



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

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

2

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4

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17

18 **Author contribution:**

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

22

23 **Short title:** Glucagon antagonism and GIP receptor activation

24

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26 insulin secretion; insulin sensitivity, glucagon receptor antagonist, GIP receptor agonist, diet  
27 induced obese diabetic mice Word count 6956

## 28 **ABSTRACT**

29 Ablation of glucagon receptor signalling represents a potential treatment option for type 2  
30 diabetes (T2DM). Additionally, activation of glucose-dependent insulintropic polypeptide  
31 (GIP) receptor signalling also holds therapeutic promise for T2DM. Therefore, the present  
32 study examined both independent and combined metabolic actions of  
33 desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon (glucagon receptor antagonist) and D-Ala<sup>2</sup>GIP (GIP  
34 receptor agonist), in diet induced obese mice. Glucagon receptor binding has been linked to  
35 alpha-helical structure and desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon displayed enhanced alpha-  
36 helical content compared to native glucagon. In clonal pancreatic BRIN-BD11 beta-cells,  
37 desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon was devoid of any insulintropic or cAMP generating  
38 actions, and did not impede D-Ala<sup>2</sup>GIP-mediated (p<0.01 to p<0.001) effects on insulin and  
39 cAMP production. Twice daily injection of desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon or D-  
40 Ala<sup>2</sup>GIP alone, and in combination, in high fat fed mice failed to affect body weight or  
41 energy intake. Circulating blood glucose levels were significantly (p<0.05 to p<0.01)  
42 decreased by all treatments regimens, with plasma and pancreatic insulin elevated (p<0.05 to  
43 p<0.001) in all mice receiving D-Ala<sup>2</sup>GIP. Interestingly, plasma glucagon concentrations  
44 were decreased (p<0.05) by sustained glucagon inhibition (day 28), but increased (p<0.05) by  
45 D-Ala<sup>2</sup>GIP therapy, with combined treatment resulting in glucagon concentration similar to  
46 saline controls. All treatments improved (p<0.01) intraperitoneal and oral glucose tolerance,  
47 and peripheral insulin sensitivity. D-Ala<sup>2</sup>GIP treated mice showed increased glucose-induced  
48 insulin secretion in response to intraperitoneal and oral glucose. Metabolic rate and  
49 ambulatory locomotor activity were increased (p<0.05 to p<0.001) in all

50 desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon treated mice. These studies highlight the potential of  
51 glucagon receptor inhibition alone, and in combination with GIP receptor activation, for  
52 T2DM treatment.

53

#### 54 **INTRODUCTION**

55 Through advances in our understanding of the pathways involved glucose homeostasis, and  
56 an appreciation that type 2 diabetes (T2DM) is a bi-hormonal disorder, it is clear that  
57 abnormalities of insulin secretion and action in T2DM are present in the setting of glucagon  
58 excess (Unger & Cherrington, 2012). Thus, improved control of glucagon signalling  
59 represents a rational therapeutic target for T2DM. In agreement with this, early proof-of-  
60 concept studies using the orally available glucagon receptor antagonist, Bay 27-9955, have  
61 shown initial promise in humans (Petersen & Sullivan, 2001). Additionally, more recent  
62 clinical trials with similar orally available glucagon receptor inhibitors, MK-0893 and LY-  
63 2409021, reveal further potential for the treatment of T2DM (Xiong *et al.* 2012; Kelly *et al.*  
64 2015). A separate, but somewhat comparable approach, to reduce glucagon receptor  
65 expression through use of antisense oligonucleotides, has also reached Phase 2 clinical trials  
66 (Sehgal *et al.* 2013). However, the ultimate approval of these types of low molecular weight  
67 therapies will depend upon specificity and off-target effects, toxicity and potential for  
68 immune responses (Peng *et al.* 2014; Lefebvre *et al.* 2015; Kelly *et al.* 2015).

69 Therefore, we have recently characterised the novel peptide-based glucagon receptor  
70 antagonist, desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon (O'Harte *et al.* 2014), that should represent  
71 a more specific approach to inhibit glucagon receptor action. Indeed, this peptide analogue  
72 induced significant improvements in metabolic control following a chronic dosing regimen in  
73 diet induced obese (DIO) as well as in *ob/ob* diabetic mice (O'Harte *et al.* 2014). Importantly,  
74 we did not observe any evidence of adverse effects, and further studies in normal mice

75 indicate that this peptide-based glucagon receptor antagonist represents a safe and effective  
76 treatment option for T2DM (Franklin *et al.* 2014). Interestingly, Mu and colleagues reported  
77 that co-administration of the glucagon antagonist, Cpd-A, with a dipeptidylpeptidase-4 (DPP-  
78 4) inhibitor in diabetic mice resulted in additional improvements in glycaemic control when  
79 compared to either treatment alone (Mu *et al.* 2011). It follows that combined therapy of a  
80 glucagon receptor antagonist with an incretin based drug could offer an advantageous  
81 approach for the treatment of T2DM.

82         The incretin hormones, glucose-dependent insulintropic polypeptide (GIP) and  
83 glucagon-like peptide-1 (GLP-1), are recognised to account for approximately 50-70% of  
84 insulin secretion following a meal (Nauck *et al.* 1986). However, this incretin contribution to  
85 postprandial insulin release falls to less than 20% in T2DM (Nauck *et al.* 1986). The  
86 reduction is attributable to decreased GLP-1 release (Vilsboll *et al.* 2001) and resistance to  
87 the insulintropic actions of GIP in T2DM (Nauck *et al.* 1993). Accordingly, enzymatically  
88 stable GLP-1 mimetics that enhance circulating physiological levels of GLP-1 have gained  
89 notable success in the T2DM clinic (Gupta 2013; Chaplin & Joseph 2014), whereas GIP  
90 mimetics are yet to reach the clinic due to insensitivity in T2DM patients (Nauck *et al.* 1993).  
91 More encouragingly, GIP resistance in T2DM appears to be reversible in both animals and  
92 man through tight glycaemic control or combinational drug therapy (Meneilly *et al.* 2003;  
93 Piteau *et al.* 2007; Højberg *et al.* 2009). In addition to this, there is a suggestion that GIP,  
94 unlike GLP-1, can promote glucagon release (Meier & Nauck 2004, 2015) which would  
95 further detract from its therapeutic efficacy in T2DM. Thus, co-administration of a specific  
96 glucagon receptor antagonist (O'Harte *et al.* 2014), with a stable long-acting GIP mimetic,  
97 such as D-Ala<sup>2</sup>GIP (Hinke *et al.* 2002; Gault *et al.* 2003), should offer a meaningful  
98 therapeutic advantage.

99 To evaluate the potential of combined glucagon receptor inhibition and GIP receptor  
100 activation in T2DM, we have investigated the effects of sub-chronic treatment with the peptide-  
101 based glucagon receptor antagonist, desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon, and D-Ala<sup>2</sup>GIP in  
102 DIO mice fed a high fat diet. The results provide experimental evidence that GIP mimetics may  
103 prove to be surprisingly useful for the treatment of T2DM when combined with a glucagon  
104 receptor antagonist.

## 105 MATERIALS AND METHODS

### 106 *Peptide synthesis*

107 Glucagon, D-Ala<sup>2</sup>GIP and desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon were produced (>95%  
108 purity) by Fmoc solid-phase peptide synthesis and purchased from GL Biochem Ltd.  
109 (Shanghai, China). All peptides were further characterized in-house using matrix-assisted  
110 laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry, as previously  
111 described (O'Harte *et al.* 2013).

112

### 113 *Circular dichroism (CD)*

114 CD spectra for glucagon and desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon were acquired at the far-  
115 UV region (190-250 nm) using a JASCO J-810 spectropolarimeter. Peptide samples were  
116 prepared by dissolving the analogues in water or in 20 mmol/l phosphate buffer at pH 7.0 to a  
117 final concentration of 30 µM and the concentrations of trifluoroethanol (TFE) used was 15%,  
118 30%, 50% and 70% for each peptide. Parameters used for CD experiments were response  
119 time of 2 s, bandwidth of 1 nm, scanning speed of 50 nm/min and a data pitch of 0.2 nm. All  
120 spectra were acquired at 25°C by accumulation of 15 scans in a 1 mm quartz cell, and the  
121 baseline corrected. Calculation of alpha-helical and beta-sheet content was carried out by the  
122 K2D3 program using the DICHROWEB web interface (Louis-Jeune *et al.* 2012).

123

124 *Acute in vitro insulin release and cAMP measurements*

125 BRIN-BD11 cells were cultured in RPMI-1640 culture medium containing, 10% v/v FBS,  
126 and 11.1 mmol l<sup>-1</sup> glucose, and were seeded at a density of 1x10<sup>5</sup> cells/well in 24 well plates  
127 for insulin release studies or 8x10<sup>4</sup> cells/well in 96 well plates for cAMP studies. Cells were  
128 allowed to attach overnight at 37°C in a LEEC incubator (Laboratory Technical Engineering,  
129 Nottingham, UK) in an atmosphere of 5% CO<sub>2</sub> and 95% air. Prior to insulin and cAMP  
130 studies, the tissue culture medium was removed and cells were pre-incubated with 1 ml KRB  
131 buffer (pH 7.4) supplemented with bovine serum albumin (0.5% w/v), containing 1.1 mmol/l  
132 glucose at 37°C for 40 min. Test incubations were conducted at 5.6 mmol l<sup>-1</sup> glucose over a  
133 20 min incubation period, using individual and combined peptide treatments as shown in the  
134 Figures. For insulin release supernatants were removed and frozen at -20°C prior to  
135 measurement of insulin by radioimmunoassay (Flatt & Bailey, 1981). For cAMP  
136 measurements cells were lysed and total cAMP content was determined using a commercially  
137 available chemiluminescent cAMP immunoassay kit (R&D Systems Europe Ltd., Abingdon,  
138 UK).

139

140 *Animals*

141 NIH Swiss male mice (Harlan Ltd., Oxon, UK) were used at 18 weeks of age. The animals  
142 were housed individually in an air-conditioned room at 22 ± 2°C with a 12 h light:12 h dark  
143 cycle (lights off between 20:00 and 08:00 h). All animals had free access to drinking water  
144 and a high fat (45% fat, 35% carbohydrate and 20% protein, Dietex International Ltd.  
145 Witham, Essex, UK) diet for 100 days prior to commencement of studies. Obesity and  
146 glycaemic dysregulation were clearly manifested compared to age matched mice maintained  
147 on normal laboratory chow (10% fat, 30% protein and 60% carbohydrate, Trouw Nutrition,  
148 Cheshire, UK) as verified by body weight and blood glucose analyses. All experiments were

149 conducted in accordance with the UK Animals (Scientific Procedures) Act 1986, under  
150 project licences approved by the local ethical committee.

151

### 152 *Study Design*

153 Mice received twice daily intraperitoneal (i.p.) injections of saline (0.9% NaCl w/v) at 10:00  
154 and 16.30 h, over a 6 day acclimatisation period. Following this, mice received twice daily  
155 i.p. administration (10:00 and 16.30 h) of saline vehicle (0.9% NaCl w/v) or  
156 desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon alone, D-Ala<sup>2</sup>GIP alone or a combination of both  
157 peptides (all treatments at 25 nmol kg<sup>-1</sup> body weight) over a 28 day period. Doses were  
158 chosen based on our previous extensive *in vivo* assessments with glucagon antagonist and  
159 GIP agonist peptides (Martin *et al.* 2013; O'Harte *et al.* 2014). Food intake was monitored  
160 daily, whereas body weight, circulating blood glucose and plasma insulin concentrations  
161 were assessed at 3-4 day intervals in non-fasted mice at 09.00 h prior to the normal morning  
162 10:00 h peptide administration. At the end of the treatment period, oral and i.p. (18 mmol kg<sup>-1</sup>  
163 bw) glucose tolerance tests were performed in overnight fasted mice. In addition, an insulin  
164 sensitivity (10 U kg<sup>-1</sup> bw) test was also performed in non-fasted mice. At termination,  
165 pancreatic tissue was excised and insulin content measured following extraction with 5 ml g<sup>-1</sup>  
166 of ice-cold acid ethanol (75% ethanol, 23.5% water, 1.5% concentrated HCl).

167

### 168 *Measurement of metabolic rate and locomotor activity*

169 Metabolic rate and locomotor activity were measured using an Oxymax Complex Laboratory  
170 Animal Monitoring System or CLAMS (Columbus Instruments, OH, USA) on day 28. Mice  
171 were acclimatised to the air tight metabolic chambers for 18 h prior to commencement of  
172 observations. Oxygen consumption and carbon dioxide production were monitored for 30 sec  
173 at 15 min intervals over a period of 24 h and respiratory exchange ratios (RER's) were



174 produced to calculate energy expenditure using the following equation;  $(3.815 + 1.232 \times$   
175  $RER) \times VO_2$ . Ambulatory locomotor activity was assessed using the optical beams (Opto  
176 M3, Columbus Instruments, OH, USA). Consecutive photo-beam breaks were scored as an  
177 ambulatory movement. Activity counts in X- and Z- axis were recorded each minute for 24 h.  
178

### 179 *Biochemical analyses*

180 Blood samples were collected from the cut tip on the tail vein of conscious mice into chilled  
181 fluoride/heparin glucose micro-centrifuge tubes (Sarstedt, Numbrecht, Germany) at the time  
182 points indicated in the Figures. Blood glucose was measured directly using a hand-held  
183 Ascencia Contour blood glucose meter (Bayer Healthcare, Newbury, Berkshire, UK). For  
184 plasma insulin analysis, blood samples were immediately centrifuged using a Beckman  
185 microcentrifuge (Beckman Instruments, Galway, Ireland) for 1 min at  $13,000 \times g$  and stored  
186 at  $-20^\circ\text{C}$ . Plasma and pancreatic insulin was assayed by a modified dextran-coated charcoal  
187 RIA (Flatt & Bailey, 1981). In addition, a terminal blood sample was also collected for the  
188 measurement of plasma glucagon via a sandwich immunoassay using a commercially  
189 available kit (Meso Scale Discovery, Gaithersburg, Maryland, USA).

190

### 191 *Statistical analyses*

192 Results are expressed as means  $\pm$  SEM and data compared using ANOVA, followed by a  
193 Student–Newman–Keuls *post hoc* test. Incremental areas under plasma glucose and insulin  
194 curves (AUC) were calculated using a computer-generated program (Prism 5, CA, USA)  
195 employing the trapezoidal rule with baseline subtraction.  $p < 0.05$  was considered to be  
196 significantly different.

197

## 198 **RESULTS**

199 *Circular dichroism analysis of peptides*

200 In aqueous conditions all peptides had an overall random structure. Upon addition of TFE,  
201 two negative dichroic bands at 208 nm and 222 nm were observed indicating the formation of  
202 alpha-helical conformations within the peptide analogues. The alpha-helical content of  
203 glucagon was calculated at 26-31% at high TFE concentrations (Fig 1A). As the  
204 concentration of TFE increased, desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon revealed an overall  
205 trend of increasing alpha-helical concentration with decreased beta-sheet content (Fig 1B). In  
206 comparison to native glucagon (Fig 1A), desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon had increased  
207 alpha-helical content at 15-70% TFE concentrations (Fig 1B).

208

209 *Effects of desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon and D-Ala<sup>2</sup>GIP on acute insulin secretion*  
210 *and cAMP production in BRIN-BD11 cells*

211 The acylated glucagon receptor antagonist, desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon, had no  
212 significant stimulatory effect on either insulin secretion (Fig 2A) or cAMP production (Fig  
213 2B) in BRIN-BD11 cells. However, the stable GIP agonist, D-Ala<sup>2</sup>GIP, induced a significant  
214 (p<0.01 to p<0.001) concentration-dependant (10<sup>-8</sup> to 10<sup>-6</sup> M) increase in insulin secretion  
215 when compared with a 5.6 mmol l<sup>-1</sup> glucose control (Fig 2A). Co-incubation of  
216 desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon (10<sup>-7</sup> M) with D-Ala<sup>2</sup>GIP had no effect on D-Ala<sup>2</sup>GIP-  
217 mediated insulin release (Fig 2A). Furthermore, desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon (10<sup>-7</sup>  
218 M) had no inhibitory effect on D-Ala<sup>2</sup>GIP-induced (p<0.01) cAMP production (Fig 2B).

219

220 *Effects of 28-days administration of desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon and D-Ala<sup>2</sup>GIP on*  
221 *metabolic parameters in high fat diet-induced obese mice*

222 Twice daily treatment with desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon or D-Ala<sup>2</sup>GIP alone, and in  
223 combination, for 28 days had no significant effect on body weight (Fig 3A) or food intake

224 (Fig 3C). However, total body fat mass was significantly ( $p < 0.01$  to  $p < 0.001$ ) reduced in all  
225 treatment groups (Fig 3B), specifically saline control, desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon  
226 and D-Ala<sup>2</sup>GIP treated high fat mice had body fat masses of  $40.3 \pm 0.6\%$ ,  $33.8 \pm 0.8\%$  and  
227  $37.7 \pm 0.5\%$ , respectively, compared to a body fat mass of  $37.8 \pm 0.5\%$  in lean control mice'.  
228 In addition, D-Ala<sup>2</sup>GIP treated mice and those given the combination of both peptides had  
229 increased ( $p < 0.01$ ) body fat percentage compared to desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon  
230 treatment alone (Fig 3B). Furthermore, a significant ( $p < 0.05$  to  $p < 0.001$ ) decrease in  
231 circulating blood glucose was observed in all three treatment groups from day 10 onwards  
232 when compared to saline controls (Fig 3D). In addition, D-Ala<sup>2</sup>GIP induced a highly  
233 significant ( $p < 0.05$  to  $p < 0.001$ ) increase in circulating insulin on day 28 compared to all other  
234 groups (Fig 3E), whereas desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon monotherapy had no effect  
235 on plasma insulin levels (Fig 3E). D-Ala<sup>2</sup>GIP mediated elevations in plasma insulin were  
236 partially restrained by combined desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon therapy, although  
237 values still remained significantly ( $p < 0.05$ ) higher compared to desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-  
238 glucagon alone from days 17 through to 28 (Fig 3E). Circulating plasma glucagon  
239 concentrations were significantly ( $p < 0.01$ ) elevated in D-Ala<sup>2</sup>GIP mice compared to saline  
240 and desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon treated mice at the end of the study, whereas  
241 desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon treatment alone resulted in a significant ( $p < 0.05$ )  
242 reduction in glucagon concentrations (Fig 3F). Combined administration of both peptides  
243 resulted in no significant change in plasma glucagon concentrations compared to high fat  
244 control mice (Fig. 3F). Thus, desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon prevented the significant  
245 ( $p < 0.05$ ) augmentation of circulating glucagon induced by D-Ala<sup>2</sup>GIP treatment alone. Interestingly,  
246 the insulin:glucagon molar ratios on day 28 were 23:1, 71:1 and 44:1 in  
247 desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon, D-Ala<sup>2</sup>GIP and the combined treatment groups,  
248 respectively, compared to 17:1 in saline treated controls.

249

250 *Effects of 28-days administration of desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon and D-Ala<sup>2</sup>GIP on*  
251 *glucose tolerance and insulin sensitivity in high fat diet-induced obese mice*

252 All treatment groups had significantly ( $p<0.05$ - $p<0.01$ ) reduced blood glucose excursions  
253 during an i.p. glucose tolerance test when compared to saline controls (Fig 4A,B). In  
254 addition, D-Ala<sup>2</sup>GIP treatment was associated with a significantly ( $p<0.01$ ) enhanced overall  
255 insulinotropic response in comparison to control mice (Fig 4C,D). Similarly, during an oral  
256 glucose challenge, blood glucose levels were significantly ( $p<0.01$ ) reduced 30 and 60 min  
257 post administration in all treatment groups (Fig 5A). In harmony with observations following  
258 an i.p. glucose load, D-Ala<sup>2</sup>GIP treatment significantly enhanced the individual ( $p<0.05$  to  
259  $p<0.001$ ) and overall ( $p<0.05$ ) insulin secretory response following oral glucose delivery  
260 when compared to all other groups of mice (Fig 5C,D). Likewise, combined treatment of D-  
261 Ala<sup>2</sup>GIP with desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon also enhanced ( $p<0.05$ ) the overall  
262 insulin secretory response (Fig 5C,D). As shown in Figure 6, treatment with D-Ala<sup>2</sup>GIP  
263 alone, or in combination with desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon, significantly ( $p<0.01$ )  
264 improved the glucose-lowering action of exogenous insulin at 30 and 60 min post insulin  
265 injection when compared to saline controls (Fig 6A). Treatment with  
266 desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon alone also resulted in a significant ( $p<0.01$ ) reduction  
267 in blood glucose levels at 60 min post insulin injection (Fig 6A). Moreover, the overall  
268 glucose-lowering effect of insulin was significantly ( $p<0.05$  to  $p<0.01$ ) enhanced in all  
269 treatment groups (Fig 6B). Interestingly, pancreatic insulin content was significantly ( $p<0.05$   
270 to  $p<0.01$ ) higher in mice treated with D-Ala<sup>2</sup>GIP alone, or in combination with  
271 desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon, when compared to saline controls or  
272 desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon treatment alone (Fig 6C).

273

274 *Effects of 28-days administration of desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon and D-Ala<sup>2</sup>GIP on*  
275 *metabolic rate and locomotor activity in high fat diet-induced obese mice*

276 Treatment with desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon alone, and in combination with D-  
277 Ala<sup>2</sup>GIP, significantly (p<0.001) increased energy expenditure during the dark phase  
278 compared to saline-treated controls and D-Ala<sup>2</sup>GIP treatment alone (Fig 7A). Respiratory  
279 exchange ratio was not different between groups of mice (Fig 7B). Ambulation, as assessed  
280 by X-beam breaks, was significantly (p<0.05) elevated in mice treated with  
281 desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon alone, or in combination with D-Ala<sup>2</sup>GIP (Fig 7C). D-  
282 Ala<sup>2</sup>GIP treatment did not affect x-beam breaks when compared to control mice (Fig 7C). All  
283 three treatment groups had significantly (p<0.05 to p<0.001) increased numbers of Z-beam  
284 breaks compared to controls, with the combination treatment group also displaying increased  
285 Z-beam breaks when compared to individual treatment regimens (Fig7D). Energy  
286 expenditure, ambulatory activity and Z-beam breaks were not significantly different between  
287 groups during the light phase (data not shown).

288

## 289 **DISCUSSION**

290 Notwithstanding encouraging preclinical data (Bagger *et al.* 2011; Trujillo & Nuffer 2014),  
291 the progression of monotherapy glucagon antagonist or GIP agonist based therapies to the  
292 clinic is lacking. This is despite knowledge that a potential major beneficial effect of the most  
293 widely used antidiabetic drug, metformin, is mediated through inhibition of glucagon action  
294 (Pernicova & Korkonits 2014). Furthermore, recent studies have shown that targeting  
295 multiple regulatory hormone receptors may be a viable treatment option for T2DM (Patel *et*  
296 *al.* 2013; Trevaskis *et al.* 2013; Skarbaliene *et al.* 2015). As such, the dual activation of  
297 incretin-related pathways coupled with glucagon receptor blockade significantly improves  
298 metabolic control in diabetes (Claus *et al.* 2007; Mu *et al.* 2011). Given that a documented

299 therapeutic drawback of GIP mimetics relates to elevation of glucagon levels (Meier &  
300 Nauck 2004, 2015), combined therapy with a specific glucagon antagonist would seem  
301 logical. Here we assessed the benefits of combining the glucagon receptor  
302 antagonist desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon (O'Harte *et al.* 2014) with the well  
303 characterised DPP-4 resistant GIP analogue, D-Ala<sup>2</sup>GIP (Hinke *et al.* 2002; Widenmaier *et*  
304 *al.* 2010). We aimed to prove the concept that desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon could  
305 counter GIP-related elevations of blood glucagon levels.

306 Structure function studies with native glucagon have shown that the C-terminal  
307 portion of peptide, which exhibits an alpha-helical conformation, is involved in receptor  
308 recognition, with the N-terminal more important for receptor signalling (Sturm *et al.* 1998).  
309 In the present study, desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon had an increased  $\alpha$ -helical content  
310 when compared with native glucagon, a trait that is strongly associated with increased  
311 receptor binding potency (Krstenansky *et al.* 1988). As previously shown by the leading  
312 synthetic peptide groups of Hruby and Merrifield, and later independently confirmed in our  
313 laboratory, His<sup>1</sup>, Gly<sup>4</sup> and Asp<sup>9</sup> are essential for normal agonist activity of glucagon at the  
314 level of the receptor (Hruby 1982; Unson *et al.* 1991, 1993; Ahn *et al.* 2001; O'Harte *et al.*  
315 2013, McShane *et al.* 2014; Franklin *et al.* 2014). In complete harmony with this structural  
316 data, desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon is known to inhibit glucagon-induced elevations  
317 of cAMP generation and insulin secretion (O'Harte *et al.* 2013). Both the glucagon and GIP  
318 receptors belong to the same family of G-protein coupled receptors (Brubaker & Drucker,  
319 2002) and share considerable structural homology (Kogire *et al.* 1992). However,  
320 desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon did not adversely hinder the insulintropic and cAMP  
321 potentiating effect of D-Ala<sup>2</sup>GIP (Martin *et al.* 2013), further confirming specificity.

322 As would be expected, twice daily treatment with either desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-  
323 glucagon or D-Ala<sup>2</sup>GIP in high fat fed mice reproduced many of the beneficial effects

324 previously noted with sustained GIP receptor activation (Kerr *et al.* 2009; Porter *et al.* 2011)  
325 or glucagon receptor blockade (Lotfy *et al.* 2014; McShane *et al.* 2014; O'Harte *et al.* 2014).  
326 This included significant reductions in circulating blood glucose levels and improvements in  
327 peripheral glucose disposal. Beneficial effects of both treatment regimens were independent  
328 of alterations in body weight or energy intake. Previous studies have indicated that glucagon  
329 can decrease food intake (Habegger *et al.* 2010; Kosinski *et al.* 2012), however our studies  
330 with peptide-based glucagon antagonists suggest that contrasting elevations of energy intake  
331 do not occur with glucagon receptor inhibition (Franklin *et al.* 2014; McShane *et al.* 2014;  
332 O'Harte *et al.* 2014). This probably reflects the complex neural pathways and plasticity  
333 involved in the regulation of feeding and energy balance (Dockray & Burdyga 2011).  
334 Combined therapy with desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon and D-Ala<sup>2</sup>GIP did not result  
335 in discernible benefits on blood glucose or glucose disposal when compared to either  
336 treatment alone. This likely reflects the good efficacy of each treatment alone and the  
337 relatively high doses employed, which could preclude additive action. Indeed, circulating  
338 blood glucose levels were around 5-6 mmol/l in each treatment group by the end of the study.  
339 As has been proposed, circulating glucagon levels were significantly elevated by D-Ala<sup>2</sup>GIP  
340 treatment (Meier & Nauck 2004, 2015), but this detrimental effect was completely annulled  
341 by concurrent administration of desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon. Moreover, circulating  
342 insulin concentrations were reduced in mice treated with a combination of  
343 desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon and D-Ala<sup>2</sup>GIP, when compared to D-Ala<sup>2</sup>GIP alone,  
344 implying improved insulin action in these mice, since ambient glucose levels were essentially  
345 similar. Indeed, pancreatic insulin stores and the insulin:glucagon ratio were substantially  
346 augmented by dual therapy on day 28 when compared to desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-  
347 glucagon treatment alone, which also points towards decreased insulin demand in the  
348 combined treatment group. Plasma glucagon levels were actually reduced by

349 desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon monotherapy, which is somewhat unexpected given  
350 previous observations (Bagger *et al.* 2011), and therefore does require further investigation.

351 Interestingly, total body fat mass was lowered in all treatment groups without change  
352 in overall body weight, suggesting a possible shift towards the use of stored fat as an energy  
353 source. Somewhat surprisingly, although in agreement with increased fat utilisation, energy  
354 expenditure was increased during the dark phase in all mice receiving  
355 desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon treatment. Thus, glucagon receptor activation is  
356 generally associated with enhanced energy expenditure (Campbell & Drucker 2015) implying  
357 that counter-regulatory mechanisms may be important for the benefits of sustained glucagon  
358 receptor inhibition in the present study. However, respiratory exchange ratio was unaltered  
359 between groups with values of approximately 0.85, indicating a similar combination of fat  
360 and carbohydrates utilisation. Interestingly, mice with genetic knock out of synaptotagmin-7,  
361 a regulator of glucagon and insulin secretion, present with reduced circulating glucagon  
362 levels and increased energy expenditure (Lou *et al.* 2011), in harmony with the current  
363 findings. Ambulatory locomotion was also elevated only in mice where glucagon receptor  
364 action was inhibited. The overall significance of these centrally mediated effects requires  
365 further detailed elucidation. Moreover, the passage of both desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-  
366 glucagon and D-Ala<sup>2</sup>GIP through the blood brain barrier also requires investigation.

367 Improvements in glucose tolerance and glucose-stimulated insulin release are a  
368 previously reported feature of D-Ala<sup>2</sup>GIP treatment in high fat fed mice (Gault *et al.* 2011).  
369 Indeed, studies suggest that high fat feeding increases islet GIP receptor expression (Harada  
370 *et al.* 2008; Moffett *et al.* 2015). Thus, the GIP analogue may be able to independently  
371 overcome any potential GIP-resistance (Nauck *et al.* 1993) in this mouse model of T2DM.  
372 Similarly, improvements in response to both oral and intraperitoneal glucose challenge was  
373 observed in desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon mice, consistent with previous studies



374 (O'Harte *et al.* 2013). This was despite any obvious increase of glucose-stimulated insulin  
375 secretion in desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon treated mice. In agreement with this,  
376 peripheral insulin sensitivity was dramatically improved by desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-  
377 glucagon, however this was also the case for all treatment paradigms. Thus, improved insulin  
378 action might simply be a reflection of decreased glucose toxicity in each treatment group, due  
379 to lower circulating blood glucose concentrations. This might also be a reason for the lack of  
380 benefit of the combined treatment regimen. More interestingly, D-Ala<sup>2</sup>GIP-induced  
381 elevations of insulin secretion appeared to be blunted by co-administration of  
382 desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon following intraperitoneal glucose, but much less so  
383 following oral glucose administration. This would suggest D-Ala<sup>2</sup>GIP treatment alone, and in  
384 combination with desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon, enhances the incretin axis in high  
385 fat fed mice (Moffett *et al.* 2015). Indeed, this could be linked to augmented secretion and/or  
386 action of GLP-1 following an oral glucose challenge in these mice, as suggested previously  
387 (Parker *et al.* 2002; Gelling *et al.* 2003).

388           In conclusion, the present study indicates that twice daily injection of either D-  
389 Ala<sup>2</sup>GIP or desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon is an effective means of improving  
390 diabetic control in obese-diabetic high fat fed mice. There was some limited evidence for  
391 benefits following combined treatment, but this requires further detailed study to assess the  
392 relative importance. As such, studies utilising various concentration and ratios of individual  
393 peptides could be interesting and might reveal further benefits. Importantly however,  
394 combined therapy of desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon with D-Ala<sup>2</sup>GIP did completely  
395 annul GIP-induced elevations of circulating glucagon levels and augment pancreatic insulin  
396 stores, confirming proof of concept. Furthermore, it may be interesting to examine the  
397 metabolic benefits of sustained glucagon inhibition in combination with GLP-1 receptor  
398 activation, or in other animal models of diabetes. Taken together the data presented here

399 provide evidence for the usefulness of peptide-based GIP receptor agonist and glucagon  
400 receptor antagonist therapies for the treatment of T2DM.

401

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405

406 **Conflict of Interest:**

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

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## Figure legends

**Figure 1. Circular dichroism spectra of (A) glucagon and (B) desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>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<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon and D-Ala<sup>2</sup>GIP.** (A) BRIN-BD11 cells were exposed to varying concentrations ( $10^{-12}$  –  $10^{-6}$  M) of desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon, D-Ala<sup>2</sup>GIP and D-Ala<sup>2</sup>GIP in the presence of  $10^{-7}$  M desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>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  $\pm$  SEM (n=8) where \*\*p<0.01, \*\*\*p<0.001 compared with 5.6 mmol glucose control.  $\Delta\Delta$ p<0.01,  $\Delta\Delta\Delta$ p<0.001 compared with desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon alone.

**Figure 3. Effects of twice daily treatment with desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon and D-Ala<sup>2</sup>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<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon, D-Ala<sup>2</sup>GIP or desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon in combination with D-Ala<sup>2</sup>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  $\pm$  SEM for 8 mice. \*p<0.05, \*\*p<0.01 and \*\*\*p<0.001 compared with saline group.  $\Delta$ p<0.05,  $\Delta\Delta$ p<0.01 and  $\Delta\Delta\Delta$ p<0.001 compared with desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon treatment alone.  $^+$ p<0.05 and  $^{++}$ p<0.01 compared with combined treatment group.

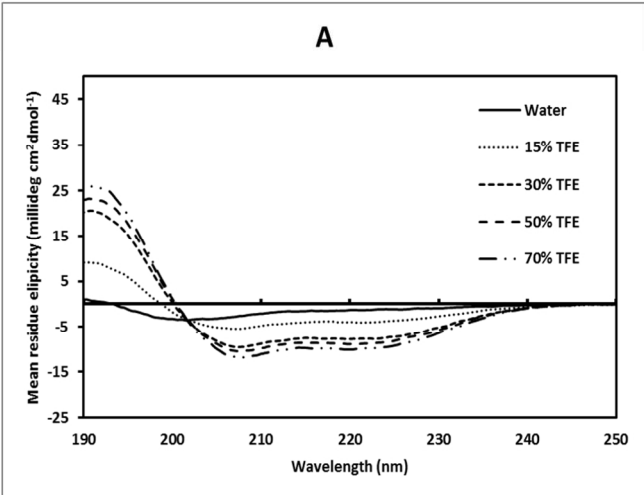
**Figure 4. Effects of twice daily treatment with desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon and D-Ala<sup>2</sup>GIP alone, or in combination on intraperitoneal glucose tolerance and plasma insulin response to glucose in high fat mice.** Tests were conducted after 28 days twice daily treatment with saline vehicle (0.9% w/v NaCl), desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon, D-Ala<sup>2</sup>GIP or desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon in combination with D-Ala<sup>2</sup>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. <sup>Δ</sup>p<0.05 compared with desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon treatment alone.

**Figure 5. Effects of twice daily treatment with desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon and D-Ala<sup>2</sup>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<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon, D-Ala<sup>2</sup>GIP or desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon in combination with D-Ala<sup>2</sup>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. <sup>Δ</sup>p<0.05 and <sup>ΔΔ</sup>p<0.01 compared with desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon treatment alone. <sup>+</sup>p<0.05 compared with combined treatment group.

**Figure 6. Effects of twice daily treatment with desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon and D-Ala<sup>2</sup>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<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon, D-Ala<sup>2</sup>GIP or desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon in combination with D-Ala<sup>2</sup>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  $\pm$  SEM for 8 mice. \*p<0.05 and \*\*p<0.01 compared with saline-treated control group.  $\Delta$ p<0.05 compared with desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon treatment alone.  $^+$ p<0.05 compared with combined treatment group.

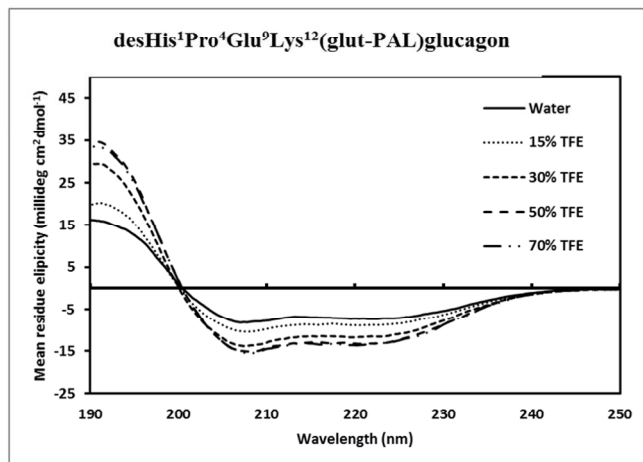
**Figure 7. Effects of twice daily treatment with desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon and D-Ala<sup>2</sup>GIP alone, or in combination on metabolic rate and locomotor activity in high fat mice.** Parameters were measured after 28 days twice daily treatment with saline vehicle (0.9% w/v NaCl), desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon, D-Ala<sup>2</sup>GIP or desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon in combination with D-Ala<sup>2</sup>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  $\pm$  SEM for 6 mice. \*p<0.05 and \*\*\*p<0.001 compared with saline group.  $\Delta$ p<0.05 and  $\Delta\Delta\Delta$ p<0.001 compared with desHis<sup>1</sup>Pro<sup>4</sup>Glu<sup>9</sup>(Lys<sup>12</sup>PAL)-glucagon treatment alone.  $^+$ p<0.05,  $^{++}$ p<0.01 and  $^{+++}$ p<0.001 compared with combined treatment group.

Figure 1



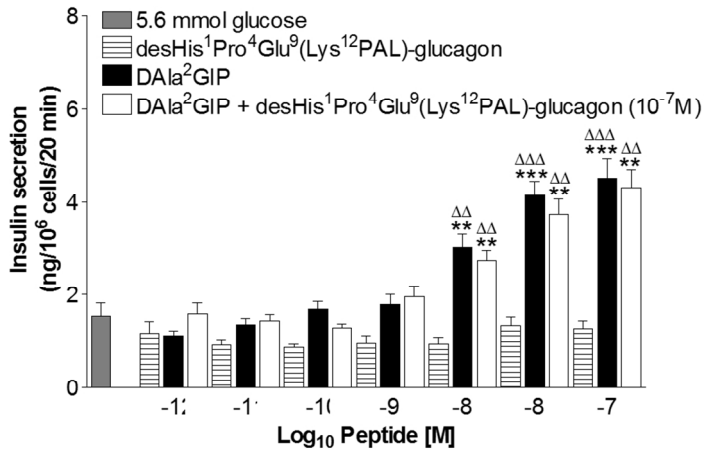
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Figure 1 B



338x190mm (96 x 96 DPI)

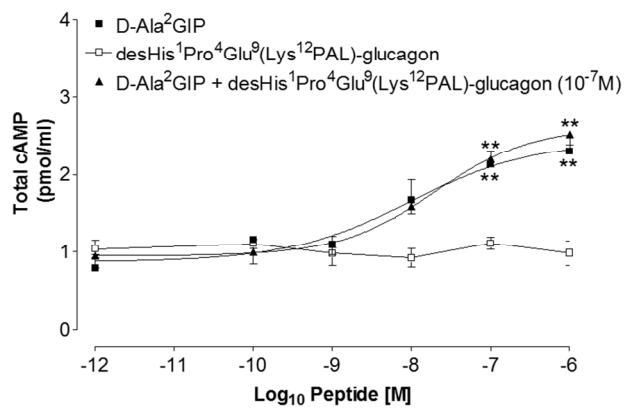
Figure 2A



338x190mm (96 x 96 DPI)



Figure 2B



338x190mm (96 x 96 DPI)

Figure 3A

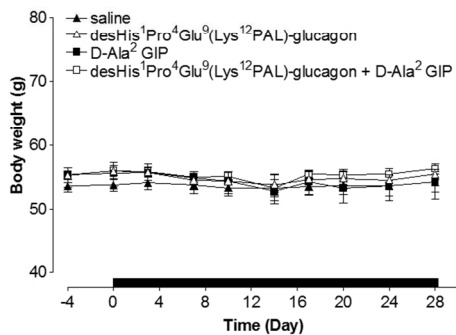
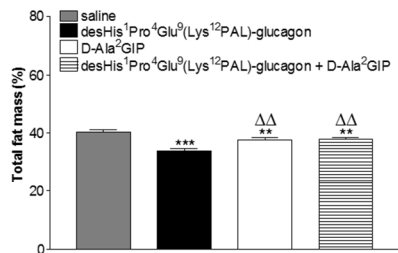


Figure 3B



338x190mm (96 x 96 DPI)

Figure 3C

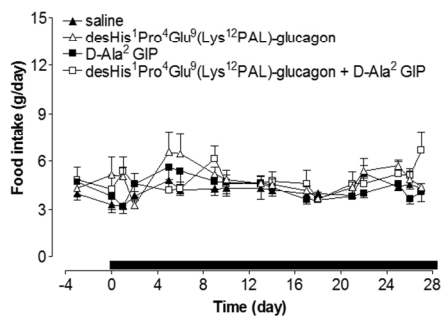
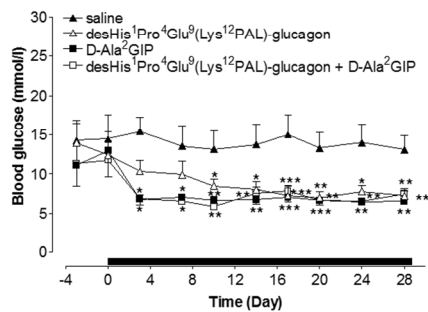


Figure 3D



338x190mm (96 x 96 DPI)

Figure 3E

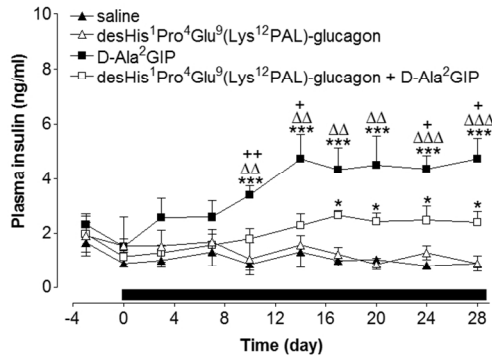
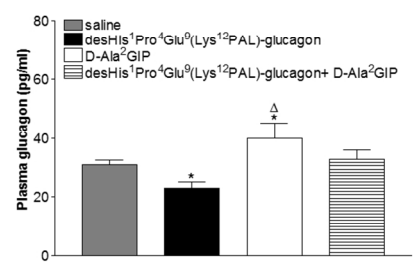


Figure 3F



338x190mm (96 x 96 DPI)

Figure 4A

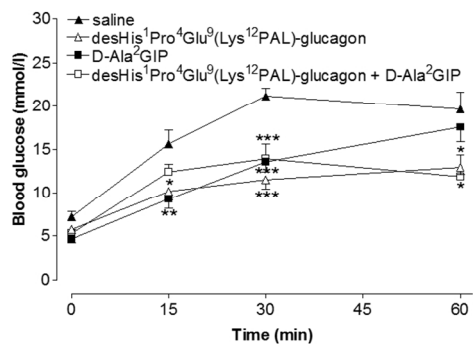
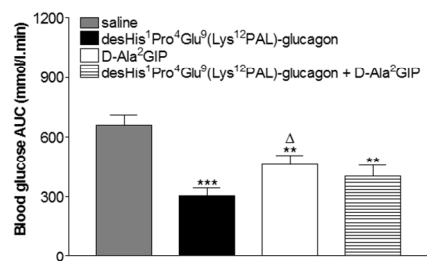


Figure 4B



338x190mm (96 x 96 DPI)

Figure 4C

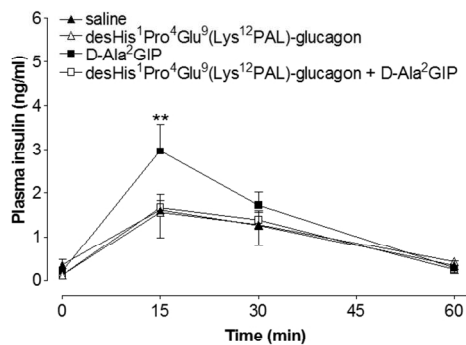
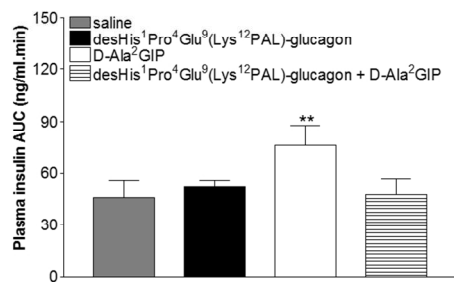


Figure 4D



338x190mm (96 x 96 DPI)

Figure 5A

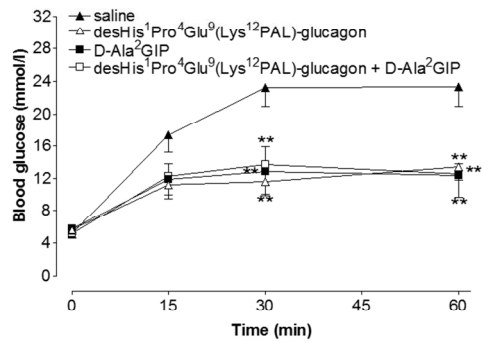
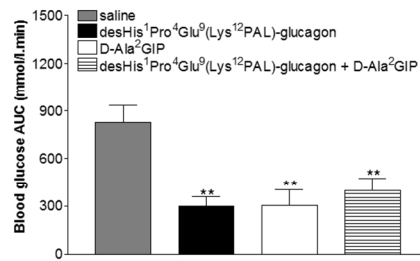


Figure 5B



338x190mm (96 x 96 DPI)

Figure 5C

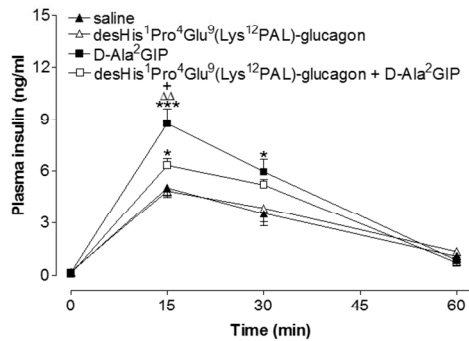
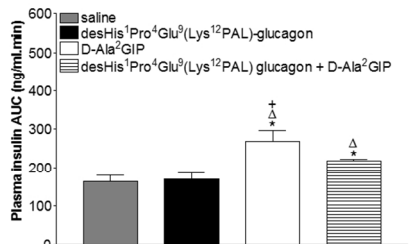


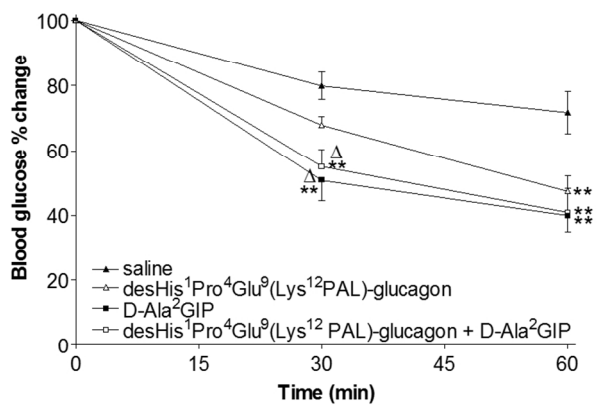
Figure 5D



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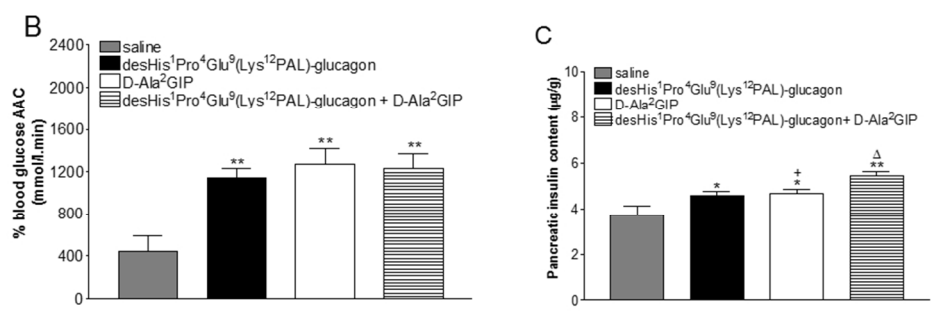


Figure 6A



338x190mm (96 x 96 DPI)

Figure 6B + 6C



254x190mm (96 x 96 DPI)

Figure 7A

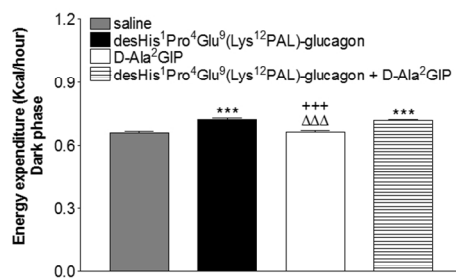
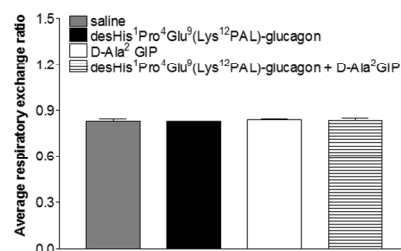


Figure 7B



338x190mm (96 x 96 DPI)

Figure 7C

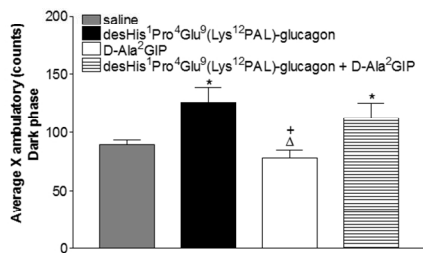
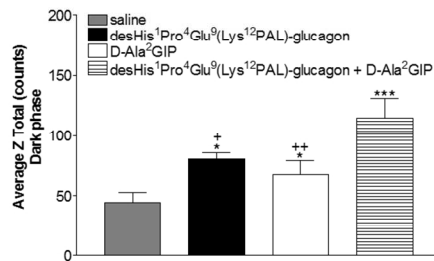


Figure 7D



338x190mm (96 x 96 DPI)