RESEARCH

Ψ -Xenin-6 enhances sitagliptin effectiveness, but does not improve glucose tolerance

Sarah L Craig¹, Victor A Gault¹, Gerd Hamscher² and Nigel Irwin¹¹SAAD Centre for Pharmacy and Diabetes, University of Ulster, Coleraine, Northern Ireland, UK²Institute of Food Chemistry and Food Biotechnology, Justus Liebig University Giessen, Giessen, GermanyCorrespondence should be addressed to N Irwin: n.irwin@ulster.ac.uk

Abstract

Recent studies have characterised the biological properties and glucose-dependent insulinotropic polypeptide (GIP) potentiating actions of an enzymatically stable, C-terminal hexapeptide fragment of the gut hormone xenin, namely Ψ -xenin-6. Given the primary therapeutic target of clinically approved dipeptidyl peptidase-4 (DPP-4) inhibitor drugs is augmentation of the incretin effect, the present study has assessed the capacity of Ψ -xenin-6 to enhance the antidiabetic efficacy of sitagliptin in high fat fed (HFF) mice. Individual administration of either sitagliptin or Ψ -xenin-6 alone for 18 days resulted in numerous metabolic benefits and positive effects on pancreatic islet architecture. As expected, sitagliptin therapy was associated with elevated circulating GIP and GLP-1 levels, with concurrent Ψ -xenin-6 not elevating these hormones or enhancing DPP-4 inhibitory activity of the drug. However, combined sitagliptin and Ψ -xenin-6 therapy in HFF mice was associated with further notable benefits, beyond that observed with either treatment alone. This included body weight change similar to lean controls, more pronounced and rapid benefits on circulating glucose and insulin as well as additional improvements in attenuating gluconeogenesis. Favourable effects on pancreatic islet architecture and peripheral insulin sensitivity were more apparent with combined therapy. Expression of hepatic genes involved in gluconeogenesis and insulin action were partially, or fully, restored to normal levels by the treatment regimens, with beneficial effects more prominent in the combination treatment group. These data demonstrate that combined treatment with Ψ -xenin-6 and sitagliptin did not alter glucose tolerance but does offer some metabolic advantages, which merit further consideration as a therapeutic option for type 2 diabetes.

Key Words

- ▶ beta-cell
- ▶ diabetes
- ▶ DPP-4
- ▶ insulin
- ▶ xenin

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Introduction

Dipeptidyl peptidase-4 (DPP-4) inhibitors are an orally available class of drugs, clinically approved for the treatment of type 2 diabetes mellitus (T2DM) (Scott 2017). The primary therapeutic benefit of DPP-4 inhibitors in T2DM relates to preventing degradation and subsequent loss of bioactivity of the endogenous intestinal-derived incretin hormones, glucagon-like peptide-1 (GLP-1) and glucose-dependent

insulinotropic polypeptide (GIP) (Deacon 2019). Thus, GLP-1 and GIP stimulate glucose-dependent insulin secretion and improve glycaemic status, resulting in reduced glycated haemoglobin concentrations (Baggio & Drucker 2007). These hormones also exert a number of extrapancreatic actions that have beneficial glucose homeostatic effects (O'Harte *et al.* 1998, Baggio & Drucker 2007).

However, a key pathophysiological aspect of T2DM relates to an impaired incretin effect (Holst & Ørskov 2004). As such, circulating levels of GLP-1 are believed to be reduced in T2DM, while the insulinotropic effects of GIP are severely diminished (Knop *et al.* 2007). DPP-4 inhibition will increase circulating concentrations of biologically active GLP-1 (Ahrén 2007), but does not correct GIP insensitivity in T2DM. In this regard, a related 25 amino acid gut-derived hormone known as xenin has been shown to augment the biological actions of GIP *in vitro* and *in vivo* (Wice *et al.* 2010, Martin *et al.* 2012, Gault *et al.* 2015a). In addition, xenin also independently stimulates insulin secretion and suppresses appetite (Taylor *et al.* 2010, Martin *et al.* 2012, 2014, 2016, Gault *et al.* 2015b, Craig *et al.* 2018), which would be further advantageous in T2DM. Unlike GIP and GLP-1, xenin is not degraded by DPP-4 (Taylor *et al.* 2010), and thus DPP-4 inhibitors do not alter circulating xenin levels. However, enzymatic degradation fragment peptides of xenin are present in plasma (Martin *et al.* 2012), with some of these shown to retain the parent peptide biological action profile (Martin *et al.* 2014, 2016, Parthsarathy *et al.* 2016, Craig *et al.* 2019). In this regard, xenin-6 appears to be the shortest xenin form that recapitulates the key physiological actions of xenin at the level of the endocrine pancreas (Craig *et al.* 2019).

Recent studies in our laboratory (Craig *et al.* 2019) have followed up on the initial characterisation of an enzymatically stabilised xenin-6 form, namely Ψ -xenin-6 (Feurle *et al.* 2003). Ψ -Xenin-6 is a C-terminal hexapeptide of xenin with a reduced pseudopeptide bond (CH₂NH) positioned between the first two N-terminal amino acid residues that possesses a significantly extended pharmacokinetic profile (Feurle *et al.* 2003). In our hands, Ψ -xenin-6 stimulated insulin secretion from *in vitro* and *ex vivo* systems, promoted beta-cell proliferation and survival, augmented glucose homeostasis, insulin secretion and satiety in rodents as well as amplified the insulinotropic actions of GIP (Craig *et al.* 2019). Thus, combination therapy with a DPP-4 inhibitor and Ψ -xenin-6 represents an exciting potential therapeutic option for T2DM. Recent positive advances in the field of peptide hormone therapeutics and T2DM relate to the targeting of multiple receptor signalling pathways (Bhat *et al.* 2013, Irwin *et al.* 2015, Khajavi *et al.* 2017, Coskun *et al.* 2018, Frias *et al.* 2018). Such strategies linked to observations of rapid remission of T2DM subsequent to certain types of bariatric surgeries, being directly associated with distinct changes in the secretion and action of numerous gut-derived peptide hormones (Vincent & Le Roux 2008, Knop & Taylor 2013).

In the current study we have created a novel and targeted combinational therapeutic approach by employing Ψ -xenin-6 administration alongside the established DPP-4 inhibitor sitagliptin. We hypothesised that the complementary biological actions of both compounds would significantly improve diabetic status beyond that observed with either treatment alone. Thus, the major benefit of sitagliptin in diabetes therapy relates specifically to GLP-1 induced effects (Bennett 2018), whereas Ψ -xenin-6 will promote additional antidiabetic actions of the increased GIP levels encouraged by sitagliptin. As such, our primary objective was to examine the therapeutic efficacy of a 18-day combined administration of Ψ -xenin-6 and sitagliptin in high fat fed (HFF) mice. Effects on body weight, energy intake, circulating glucose, insulin, GIP and GLP-1, as well as glucose and pyruvate tolerance, peripheral insulin sensitivity, expression of key genes involved in liver glucose metabolism and insulin action, as well as DPP-4 activity and pancreatic islet morphology were assessed.

Research design and methods

Animals studies

Animal studies were conducted in male C57BL/6 mice (10–12 weeks old, Envigo Ltd, London, UK, $n=8$). Prior to commencement of experiments, mice were given free access to high fat diet (45% fat, 20% protein and 35% carbohydrate, Special Diet Services, Witham, Essex, UK) for 4 weeks. During this 4-week period, HFF mice then received a low dose streptozotocin (STZ) injection (4 h fast, 50 mg/kg bw, i.p., in sodium citrate buffer, pH 4.5) on week 2 of high fat feeding, with a second STZ injection (4 h fast, 75 mg/kg bw, i.p., in sodium citrate buffer, pH 4.5) on week 3. Mice exhibiting blood glucose concentrations greater than 11.1 mM at the end of the 4-week period were then recruited onto the study and termed 'HFF mice' throughout. It would have been interesting to investigate pancreatic islet architecture at this point, prior to treatment initiation, but this was not possible in the current study. An additional group of mice ($n=8$) were maintained on normal laboratory chow throughout (10% fat, 30% protein and 60% carbohydrate, Trouw Nutrition, Ashbourne, Derbyshire, UK). These mice received buffer saline injection instead of STZ and were employed as gold standard controls. All mice were housed individually in an air-conditioned room at 22±2°C with a 12 h light: 12 h darkness cycle and experiments were carried out in accordance with the UK Animals Scientific Procedures Act

1986. Studies were approved by the University of Ulster Animal Welfare and Ethical Review Body (AWERB). No adverse effects were observed after drug treatment.

Experimental design

HFF mice ($n=8$) received oral sitagliptin phosphate monohydrate once daily (50 mg/kg bw; ApexBio Technology, Houston, TX, USA; >97% purity; MW 523.3; CAS No. 654671-77-9), intraperitoneal (i.p.) Ψ -xenin-6 twice daily (25 nmol/kg bw, Saxon Biochemicals, Hannover, Germany) or a combination of both compounds using the same dosing regimen for 18 days. All twice daily treatments were administered at 09:30 h and 17:00 h, with once daily treatments delivered at 09:30 h. Saline vehicle (0.9% w/v, NaCl; i.p. or oral) was administered as control at each time point either orally or intraperitoneally, as appropriate. Doses of sitagliptin and Ψ -xenin-6 were chosen based on previous published studies in mice (Gault *et al.* 2015b, Craig *et al.* 2019). Lean control mice ($n=8$) were also administered saline vehicle twice daily (orally at 09:30 h and i.p. at 17:00 h) throughout the study. Cumulative energy intake, body weight, circulating glucose and insulin concentrations were assessed at regular intervals during the study. At the end of the treatment period, glucose tolerance (18 mmol/kg bw; i.p.; 18 h-fasted mice), insulin sensitivity (25 U/kg bovine insulin; i.p.; non-fasted mice) and pyruvate tolerance (2 g/kg sodium pyruvate; i.p.; 18 h-fasted mice) were assessed. HOMA-IR, fasting glucose (mmol/L) $\times$ fasting insulin (mU/L)/22.5, was also calculated as a surrogate marker of insulin resistance. Terminal analyses included extraction of pancreatic tissue with appropriate processing for measurement of hormone content following acid/ethanol protein extraction or islet architecture, as described previously (Khan *et al.* 2017). Tissue blocks were sectioned (7 μ m) using a Shandon Finesse 325 microtome (Thermo Scientific) and picked for staining at intervals of ten sections. Approximately 60–80 islets were analysed per group. In addition, liver tissue was processed for hepatic gene expression by quantitative PCR after total RNA extraction, as described previously (Moffett *et al.* 2013). RealTime ready quantitative PCR target-specific primers (Roche Diagnostics) included: phosphoenolpyruvate carboxykinase (*Pepck*), glucose 6-phosphatase (*G6pase*), insulin receptor substrate-1 (*Irs1*), fibroblast growth factor 21 (*Fgf21*) and serine-threonine protein kinase (*Akt1*). β -Actin (*Actb*) was used as an internal control for normalisation. PCR conditions were 95°C for 10 min, followed by cDNA amplification for 45 cycles with 95°C denaturation for 10 s, 60°C annealing

for 30 s and 72°C elongation for 1 s, followed by 30 s cooling at 40°C. Relative quantification using the $2^{-\Delta\Delta CT}$ (two delta, delta comparative test) method was used to calculate differences in gene expression between samples.

Biochemical analysis

Blood samples were collected from the cut tip of the tail vein of conscious mice into chilled fluoride/heparin glucose microcentrifuge tubes (Sarstedt, Numbrecht, Germany). Blood glucose was measured directly using a Contour blood glucose meter. For plasma insulin analysis, blood samples were collected into chilled fluoride/heparin glucose microcentrifuge tubes (Sarstedt, Numbrecht, Germany) and immediately centrifuged using a Beckman microcentrifuge (Beckman Instruments) for 1 min at 13,000 g and stored at -20°C . Plasma and pancreatic insulin were then assayed using a modified dextran-coated charcoal RIA (Flatt & Bailey 1982). Total GLP-1 and GIP plasma concentrations were determined using specific enzyme linked immunoassays (GLP-1 Total ELISA, EZGLP-1T-36K, Millipore; Rat/mouse GIP (Total) ELISA, EZRMGIP-55K, Millipore), following manufacturer's instructions. DPP-4 activity of terminal plasma, as well as sitagliptin and Ψ -xenin-6 alone and in combination *in vitro* (both at 10^{-6} M), were determined using a fluorometric assay that measures free 7-amino-4-methyl-coumarin (AMC) liberated from Gly-Pro-AMC substrate, as described in more detail previously (Lindsay *et al.* 2005). Briefly, 10 μ L of plasma ($n=8$) or peptide ($n=4$) sample, where appropriate, was incubated with 30 μ L of Tris-HCl buffer (0.02 M, pH 8.0) containing 0.10 M NaCl and 1 mM EDTA and 50 μ L of H-Gly-Pro-AMC (200 μ M). Following 5 min incubation at 37°C , the reaction with the peptide samples was initiated by addition of 10 μ L DPP-4 (8 mU mL $^{-1}$). The change in fluorescence was monitored over a 30-min period using a plate reader at excitation and emission wavelengths of 360 and 460 nm, respectively.

Statistical analysis

Statistical analysis was completed using GraphPad PRISM (Version 5). Results are expressed as means $\pm$ S.E.M. and data compared using repeated measures ANOVA followed by the Student-Newman-Keuls post-hoc test. The unpaired Student *t*-test was used where appropriate. Area under curve (AUC) and area above curve (AAC) analyses were calculated using appropriate baseline subtraction. Groups of data were considered to be significantly different if $P < 0.05$.

Results

Effects of Ψ -xenin-6, sitagliptin or a combination of both compounds on body weight, energy intake, glucose and insulin concentrations in HFF mice

Increases of body weight were significantly ($P < 0.01$ to $P < 0.001$) reduced in HFF mice treated with Ψ -xenin-6, sitagliptin or a combination of both drugs by day 18, when compared to HFF saline controls (Fig. 1A). Notably, in the combination treatment group, but not with monotherapy, percentage body weight increase was not significantly different from lean control mice (Fig. 1A). Energy intake was elevated ($P < 0.01$ to $P < 0.001$) in all HFF mice compared to lean controls, but not significantly different between HFF groups (Fig. 1B). Non-fasting glucose levels were reduced to lean control levels in a time-dependent manner by all treatments, but this was more prominent in the combined treatment group with benefits evident from day 12 (Fig. 1C), with only the combined treatment group

significantly reduced ($P < 0.05$) on day 18 compared to HFF mice (Fig. 1C). In addition, all HFF treatment groups exhibited progressive increases in insulin concentrations, with levels significantly ($P < 0.05$ to $P < 0.001$) elevated compared to HFF saline controls from day 7 onward (Fig. 1D). Moreover, in HFF mice treated with Ψ -xenin-6 in combination with sitagliptin, circulating insulin was not significantly different to lean control mice by day 18 (Fig. 1D) and were significantly increased ($P < 0.05$) compared to all other treatment groups (Fig. 1D).

Effects of Ψ -xenin-6, sitagliptin or a combination of both compounds on glucose and pyruvate tolerance, insulin sensitivity as well as pancreatic insulin content in HFF mice

Treatment of HFF mice with Ψ -xenin-6, sitagliptin or a combination of both drugs for 18 days resulted in reductions ($P < 0.05$ to $P < 0.01$) of individual and overall

Figure 1

Effects of 18-day treatment with Ψ -xenin-6, sitagliptin or a combination of both compounds on (A) body weight, (B) energy intake, (C) glucose and (D) insulin concentrations in HFF mice. Parameters were measured during 18 days of treatment with sitagliptin (50 mg/kg bw, p.o., once daily), Ψ -xenin-6 (25 nmol/kg bw, i.p. twice daily) or a combination of both compounds using the same dosing regimens. Values are mean $\pm$ s.e.m. for eight mice. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to lean controls. $\Delta P < 0.05$, $\Delta\Delta P < 0.01$ and $\Delta\Delta\Delta P < 0.001$ compared to HFF saline controls. $\Omega P < 0.05$ compared to sitagliptin alone. $\Phi P < 0.05$ compared to Ψ -xenin-6 alone.

Figure 2

Effects of 18-day treatment with Ψ -xenin-6, sitagliptin or a combination of both compounds on glucose tolerance and insulin secretion in HFF mice. Parameters were assessed following 18 days of treatment with sitagliptin (50 mg/kg bw, p.o., once daily), Ψ -xenin-6 (25 nmol/kg bw, i.p. twice daily) or a combination of both compounds using the same dosing regimens. (A and C) Glucose (18 mmol/kg bw) was administered by i.p. injection at t = 0 min in 18-h fasted mice. (B and D) Blood glucose and plasma insulin AUC values for 0–60 min post injection are shown. Values are mean $\pm$ s.e.m. for eight mice. * P < 0.05, ** P < 0.01 and *** P < 0.001 compared to lean controls. ΔP < 0.05 and $\Delta\Delta P$ < 0.01 compared to HFF saline controls.

glucose levels when compared to saline HFF controls following an i.p. glucose load (Fig. 2A and B). However, glucose disposal was still impaired in all HFF mice when compared to lean controls (Fig. 2A and B). Interestingly, corresponding glucose-induced insulin levels were not significantly different between all groups of mice at each of the observation time points (Fig. 2C). However, overall 0–60 min AUC insulin concentrations were elevated (P < 0.01) in saline treated HFF mice when compared to all other groups of mice (Fig. 2D). When challenged with a pyruvate load on day 18, only combined treatment with Ψ -xenin-6 and sitagliptin resulted in a significant decrease (P < 0.05 to P < 0.001) in glucose levels when compared to HFF control mice at 30, 60 and 120 min post-injection (Fig. 3A). Overall 0–120 min blood glucose AUC values were reduced (P < 0.05 to P < 0.001) by all treatment interventions, but still elevated (P < 0.01 to P < 0.001) when compared to lean control mice (Fig. 3B). However, combination treatment was significantly reduced (P < 0.05) when compared to sitagliptin monotherapy (Fig. 3B). Peripheral insulin sensitivity was improved (P < 0.05 to P < 0.001) by all treatment interventions (Fig. 4A), although administration of sitagliptin alone, or in combination with Ψ -xenin-6, resulted in similar glucose-lowering effects of exogenous insulin as observed in lean control mice (Fig. 4A). HOMA-IR was also significantly (P < 0.05 to P < 0.01) improved in all HFF treatment groups and not different from lean control mice (Fig. 4B). Pancreatic insulin content was similar to lean control mice in all HFF treatment groups and significantly

elevated (P < 0.001) when compared to saline treated HFF mice (Fig. 4C).

Effects of Ψ -xenin-6, sitagliptin or a combination of both compounds on pancreatic islet histology in HFF mice

Images of pancreatic islets stained for insulin and glucagon from all groups of mice are shown in Fig. 5A, B, C, D and E. Visual inspection of these images illustrates dramatically reduced insulin staining in HFF saline treated mice when compared to lean controls (Fig. 5A and B). Following appropriate quantification, it was revealed that HFF saline mice had significantly reduced overall islet (P < 0.05) and beta-cell (P < 0.01) areas when compared to lean controls (Fig. 5F and G). All treatment interventions returned islet and beta-cell area to normal levels (Fig. 5F and G). Alpha-cell area was increased in HFF saline (P < 0.001) and sitagliptin (P < 0.05) treated mice, but similar to lean controls in HFF mice treated with Ψ -xenin-6 alone or in combination with sitagliptin (Fig. 5H). Beta to alpha-cell ratios were significantly (P < 0.001) increased by all treatments when compared to HFF controls and by Ψ -xenin-6 alone or in combination with sitagliptin, when compared to sitagliptin alone (P < 0.001), but still reduced (P < 0.001) when compared to lean control mice (Fig. 5I). Islet size distribution was comparatively similar in all groups of mice, but all HFF mice administered treatment did have reduced (P < 0.05 to P < 0.01) numbers of smaller sized islets when compared to saline HFF controls (Fig. 5J).

Figure 3

Effects of 18-day treatment with Ψ -xenin-6, sitagliptin or a combination of both compounds on pyruvate tolerance in HFF mice. Parameters were assessed following 18 days of treatment with sitagliptin (50 mg/kg bw, p.o., once daily), Ψ -xenin-6 (25 nmol/kg bw, i.p. twice daily) or a combination of both compounds using the same dosing regimens. (A) Pyruvate (2 g/kg bw sodium pyruvate) was administered by i.p. injection at $t = 0$ min in 18-h fasted mice. (B) Blood glucose AUC values for 0–120 min post injection are shown. Values are mean $\pm$ S.E.M. for eight mice. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to lean controls. $\Delta P < 0.05$, $\Delta\Delta P < 0.01$ and $\Delta\Delta\Delta P < 0.001$ compared to HFF saline controls. $\Omega P < 0.05$ compared to sitagliptin alone.

Effects of Ψ -xenin-6, sitagliptin or a combination of both compounds on expression of hepatic genes involved in gluconeogenesis and insulin action in HFF mice

As would be expected, high fat feeding increased ($P < 0.01$ and $P < 0.05$, respectively) hepatic expression of *Pepck* and *G6pase* (Fig. 6A and B). All treatment interventions significantly ($P < 0.05$) reduced *Pepck* mRNA levels (Fig. 6A), but only combined Ψ -xenin-6 and sitagliptin therapy reduced ($P < 0.05$) *G6pase* expression, and returned expression of both genes to lean control levels (Fig. 6A and B). In addition, *Irs1* hepatic expression levels were increased ($P < 0.01$) in all HFF treatment groups when compared to saline controls (Fig. 6C). Interestingly, only

combination therapy increased ($P < 0.001$) *Fgf21* and decreased ($P < 0.01$) *Akt1*, hepatic mRNA levels compared to HFF control mice (Fig. 6D and E). Indeed, expression levels of both genes were similar to lean control mice in the combined Ψ -xenin-6 and sitagliptin treatment group (Fig. 6D and E).

Effects of Ψ -xenin-6, sitagliptin or a combination of both compounds on GLP-1 and GIP concentrations as well as DPP-4 activity in HFF mice

Following the 18-day treatment regimen, all sitagliptin treated mice had significantly ($P < 0.01$ and $P < 0.001$) increased circulating total GIP concentrations (Fig. 7A). Interestingly, Ψ -xenin-6 therapy also increased ($P < 0.01$) GIP levels, but did not affect GLP-1 concentrations (Fig. 7A and B). However, the combination treatment group did not present with GIP levels greater than with those with sitagliptin therapy alone (Fig. 7A). High fat feeding reduced ($P < 0.001$) circulating GLP-1, but this was restored to normal lean control levels in all sitagliptin treated mice (Fig. 7B). Plasma DPP-4 activity was significantly ($P < 0.05$) reduced in all HFF mice treated with sitagliptin when compared to HFF control mice (Fig. 7C). DPP-4 activity in the Ψ -xenin-6 treatment group was not significantly altered when compared to lean or high fat control mice (Fig. 7C). To confirm *in vivo* observations, DPP-4 activity of sitagliptin, Ψ -xenin-6 and a combination of both compounds was evaluated in the *in vitro* setting (Fig. 7D). In harmony with data from our mouse study, Ψ -xenin-6 (10^{-6} M) had no effect on DPP-4 activity and did not augment sitagliptin-induced reductions of DPP-4 activity (Fig. 7D).

Discussion

Although DPP-4 inhibitors have undoubted therapeutic benefits in T2DM (Elrishi *et al.* 2007), their overall effectiveness is perhaps less striking than other clinically approved drugs (Rosenstock *et al.* 2010). Taking into consideration that the primary pharmacological benefit of DPP-4 inhibition is enhancement of the incretin effect (Gallwitz 2013), known to be severely perturbed in T2DM (Nauck *et al.* 1993), this is perhaps not overly surprising. In the current study, we sought to address this problem through concurrent administration of the DPP-4 inhibitor, sitagliptin, with the enzymatically stable xenin form, Ψ -xenin-6 (Feurle *et al.* 2003). Ψ -xenin-6 represents a compound known to correct the underlying

Figure 4

Effects of 18-day treatment with Ψ -xenin-6, sitagliptin or a combination of both compounds on insulin sensitivity and pancreatic insulin content in HFF mice. Parameters were assessed following 18 days of treatment with sitagliptin (50 mg/kg bw, p.o., once daily), Ψ -xenin-6 (25 nmol/kg bw. i.p. twice daily) or a combination of both compounds using the same dosing regimens. (A) Insulin (25 U/kg bw) was administered by i.p. injection at t = 0 min in non-fasted mice. Blood glucose AAC values for 0–60 min post injection are shown in the inset. (B) HOMA-IR was calculated by the following equation: fasting glucose (mmol/L) $\times$ fasting insulin (mU/L)/22.5. (C) Pancreatic insulin content was measured by RIA following acid-ethanol extraction. Values are mean $\pm$ s.e.m. for eight mice. * P < 0.05 and *** P < 0.001 compared to lean saline controls. ΔP < 0.05 and $\Delta\Delta P$ < 0.001 compared to HFF saline controls. ΦP < 0.01 compared to Ψ -xenin-6 alone.

Figure 5

Effects of 18-day treatment with Ψ -xenin-6, sitagliptin or a combination of both compounds on pancreatic islet architecture in HFF mice. Parameters were assessed following 18 days of treatment with sitagliptin (50 mg/kg bw, p.o., once daily), Ψ -xenin-6 (25 nmol/kg bw. i.p. twice daily) or a combination of both compounds using the same dosing regimens. (A, B, C, D and E) Representative images (20 $\times$) of islets showing insulin (red) and glucagon (green) immunoreactivity from each treatment group. (F) Islet, (G) beta- and (H) alpha-cell areas, as well as (I) beta:alpha area cell area ratio and (J) islet size distribution were measured using CellF image analysis software. Values are mean $\pm$ s.e.m. for eight mice. * P < 0.05, ** P < 0.01 and *** P < 0.001 compared to lean controls. ΔP < 0.05, $\Delta\Delta P$ < 0.01 and $\Delta\Delta\Delta P$ < 0.001 compared to HFF saline controls. $\Omega\Omega\Omega P$ < 0.001 compared to sitagliptin alone. A full color version of this figure is available at <https://doi.org/10.1530/JOE-19-0557>.

Figure 6

Effects of 18-day treatment with Ψ -xenin-6, sitagliptin or a combination of both compounds on hepatic gene expression in HFF mice. Parameters were assessed following 18 days of treatment with sitagliptin (50 mg/kg bw, p.o., once daily), Ψ -xenin-6 (25 nmol/kg bw, i.p. twice daily) or a combination of both compounds using the same dosing regimens. mRNA expression of (A) phosphoenolpyruvate carboxykinase (*Pepck*), (B) glucose 6-phosphatase (*G6pase*), (C) insulin receptor substrate-1 (*Irs1*), (D) fibroblast growth factor 21 (*Fgf21*) and (E) serine-threonine protein kinase (*Akt1*) were determined relative to control gene expression (β -actin). Values are mean $\pm$ S.E.M. for eight mice. * P < 0.05, ** P < 0.01 and *** P < 0.001 compared with lean controls. ΔP < 0.05, $\Delta\Delta P$ < 0.01 and $\Delta\Delta\Delta P$ < 0.001 compared to HFF saline controls. ΩP < 0.01 compared to sitagliptin alone. ΦP < 0.01 compared to Ψ -xenin-6 alone.

incretin defect in T2DM, specifically GIP insensitivity (Craig *et al.* 2019).

As would be expected, sitagliptin and Ψ -xenin-6 therapy induced a number of benefits on metabolism in HFF mice. This is interesting, given that Ψ -xenin-6 was administered at a reduced molar concentration when compared to sitagliptin and because HFF mice

were also administered two low doses of STZ prior to treatment intervention. Such benefits included reduced glucose levels as well as improved glucose tolerance and insulin sensitivity, with notable improvements in pancreatic islet architecture. Indeed, assessment of peripheral insulin sensitivity would have been even more positive, but for normal adaptive responses in

Figure 7

Effects of Ψ -xenin-6, sitagliptin or a combination of both compounds on total GIP and GLP-1 concentrations as well as DPP-4 activity in HFF mice. (A, B and C) Parameters were assessed following 18 days of treatment with sitagliptin (50 mg/kg bw, p.o., once daily), Ψ -xenin-6 (25 nmol/kg bw, i.p. twice daily) or a combination of both compounds using the same dosing regimens. Total plasma (A) GIP and (B) GLP-1 concentrations were determined using commercially available ELISA kits. (C) DPP-4 activity was measured in plasma using established procedures (Lindsay *et al.* 2005). (D) DPP-4 activity of all test compounds (10^{-6} M) was also determined in the *in vitro* setting. Values are mean $\pm$ S.E.M. for (A, B and C) eight mice or (D) four experimental replicates. * P < 0.05, ** P < 0.01 and *** P < 0.001 compared with lean or vehicle control, as appropriate. ΔP < 0.05, $\Delta\Delta P$ < 0.01 and $\Delta\Delta\Delta P$ < 0.001 compared to HFF saline controls. ΩP < 0.05 and $\Omega\Omega P$ < 0.001 compared to sitagliptin alone. ΦP < 0.05 and $\Phi\Phi P$ < 0.001 compared to Ψ -xenin-6 alone.

these mice to prevent glucose concentrations falling toward life threatening hypoglycaemia levels, following exogenous insulin injection. As expected, positive effects of sitagliptin were linked to elevated circulating GIP and GLP-1 concentrations (Deacon *et al.* 1998). Interestingly, Ψ -xenin-6 also increased GIP levels, which may be somewhat unexpected. As such, the secretory dynamics of intestinal K-cells are well established (Gribble & Reimann 2019), although whether xenin exerts a positive autocrine effect on GIP secretion from K-cells is currently unknown. More importantly, elevated GIP and GLP-1 concentrations of the combined treatment group were not greater than with sitagliptin alone, confirming that benefits of combination therapy are not linked to increased incretin hormone levels.

Despite administration of the specific beta-cell toxin STZ, each of the treatments, namely sitagliptin, Ψ -xenin-6 or a combination of both compounds, returned overall islet and beta-cell areas, as well as pancreatic insulin content, to normal levels. It is intriguing to note that, in a related HFF mouse model with STZ-induced defects of insulin secretion, similar benefits of pancreatic islet architecture were observed following sustained administration of an analogue of sitagliptin, namely des-fluoro-sitagliptin (Mu *et al.* 2006). In the study by Mu and co-workers, des-fluoro-sitagliptin was administered for 11 weeks in the diet, as opposed to 18 days oral gavage in the current investigation. Thus, the oral gavage route ensures consistent dosing, drug delivery and faster onset of action, which may not always be the case with drug-supplemented animal diets. Whether augmented pancreatic insulin content in the current study was due to increases in beta-cell proliferation, reductions of beta-cell apoptosis or increased insulin production within beta-cells still needs to be determined. However, the beneficial impact of such pancreatic changes was clearly apparent on circulating insulin concentrations, with steady augmentations being evident in the combined treatment group. In keeping with this, GIP, GLP-1 and xenin have been shown to independently stimulate insulin secretion (Schmidt *et al.* 1985, Taylor *et al.* 2010), as well as promote pancreatic beta-cell growth and survival (Vilsbøll & Holst 2004, Drucker 2006, Khan *et al.* 2017). In addition to this, intra-islet synthesis and secretion of GLP-1 have previously been shown to play a fundamental role in appropriate pancreatic islet adaptations to various diabetes-like disease states (Moffett *et al.* 2014, Vasu *et al.* 2014). As such, it would have been interesting to assess pancreatic islet levels of both GLP-1 and GIP in the current study. Nonetheless, dual treatment with sitagliptin and Ψ -xenin-6 evoked

rapid improvements in glycaemic status, beyond that observed with either treatment alone. Such benefits were more apparent in the fed, as opposed to fasted, state, as evidenced during glucose tolerance testing that revealed no differences between the treatment regimens. This could simply be a consequence of the primary metabolic benefits of GIP, GLP-1 and xenin being more associated with the postprandial setting (Chowdhury *et al.* 2014, Irwin & Flatt 2015). Lack of benefit of combined treatment intervention on glucose tolerance and related insulin secretion is somewhat surprising and may require further investigation.

Although benefits on circulating insulin and pancreatic morphology were evident, none of the treatments increased the insulin response to exogenous glucose on day 18. Furthermore, there was a significant improvement in peripheral insulin sensitivity in all treated HFF mice, suggesting that improvements in glycaemic control were not solely a result of pancreatic-related benefits and could be related to the weight loss induced by each treatment. Indeed, hepatic expression of *Irs1* was augmented to a similar level by all treatment interventions. Moreover, combined sitagliptin and Ψ -xenin-6 therapy also increased *Fgf21* expression in the liver and returned mRNA levels of hepatic *Akt1* to that of lean controls, both of which would improve insulin action (Kim *et al.* 2000, Emanuelli *et al.* 2014). However, whether these positive changes in gene expression are independent of body weight loss still needs to be determined. Moreover, further investigation of the FGF21-adiponectin axis (Hui *et al.* 2016), in relation to the established crosstalk between the liver and adipose tissue that is known to be important for overall glucose homeostasis (Ye *et al.* 2017), would also be of interest here. In keeping with insulin action, hepatic gluconeogenesis was decreased in all treatment groups, but particularly by combined therapy, as established through assessment of pyruvate tolerance. In this respect, hyperinsulinaemic clamp studies may have given a more consistent readout of hepatic glucose production, but are beyond the scope of the current study. Nonetheless, irregular carbohydrate metabolism and inappropriate pyruvate flux are well known to be associated with diabetes and obesity (Gao *et al.* 2009, Singla *et al.* 2010). To further support the notion that sitagliptin and Ψ -xenin-6 diminish hepatic gluconeogenesis, we determined expression of *Pepck* and *G6pase* in liver tissue, key signalling molecules in the gluconeogenesis pathway (Lochhead *et al.* 2000). As such, PEPCK and G6Pase are fundamental in the initial and final steps, respectively, of the gluconeogenic pathway (Hers & Hue 1983). Only combined sitagliptin and Ψ -xenin-6

treatment significantly reduced the expression of both genes, and although some obvious restraint is required in interpretation, especially since individual treatments also evoked reductions in the expression of these genes, this does suggest that the expression and subsequent biological function of these proteins could be a major factor in the improvement of gluconeogenesis. In addition, it would also have been interesting to assess plasma and tissue lipid status in these mice, as well as genes involved in lipid flux. Moreover, assessment of related protein expression would be required to confirm overall relevance of the observed changes in gene expression.

Despite knowledge of the weight neutral effects of DPP-4 inhibitors (Foley & Jordan 2010), as well as lack of effect of prolonged administration of enzymatically stable versions of xenin on body weight in diabetic mice (Gault *et al.* 2015a, Martin *et al.* 2016, Parthsarathy *et al.* 2016), both sitagliptin and Ψ -xenin-6 reduced body weight gain in the current setting. Moreover, combined treatment in HFF mice resulted in body weight change that was not different to lean controls, despite similar energy intake in all groups of HFF mice. In keeping with this, lack of effect of sitagliptin and xenin peptides on feeding has previously been observed in rodents using the same dosing regimens employed here (Taylor *et al.* 2010, Gault *et al.* 2015b, Hasib *et al.* 2017). In view of the role of obesity in the development of T2DM (Al-Goblan *et al.* 2014), and the comparable increasing prevalence of both conditions (Agha & Agha 2017), this more pronounced body weight lowering effect could represent an attractive therapeutic effect of combined therapy. As such, it is well recognised that the central pathways controlling energy balance display notable plasticity (Irwin & Flatt 2015), and thus concurrent positive modulation of signal transduction proteins linked to both compounds may be essential to yield such appreciable benefits. A reduction of body weight could also be a contributing factor to improved metabolic effects of the combination treatment (Wilding 2014), although further assessment of body composition, locomotor activity and metabolic rate, including energy expenditure, would be required to fully assess this.

Taken together, data presented within the current study support the view that the antidiabetic benefits of DPP-4 inhibitors can be augmented. Importantly, assessment of DPP-4 activity on day 18, together with *in vitro* observations, demonstrated that Ψ -xenin-6 does not augment DPP-4 inhibitory activity of sitagliptin. Given that incretin hormone levels were also not increased by dual administration of Ψ -xenin-6 and sitagliptin, it suggests that the likely benefit of combined

therapy is linked to the augmentation of GIP bioactivity by Ψ -xenin-6, as has been previously established (Craig *et al.* 2019). Observations of improved effects of combined Ψ -xenin-6 and sitagliptin treatment on body weight, circulating glucose and insulin, attenuation of gluconeogenesis, as well as improvements in pancreatic islet morphology are encouraging, especially over a relatively short treatment period of 18 days. However, it should be noted that combined therapy did not improve glucose tolerance beyond that observed with either treatment alone. Nonetheless, clinical studies already confirm some enhanced antidiabetic efficacy when using combinational therapies that incorporate DPP-4 inhibitor drugs (Goldstein *et al.* 2007, Barnett *et al.* 2015), in line with National Institute for Health and Care Excellence (NICE) T2DM treatment guidelines, corroborating proof of concept. Taking into account that Ψ -xenin-6 positively regulates the primary therapeutic target of DPP-4 inhibitors, unlike other available antidiabetic medications, the current therapeutic regimen represents an attractive novel option for T2DM that warrants further investigation.

Declaration of interest

V A G and N I are named on patents filed by the Ulster University for the exploitation of incretin-based drugs and other peptide therapeutics. The other authors have nothing to disclose.

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Author contribution statement

N I, G H and V A G contributed to the overall concept and experimental design and reviewed the manuscript. S L C researched data, contributed to data interpretation and edited the manuscript. N I and S L C wrote the manuscript. All authors approved the final version of this manuscript.

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