Blood glucose was measured directly using a hand-held Ascencia Contour blood glucose meter (Bayer Healthcare, Newbury, Berkshire, UK). For plasma insulin analysis, blood samples were immediately centrifuged using a Beckman microcentrifuge (Beckman Instruments, Galway, Ireland) for 1 min at 13,000 x 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, Maryland, USA).

Statistical analyses

Results are expressed as means ± SEM 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, CA, USA) employing the trapezoidal rule with baseline subtraction. p<0.05 was considered to be significantly different.

RESULTS
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 nm and 222 nm were observed indicating the formation of alpha-helical conformations within the peptide analogues. The alpha-helical content of glucagon was calculated at 26-31% at high TFE concentrations (Fig 1A). As the concentration of TFE increased, desHis\(^1\)Pro\(^4\)Glu\(^9\)(Lys\(^{12}\)PAL)-glucagon revealed an overall trend of increasing alpha-helical concentration with decreased beta-sheet content (Fig 1B). In comparison to native glucagon (Fig 1A), desHis\(^1\)Pro\(^4\)Glu\(^9\)(Lys\(^{12}\)PAL)-glucagon had increased alpha-helical content at 15-70% TFE concentrations (Fig 1B).

Effects of desHis\(^1\)Pro\(^4\)Glu\(^9\)(Lys\(^{12}\)PAL)-glucagon and D-Ala\(^2\)GIP on acute insulin secretion and cAMP production in BRIN-BD11 cells

The acylated glucagon receptor antagonist, desHis\(^1\)Pro\(^4\)Glu\(^9\)(Lys\(^{12}\)PAL)-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-Ala\(^2\)GIP, induced a significant (p<0.01 to p<0.001) concentration-dependent (10\(^{-8}\) to 10\(^{-6}\) M) increase in insulin secretion when compared with a 5.6 mmol l\(^{-1}\) glucose control (Fig 2A). Co-incubation of desHis\(^1\)Pro\(^4\)Glu\(^9\)(Lys\(^{12}\)PAL)-glucagon (10\(^{-7}\) M) with D-Ala\(^2\)GIP had no effect on D-Ala\(^2\)GIP-mediated insulin release (Fig 2A). Furthermore, desHis\(^1\)Pro\(^4\)Glu\(^9\)(Lys\(^{12}\)PAL)-glucagon (10\(^{-7}\) M) had no inhibitory effect on D-Ala\(^2\)GIP-induced (p<0.01) cAMP production (Fig 2B).

Effects of 28-days administration of desHis\(^1\)Pro\(^4\)Glu\(^9\)(Lys\(^{12}\)PAL)-glucagon and D-Ala\(^2\)GIP on metabolic parameters in high fat diet-induced obese mice

Twice daily treatment with desHis\(^1\)Pro\(^4\)Glu\(^9\)(Lys\(^{12}\)PAL)-glucagon or D-Ala\(^2\)GIP 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, desHis$^1$Pro$^4$Glu$^9$(Lys$^{12}$PAL)-glucagon and D-Ala$^2$GIP treated high fat mice had body fat masses of 40.3 ± 0.6%, 33.8 ± 0.8% and 37.7 ± 0.5%, respectively, compared to a body fat mass of 37.8 ± 0.5% in lean control mice.

In addition, D-Ala$^2$GIP treated mice and those given the combination of both peptides had increased (p<0.01) body fat percentage compared to desHis$^1$Pro$^4$Glu$^9$(Lys$^{12}$PAL)-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 onwards when compared to saline controls (Fig 3D). In addition, D-Ala$^2$GIP induced a highly significant (p<0.05 to p<0.001) increase in circulating insulin on day 28 compared to all other groups (Fig 3E), whereas desHis$^1$Pro$^4$Glu$^9$(Lys$^{12}$PAL)-glucagon monotherapy had no effect on plasma insulin levels (Fig 3E). D-Ala$^2$GIP mediated elevations in plasma insulin were partially restrained by combined desHis$^1$Pro$^4$Glu$^9$(Lys$^{12}$PAL)-glucagon therapy, although values still remained significantly (p<0.05) higher compared to desHis$^1$Pro$^4$Glu$^9$(Lys$^{12}$PAL)-glucagon alone from days 17 through to 28 (Fig 3E). Circulating plasma glucagon concentrations were significantly (p<0.01) elevated in D-Ala$^2$GIP mice compared to saline and desHis$^1$Pro$^4$Glu$^9$(Lys$^{12}$PAL)-glucagon treated mice at the end of the study, whereas desHis$^1$Pro$^4$Glu$^9$(Lys$^{12}$PAL)-glucagon treatment alone resulted in a significant (p<0.05) reduction in glucagon concentrations (Fig 3F). Combined administration of both peptides resulted in no significant change in plasma glucagon concentrations compared to high fat control mice (Fig. 3F). Thus, desHis$^1$Pro$^4$Glu$^9$(Lys$^{12}$PAL)-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 desHis$^1$Pro$^4$Glu$^9$(Lys$^{12}$PAL)-glucagon, D-Ala$^2$GIP and the combined treatment groups, respectively, compared to 17:1 in saline treated controls.
Effects of 28-days administration of desHis$^1$Pro$^4$Glu$^9$(Lys$^{12}$PAL)-glucagon and D-Ala$^2$GIP on glucose tolerance and insulin sensitivity in high fat diet-induced obese mice

All treatment groups had significantly (p<0.05-p<0.01) reduced blood glucose excursions during an i.p. glucose tolerance test when compared to saline controls (Fig 4A,B). In addition, D-Ala$^2$GIP treatment was associated with a significantly (p<0.01) enhanced overall insulinotropic response in comparison to control mice (Fig 4C,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, D-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 to all other groups of mice (Fig 5C,D). Likewise, combined treatment of D-Ala$^2$GIP with desHis$^1$Pro$^4$Glu$^9$(Lys$^{12}$PAL)-glucagon also enhanced (p<0.05) the overall insulin secretory response (Fig 5C,D). As shown in Figure 6, treatment with D-Ala$^2$GIP alone, or in combination with desHis$^1$Pro$^4$Glu$^9$(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 to saline controls (Fig 6A). Treatment with desHis$^1$Pro$^4$Glu$^9$(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 D-Ala$^2$GIP alone, or in combination with desHis$^1$Pro$^4$Glu$^9$(Lys$^{12}$PAL)-glucagon, when compared to saline controls or desHis$^1$Pro$^4$Glu$^9$(Lys$^{12}$PAL)-glucagon treatment alone (Fig 6C).
Effects of 28-days administration of desHis\textsuperscript{1}Pro\textsuperscript{4}Glu\textsuperscript{9}(Lys\textsuperscript{12}PAL)-glucagon and D-Ala\textsuperscript{2}GIP on metabolic rate and locomotor activity in high fat diet-induced obese mice

Treatment with desHis\textsuperscript{1}Pro\textsuperscript{4}Glu\textsuperscript{9}(Lys\textsuperscript{12}PAL)-glucagon alone, and in combination with D-Ala\textsuperscript{2}GIP, significantly (p<0.001) increased energy expenditure during the dark phase compared to saline-treated controls and D-Ala\textsuperscript{2}GIP treatment alone (Fig 7A). Respiratory exchange ratio was not different between groups of mice (Fig 7B). Ambulation, as assessed by X-beam breaks, was significantly (p<0.05) elevated in mice treated with desHis\textsuperscript{1}Pro\textsuperscript{4}Glu\textsuperscript{9}(Lys\textsuperscript{12}PAL)-glucagon alone, or in combination with D-Ala\textsuperscript{2}GIP (Fig 7C). D-Ala\textsuperscript{2}GIP treatment did not affect X-beam breaks when compared to control mice (Fig 7C). All three treatment groups had significantly (p<0.05 to p<0.001) increased numbers of Z-beam breaks compared to controls, with the combination treatment group also displaying increased Z-beam breaks when compared to individual treatment regimens (Fig 7D). Energy expenditure, ambulatory activity and Z-beam breaks were not significantly different between groups during the light phase (data not shown).

DISCUSSION

Notwithstanding encouraging preclinical data (Bagger \textit{et al.} 2011; Trujillo \& Nuffer 2014), the progression 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 \& Korkonits 2014). Furthermore, recent studies have shown that targeting multiple regulatory hormone receptors may be a viable treatment option for T2DM (Patel \textit{et al.} 2013; Trevaskis \textit{et al.} 2013; Skarbaliene \textit{et al.} 2015). As such, the dual activation of incretin-related pathways coupled with glucagon receptor blockade significantly improves metabolic control in diabetes (Claus \textit{et al.} 2007; Mu \textit{et al.} 2011). Given that a documented
therapeutic drawback of GIP mimetics relates to elevation of glucagon levels (Meier & Nauck 2004, 2015), combined therapy with a specific glucagon antagonist would seem logical. Here we assessed the benefits of combining the glucagon receptor antagonist desHis\(^1\)Pro\(^4\)Glu\(^9\)(Lys\(^{12}\)PAL)-glucagon (O’Harte et al. 2014) with the well characterised DPP-4 resistant GIP analogue, D-Ala\(^2\)GIP (Hinke et al. 2002; Widenmaier et al. 2010). We aimed to prove the concept that desHis\(^1\)Pro\(^4\)Glu\(^9\)(Lys\(^{12}\)PAL)-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 signalling (Sturm et al. 1998). In the present study, desHis\(^1\)Pro\(^4\)Glu\(^9\)(Lys\(^{12}\)PAL)-glucagon had an increased \(\alpha\)-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, His\(^1\), Gly\(^4\) and Asp\(^9\) 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, McShane et al. 2014; Franklin et al. 2014). In complete harmony with this structural data, desHis\(^1\)Pro\(^4\)Glu\(^9\)(Lys\(^{12}\)PAL)-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, desHis\(^1\)Pro\(^4\)Glu\(^9\)(Lys\(^{12}\)PAL)-glucagon did not adversely hinder the insulinotropic and cAMP potentiating effect of D-Ala\(^2\)GIP (Martin et al. 2013), further confirming specificity.

As would be expected, twice daily treatment with either desHis\(^1\)Pro\(^4\)Glu\(^9\)(Lys\(^{12}\)PAL)-glucagon or D-Ala\(^2\)GIP 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 (Lotfy 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). Combined therapy with desHis\(^1\)Pro\(^4\)Glu\(^9\)(Lys\(^{12}\)PAL)\(^{-}\)glucagon and D-Ala\(^2\)GIP did not result in discernible benefits on blood glucose or glucose disposal when compared to 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-Ala\(^2\)GIP treatment (Meier & Nauck 2004, 2015), but this detrimental effect was completely annulled by concurrent administration of desHis\(^1\)Pro\(^4\)Glu\(^9\)(Lys\(^{12}\)PAL)\(^{-}\)glucagon. Moreover, circulating insulin concentrations were reduced in mice treated with a combination of desHis\(^1\)Pro\(^4\)Glu\(^9\)(Lys\(^{12}\)PAL)\(^{-}\)glucagon and D-Ala\(^2\)GIP, when compared to D-Ala\(^2\)GIP 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 to desHis\(^1\)Pro\(^4\)Glu\(^9\)(Lys\(^{12}\)PAL)-glucagon treatment alone, which also points towards decreased insulin demand in the combined treatment group. Plasma glucagon levels were actually reduced by
desHis$^1$Pro$^4$Glu$^9$(Lys$^{12}$PAL)-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 towards the use of stored fat as an energy source. Somewhat surprisingly, although in agreement with increased fat utilisation, energy expenditure was increased during the dark phase in all mice receiving desHis$^1$Pro$^4$Glu$^9$(Lys$^{12}$PAL)-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 the present study. However, respiratory exchange ratio was unaltered between groups with values of approximately 0.85, indicating a similar combination of fat and carbohydrates utilisation. Interestingly, mice with genetic knock out of synaptotagmin-7, a regulator of glucagon and insulin secretion, present with reduced 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$^1$Pro$^4$Glu$^9$(Lys$^{12}$PAL)-glucagon and D-Ala$^2$GIP through the blood brain barrier also requires investigation.

Improvements in glucose tolerance and glucose-stimulated insulin release are a previously reported feature of D-Ala$^2$GIP 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. 2008; Moffett et al. 2015). Thus, the GIP analogue 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 was observed in desHis$^1$Pro$^4$Glu$^9$(Lys$^{12}$PAL)-glucagon mice, consistent with previous studies
(O’Harte et al. 2013). This was despite any obvious increase of glucose-stimulated insulin secretion in desHis-Pro-Glu-(Lys)-glucagon treated mice. In agreement with this, peripheral insulin sensitivity was dramatically improved by desHis-Pro-Glu-(Lys)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-Ala-GIP-induced elevations of insulin secretion appeared to be blunted by co-administration of desHis-Pro-Glu-(Lys)-glucagon following intraperitoneal glucose, but much less so following oral glucose administration. This would suggest D-Ala-GIP treatment alone, and in combination with desHis-Pro-Glu-(Lys)-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, the present study indicates that twice daily injection of either D-Ala-GIP or desHis-Pro-Glu-(Lys)-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 utilising various concentration and ratios of individual peptides could be interesting and might reveal further benefits. Importantly however, combined therapy of desHis-Pro-Glu-(Lys)-glucagon with D-Ala-GIP did completely annul GIP-induced elevations of circulating glucagon levels and augment 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.

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Conflict of Interest:

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Figure legends

Figure 1. Circular dichroism spectra of (A) glucagon and (B) desHis\textsuperscript{1}Pro\textsuperscript{4}Glu\textsuperscript{9}(Lys\textsuperscript{12}PAL)-glucagon. Spectra were observed in the far UV region under different concentrations of TFE, as indicated on the figure.

Figure 2. Insulinotropic and cAMP generating effects of desHis\textsuperscript{1}Pro\textsuperscript{4}Glu\textsuperscript{9}(Lys\textsuperscript{12}PAL)-glucagon and D-Ala\textsuperscript{2}GIP. (A) BRIN-BD11 cells were exposed to varying concentrations (10\textsuperscript{-12} – 10\textsuperscript{-6} M) of desHis\textsuperscript{1}Pro\textsuperscript{4}Glu\textsuperscript{9}(Lys\textsuperscript{12}PAL)-glucagon, D-Ala\textsuperscript{2}GIP and D-Ala\textsuperscript{2}GIP in the presence of 10\textsuperscript{-7} M desHis\textsuperscript{1}Pro\textsuperscript{4}Glu\textsuperscript{9}(Lys\textsuperscript{12}PAL)-glucagon for 20 min at 5.6 mmol glucose. (A) Extracellular insulin secretion was measured by RIA and total (B) total cAMP generation measured by ELISA. Values represent means ± SEM (n=8) where **p<0.01, ***p<0.001 compared with 5.6 mmol glucose control. ∆∆p<0.01, ∆∆∆p<0.001 compared with desHis\textsuperscript{1}Pro\textsuperscript{4}Glu\textsuperscript{9}(Lys\textsuperscript{12}PAL)-glucagon alone.

Figure 3. Effects of twice daily treatment with desHis\textsuperscript{1}Pro\textsuperscript{4}Glu\textsuperscript{9}(Lys\textsuperscript{12}PAL)-glucagon and D-Ala\textsuperscript{2}GIP alone, or in combination on body weight, fat mass, food intake, circulating blood glucose, plasma insulin and glucagon concentrations in high fat mice. (A, C-E) Parameters were measured 4 days prior to, and 28 days during (indicated by horizontal black bar) twice daily treatment with saline vehicle (0.9% w/v NaCl), desHis\textsuperscript{1}Pro\textsuperscript{4}Glu\textsuperscript{9}(Lys\textsuperscript{12}PAL)-glucagon, D-Ala\textsuperscript{2}GIP or desHis\textsuperscript{1}Pro\textsuperscript{4}Glu\textsuperscript{9}(Lys\textsuperscript{12}PAL)-glucagon in combination with D-Ala\textsuperscript{2}GIP (all at 25 nmol/kg bw) (B,F). Total body fat mass and plasma glucagon levels were assessed on day 28. Values are mean ± SEM for 8 mice. *p<0.05, **p<0.01 and ***p<0.001 compared with saline group. ∆p<0.05, ∆∆p<0.01 and ∆∆∆p<0.001 compared with desHis\textsuperscript{1}Pro\textsuperscript{4}Glu\textsuperscript{9}(Lys\textsuperscript{12}PAL)-glucagon treatment alone. +p<0.05 and ++p<0.01 compared with combined treatment group.
Figure 4. Effects of twice daily treatment with desHis$^{1}$Pro$^{4}$Glu$^{9}$ (Lys$^{12}$PAL)-glucagon and D-Ala$^{2}$GIP alone, or in combination on intraperitoneal glucose tolerance and plasma insulin response to glucose in high fat mice. Tests were conducted after 28 days twice daily treatment with saline vehicle (0.9% w/v NaCl), desHis$^{1}$Pro$^{4}$Glu$^{9}$ (Lys$^{12}$PAL)-glucagon, D-Ala$^{2}$GIP or desHis$^{1}$Pro$^{4}$Glu$^{9}$ (Lys$^{12}$PAL)-glucagon in combination with D-Ala$^{2}$GIP (all at 25 nmol/kg bw). (A,C) Glucose (18 mmol/kg bw) was administered by i.p. injection at t=0 in 18 h fasted mice. (B,D) Blood glucose and plasma insulin AUC values for 0-60 min post injection are also shown. Values are mean ± SEM for 8 mice. *p<0.05, **p<0.01 and ***p<0.001 compared with saline group. ∆p<0.05 compared with desHis$^{1}$Pro$^{4}$Glu$^{9}$ (Lys$^{12}$PAL)-glucagon treatment alone.

Figure 5. Effects of twice daily treatment with desHis$^{1}$Pro$^{4}$Glu$^{9}$ (Lys$^{12}$PAL)-glucagon and D-Ala$^{2}$GIP alone, or in combination on oral glucose tolerance and plasma insulin response to glucose in high fat mice. Tests were conducted after 28 days twice daily treatment with saline vehicle (0.9% w/v NaCl), desHis$^{1}$Pro$^{4}$Glu$^{9}$ (Lys$^{12}$PAL)-glucagon, D-Ala$^{2}$GIP or desHis$^{1}$Pro$^{4}$Glu$^{9}$ (Lys$^{12}$PAL)-glucagon in combination with D-Ala$^{2}$GIP (all at 25 nmol/kg bw). (A,C) Glucose (18 mmol/kg bw) was administered by oral gavage at t=0 in 18 h fasted mice. (B,D) Blood glucose and plasma insulin AUC values for 0-60 min post injection are also shown. Values are mean ± SEM for 8 mice. *p<0.05, **p<0.01 and ***p<0.001 compared with the saline-treated control group. ∆p<0.05 and ∆∆p<0.01 compared with desHis$^{1}$Pro$^{4}$Glu$^{9}$ (Lys$^{12}$PAL)-glucagon treatment alone. †p<0.05 compared with combined treatment group.
Figure 6. Effects of twice daily treatment with desHis$^1$Pro$^4$Glu$^9$(Lys$^{12}$PAL)-glucagon and D-Ala$^2$GIP 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), desHis$^1$Pro$^4$Glu$^9$(Lys$^{12}$PAL)-glucagon, D-Ala$^2$GIP or desHis$^1$Pro$^4$Glu$^9$(Lys$^{12}$PAL)-glucagon in combination with D-Ala$^2$GIP (all at 25 nmol/kg bw). (A) Insulin (10 U/kg bw) was administered by i.p. injection at t=0 in non-fasted mice. (B) Blood glucose AAC values for 0-60 min post injection are also shown, where 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 ± SEM for 8 mice. *p<0.05 and **p<0.01 compared with saline-treated control group. ∆p<0.05 compared with desHis$^1$Pro$^4$Glu$^9$(Lys$^{12}$PAL)-glucagon treatment alone. †p<0.05 compared with combined treatment group.

Figure 7. Effects of twice daily treatment with desHis$^1$Pro$^4$Glu$^9$(Lys$^{12}$PAL)-glucagon and D-Ala$^2$GIP 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), desHis$^1$Pro$^4$Glu$^9$(Lys$^{12}$PAL)-glucagon, D-Ala$^2$GIP or desHis$^1$Pro$^4$Glu$^9$(Lys$^{12}$PAL)-glucagon in combination with D-Ala$^2$GIP (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 ± SEM for 6 mice. *p<0.05 and ***p<0.001 compared with saline group. ∆p<0.05 and ∆∆∆p<0.001 compared with desHis$^1$Pro$^4$Glu$^9$(Lys$^{12}$PAL)-glucagon treatment alone. †p<0.05, ‡p<0.01 and §p<0.001 compared with combined treatment group.
Figure 1B

desHis¹Pro¹Glu¹Lys³³²(glut-PAL)glucagon

<table>
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<th>Condition</th>
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<tr>
<td>70% TFE</td>
<td>-30</td>
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Wavelength (nm)

338x190mm (96 x 96 DPI)
Figure 2B

- D-Ala²GIP
- desHis⁵Pro⁶Glu⁷(Lys¹⁵PAL)₂-glicagon
- D-Ala²GIP + desHis⁵Pro⁶Glu⁷(Lys¹⁵PAL)₂-glicagon (10⁻⁷M)

338x190mm (96 x 96 DPI)
Figure 3A

- saline
- desHis ProGlu(Lys) PAL-glicagon
- D-Ala5 GIP
- desHis ProGlu(Lys) PAL-glicagon + D-Ala5 GIP

Figure 3B

- saline
- desHis ProGlu(Lys) PAL-glicagon
- D-Ala5 GIP
- desHis ProGlu(Lys) PAL-glicagon + D-Ala5 GIP

338x190mm (96 x 96 DPI)
Figure 3C

Figure 3D

338x190mm (96 x 96 DPI)
Figure 3E

Plasma insulin (ng/ml)

Time (day)

-4 0 4 8 12 16 20 24 28

Figure 3F

Plasma glucagon (pg/ml)

-4 0 4 8 12 16 20 24 28

338x190mm (96 x 96 DPI)
Figure 4A

Figure 4B

Blood glucose (mmol/l)

Time (min)

Blood glucose (mg/dl)

338x190mm (96 x 96 DPI)
Figure 4C

- saline
- desHis Pro^1 Glu^2 (Lys^7) PAL glucagon
- D-Ala^9 GIP
- desHis Pro^1 Glu^2 (Lys^7) PAL glucagon + D-Ala^9 GIP

Figure 4D

- saline
- desHis Pro^1 Glu^2 (Lys^7) PAL glucagon
- D-Ala^9 GIP
- desHis Pro^1 Glu^2 (Lys^7) PAL glucagon + D-Ala^9 GIP

Plasma insulin (ng/ml) vs. Time (min)
Figure 5A

![Graph showing blood glucose (mM) over time (min) for different treatments.]

Figure 5B

![Graph showing blood glucose AUC (mmol x min) for different treatments.]

338x190mm (96 x 96 DPI)
Figure 5C

Figure 5D

Plasma insulin (nM)

Time (min)

0 15 30 45 60

Plasma insulin (nM)

338x190mm (96 x 96 DPI)
Figure 6A

![Graph showing blood glucose % change over time for different treatments.](image)

Saline, desHis^1^PrPro^6^Glu^8^Lys^12^P AL-glucagon, D-Ala^2^GIP, desHis^1^PrPro^6^Glu^8^Lys^12^P AL-glucagon + D-Ala^2^GIP.
Figure 6B + 6C
Figure 7C

Figure 7D

Average X-axillary counts

Days post

Average Z-axillary counts

Days post

338x190mm (96 x 96 DPI)