Effects of an enzymatically stable C-terminal hexapseudopeptide fragment peptide of xenin-25, Ψ-xenin-6, on pancreatic islet function and metabolism

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Abstract

Xenin-25 undergoes rapid enzyme metabolism following secretion. Early studies demonstrated bioactivity of a C-terminal hexapeptide fragment of xenin-25, namely xenin-6, which were enhanced through introduction of a reduced N-terminal peptide bond, to yield Ψ-xenin-6. The present study was undertaken to define the biological actions and potential antidiabetic properties of Ψ-xenin-6. *In vitro* enzymatic stability, insulin and glucagon secretory activity, as well as effects on beta-cell survival were determined. Studies in mice were used to assess the impact of Ψ-xenin-6 on glucose homeostasis and satiety. Ψ-xenin-6 was resistant to murine plasma degradation. In BRIN-BD11 cells and isolated murine islets, Ψ-xenin-6 significantly stimulated insulin secretion, and prominently enhanced the insulinotropic actions of GIP. Xenin-6 and Ψ-xenin-6 had no impact on glucagon secretion, although xenin-6 partially reversed the glucagonotropic action of GIP. Further *in vitro* investigations revealed that, similar to GLP-1, Ψ-xenin-6 significantly augmented proliferation of human and rodent clonal beta-cells, whilst also fully protecting against cytokine-induced beta-cell cytotoxicity, with greater potency than xenin-25 and xenin-6. When administered to mice in combination with glucose, Ψ-xenin-6 significantly reduced glucose levels and enhanced glucose-induced insulin release, with a duration of biological action beyond 8 hours. Ψ-xenin-6 also significantly enhanced the glucose-lowering action of GIP *in vivo*. In overnight fasted mice, Ψ-xenin-6 exhibited satiety actions at both 25 and 250 nmol/kg. These data demonstrates that Ψ-xenin-6 is a metabolically stable C-terminal fragment analogue of xenin-25, with a metabolic action profile that merits further study as a potential antidiabetic compound.

1. Introduction

The gut-derived hormone xenin-25, a 25 amino-acid peptide co-secreted from enteroendocrine K-cells with the incretin hormone glucose-dependent insulinotropic polypeptide (GIP), has previously been shown to reduce gastrointestinal transit and modulate energy balance (Cline et al., 2007; Leckstrom et al., 2009; Kerbel et al., 2018). Additional reports also suggest a role for xenin-25 in the regulation of pancreatic islet function and survival (Silvestre et al., 2003; Taylor et al., 2010; Martin et al., 2012; 2014; Gault et al., 2015; Parthsarathy et al., 2016; Khan et al., 2017). In this regard, xenin-25 has also been shown to potentiate the insulinotropic actions of GIP (Wice et al., 2010; 2012), with suggested therapeutic potential for diabetes (Craig et al., 2018). Notwithstanding this, the evident physiological importance of xenin-25 has been somewhat overlooked to date (Maryanovich et al., 2018), as well as the consequence of xenin-25 enzymatic degradation. For instance, numerous regulatory gut-derived peptide hormones possess a dramatically altered biological action profile following enzymatic degradation in the circulation (Deacon, 2004; Mayorov et al., 2008; Lafferty et al., 2018).

For xenin-25, a number of C-terminally truncated metabolites have been characterised including, xenin 9-25, xenin 11-25, xenin 14-25 and xenin 18-25 (Martin et al., 2014). The biological significance of xenin 9-25, xenin 11-25 and xenin 14-25 is largely unknown (Martin et al., 2014). Interestingly, xenin 18-25, also referred to as xenin-8, has been shown to recapitulate the effects of the parent peptide at the level of the endocrine pancreas (Silvestre et al., 2003; Gault et al., 2015) and duodenum (Kaji et al., 2017), as well as potentiating the biological actions of GIP (Martin et al., 2014; 2016). This is extremely encouraging in terms of potential therapeutic application of xenin, since smaller peptide size will lead to reduced production costs, promote simpler drug formulation, which together will dramatically increase the overall commercial attractiveness. Following on from this, two very early studies in the xenin field had demonstrated bioactivity of a C-terminal hexapeptide of xenin-25, xenin-20-25

or also known as xenin-6 (Feurle et al., 1996; 2003). Encouragingly, enzymatic stability and biological efficacy of this xenin hexapeptide was substantially enhanced through the introduction of a reduced pseudopeptide bond (CH$_2$NH) between Lys[20] and Arg[21] amino acid residues, to yield a Psi (Ψ) pseudopeptide analogue named Ψ-xenin-6 (Feurle et al., 2003). However, further application of these exciting observations with Ψ-xenin-6 have been missing until now.

Therefore, the present study was conducted to further characterise Ψ-xenin-6, with a view to potential therapeutic application in the field of diabetes. As such, we initially confirmed enzymatic stability of Ψ-xenin-6 in plasma. *In vitro* and *ex vivo* effects of Ψ-xenin-6 on insulin and glucagon secretion from pancreatic BRIN-BD11 beta-cells, alpha TC1.9 cells as well as isolated mouse islets, as appropriate, were then evaluated. In addition, effects on rodent and human beta-cell proliferation and protection against cytokine-induced apoptosis were also assessed. Finally, the impact of Ψ-xenin-6 on glucose homeostasis, insulin section and satiety were examined in mice. Taken together, the results support further investigation of the potential therapeutic promise of Ψ-xenin-6 for diabetes.

2. Materials and Methods

2.1 Peptide synthesis and assessment of plasma enzymatic stability

All peptides were purchased at greater than 95% purity. Native xenin-25 and xenin-6 was synthesised by GL Biochem (Shanghai, China). Ψ-xenin-6 was obtained from Saxon Biochemicals (Hannover, Germany). Before experimentation, peptides were characterised in-house using HPLC and Matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometry (MS), as previously described (Gault et al., 2003). Enzymatic stability of peptides was evaluated *in vitro* using 18 h fasted murine plasma, as outlined previously

(Martin et al., 2012). Concisely, peptides (50 μg) were incubated with mouse plasma (50 μl) in the buffering agent triethanolamine–HCl (50 mM, pH 7.8) at 37 °C for 0, 120, 240 and 360 min and degradation profiles followed by HPLC, with MALDI-TOF MS analyses of collected HPLC peaks.

2.2 In vitro and ex vivo insulin secretion

In vitro insulin releasing activity of test peptides were assessed in rodent BRIN-BD11 beta-cells, cultured and maintained as described (McClenaghan et al., 1996), using RPMI 1640 media (Gibco Life Technologies Ltd), supplemented with 10% v/v foetal bovine serum (Gibco), 1% v/v antibiotics (0.1 mg/ml streptomycin and 100 U/ml penicillin) at 37ºC in 5% atmospheric CO_2. BRIN-BD11 cells were seeded into 24-well plates (150,000 cells per well) and allowed to attach overnight at 37ºC. Prior to tests, cells were pre-incubated in Krebs–Ringer bicarbonate buffer (KRBB) (pH 7.4) supplemented with 0.5% (w/v) BSA and 1.1 mM glucose (40 min; 37 °C). In the first set of experiments, cells were incubated (20 min) with test peptides (10^{-12} – 10^{-6} M) at 5.6 and 16.7 mM glucose, as appropriate. In a second set of experiments, BRIN-BD11 cells were incubated with peptides (10^{-12} to 10^{-6} M) in the presence of GIP (10^{-6} M) at 5.6 mM glucose for 20 mins. In a final set of experiments, BRIN BD11 cells were cultured for 48 hours under glucotoxic (22.2 mM glucose) conditions and insulinotropic effects of GIP and xenin peptides (10^{-6} M) determined (n=8, 20 min incubation), as described previously from our laboratory (Pathak et al., 2014). After test incubations, aliquots of assay buffer (200 μl) were collected and stored at -20 °C prior to assessment of insulin concentrations. In a separate set of experiments, *ex vivo* insulinotropic effects of test peptides were assessed in NIH Swiss mouse islets, isolated using a collagenase-based method as previously described (McKillop et al., 2014). The experimental approach was similar to above at 16.7 mM glucose, but with a 60 min incubation period. In addition, at the end of the

experiment, 500 µl of acid-ethanol solution (1.5% [v/v] HCl, 75% [v/v] ethanol, 23.5% [v/v] H_2O) was added (18 h, 4°C) to extract total islet cellular insulin content. All samples were stored at -20 °C prior to assessment of insulin concentrations by an in-house radioimmunoassay (RIA) (Flatt and Bailey, 1982).

2.3 In vitro and ex vivo glucagon secretion

α-TC1.9 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 25 mM glucose and 2 mM L-glutamine supplemented with 10% (v/v) FCS and antibiotics (100 U/ml penicillin and 0.1 g/l streptomycin). For experimentation, cells were cultured for 48 h in 24-well plates at a cell density of 150,000 per well, to ensure attachment. Before commencement of secretory studies, cells were pre-incubated with 1 ml KRBB supplemented with 20 mM glucose for 1 h at 37°C. Following pre-incubation, cells were incubated (2 h, 37°C, 1.4 mM glucose) with test peptides (10^{-6} M) alone or in combination with GIP (10^{-6} M). Supernatant (900 µl) was removed from each well and stored at -20°C until glucagon measurement. Glucagon secretion from isolated mouse islets was conducted as described above, at 1.4 mM ambient glucose levels. Arginine (10 mM) was used as a positive control. All glucagon concentrations were measured by a commercially available chemiluminescent enzyme-linked immunosorbent assay (Millipore, Watford, UK), as previously described (Mohan et al., 2019).

2.4 Cytotoxicity

Toxicity of test peptides (10^{-6} M, 20 min) was determined by assessing levels of lactate dehydrogenase (LDH) release in BRIN-BD11 cells, as previously described (Srinivasan et al., 2014), with dimethyl sulfoxide (DMSO, Sigma-Aldrich, UK) as a positive control. Concentrations of LDH were measured using a CytoTox96 non-radioactive cytotoxicity assay

kit (Promega, Madison, WI) according to the manufacturer's protocol. This assay is highly sensitive and capable of accurately measuring the lower levels of LDH known to be present within pancreatic beta-cells (Schuit et al. 2001).

2.5 In vitro beta-cell proliferation and apoptosis

Rodent BRIN-BD11 and human 1.1B4 beta-cells were used to investigate effects of test peptides on beta-cell proliferation and protection against cytokine-induced apoptosis. GLP-1 (10^{-8} and 10^{-6} M) was employed as a positive control for all studies. Ki-67 immunostaining was used to assess effects on proliferation. Briefly, cells were seeded onto coverslips at a density of 40,000 cells per coverslip and cultured overnight (18 h) at 37 °C, in the presence of peptides (10^{-8} and 10^{-6} M). Cells were then washed with PBS, and fixed using 4% paraformaldehyde. Following antigen retrieval with citrate buffer at 90 °C for 20 min, tissues were blocked using 1.1% BSA for 30 min. Cells were then incubated with Ki-67 primary antibody, followed by Alexa Fluor® 594 secondary antibody. Coverslips were washed with PBS, mounted on slides for viewing using a fluorescent microscope (Olympus System Microscope) and photographed by DP70 camera adapter system. Proliferation frequency was expressed as percentage of total cells analysed. For analysis of the ability of test peptides to protect against cytokine-induced apoptosis, cells were seeded as above. However, cells were also exposed to a cytokine-cocktail (IL-1β 300U/ml, IFN-γ 300 U/ml, TNF-α 40 U/ml; (Vasu et al., 2014) in the presence or absence of test peptides (10^{-8} and 10^{-6} M) for 2 h, with hydrogen peroxide as an additional control. TUNEL staining (Roche Diagnostics Ltd, UK) was performed to quantify beta-cell apoptosis, as previously described (Vasu et al., 2014). Apoptosis was expressed as percentage of total cells analysed. Approximately 150 cells were analysed per group.

2.6 Animals

All animal studies were carried out using male NIH Swiss mice (12–14 weeks old, Envigo Ltd, UK), all housed individually in an air-conditioned room at 22 ± 2 °C with a 12 h light:12 h dark cycle. Animals were maintained on a standard rodent chow diet (10% fat, 30% protein and 60% carbohydrate, Trouw Nutrition, UK), with *ad libitum* access to diet and water. All animal experiments were carried out in accordance with the UK Animal Scientific Procedures Act 1986 and approved by the Ulster University Animal Welfare and Ethical Review Body (AWERB).

2.7 Acute food intake studies

Cumulative food intake was assessed in overnight fasted (18 h) mice following i.p. injection of saline vehicle (0.9% w/v NaCl) or test peptide (25 or 250 nmol/kg bw), and food intake measured at 30 min intervals for 180 mins. These doses were chosen based on the observed appetite suppressive effects of xenin-25, and related metabolites, following peripheral administration in rodents (Taylor et al. 2010; Martin et al. 2014).

2.8 Acute and persistent effects of peptides on glucose tolerance and insulin secretion

Blood glucose and plasma insulin concentrations were determined immediately prior to and 15, 30, 60 and 105 min after i.p. injection of glucose alone (18 mmol/kg bw) or in combination with test peptides (25 nmol/kg bw), as well as test peptides together with GIP (25 nmol/kg bw) in 4 h fasted mice. To assess duration of peptide action, mice were administered saline vehicle or test peptides (25 nmol/kg bw) at 2, 4, 8 or 12 h prior to an i.p. glucose challenge (18 mmol/kg bw) and blood glucose measured.

2.9 Biochemical analysis

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). Blood glucose was measured directly using a Contour blood glucose meter. For plasma insulin analysis, blood samples were collected into chilled fluoride/heparin glucose micro-centrifuge tubes (Sarstedt, Numbrecht, Germany) and immediately centrifuged using a Beckman microcentrifuge (Beckman Instruments, Galway, Ireland) for 1 min at 13,000 x g and stored at -20°C prior to insulin RIA (Flatt and Bailey, 1982).

2.10 Statistical analysis

Statistical analysis was completed using GraphPad PRISM (Version 5). Results are expressed as means ± SEM and data compared using repeated measures ANOVA followed by Student-Newman-Keuls *post-hoc* test. Unpaired Student *t*-test was used where appropriate. Incremental plasma insulin and glucose area under the curve (AUC) were calculated using the trapezoidal rule with baseline subtraction. Groups of data were considered significant if $p < 0.05$.

3. Results

3.1 Peptide characterisation and plasma enzymatic stability

In-house characterisation confirmed successful synthesis of test peptides (Table 1). In addition, Ψ-xenin-6 was completely resistant to plasma degradation following a 6 h incubation, whereas native xenin-6 had an estimated *in vitro* half-life of less than 2 h (Table 1).

3.2 Insulin secretory actions

In BRIN-BD11 cells, both xenin-6 and Ψ-xenin-6 displayed significant ($p < 0.05$ to $p < 0.001$) dose-dependent insulin secretory actions at 5.6 and 16.7 mM glucose concentrations when compared to respective glucose controls (Fig. 1A,B). Interestingly, at 5.6 mM glucose, Ψ-

xenin-6 exhibited superior ($p < 0.05$) insulinotropic effects than native xenin-6 at all peptide concentrations tested, barring 10^{-8} M, and at 10^{-6} M in 16.7 mM glucose (Fig. 1A,B). The potent insulin-releasing actions of Ψ-xenin-6, with superiority ($p < 0.01$) over native xenin-6, were then confirmed in fully functional isolated murine islets (Fig. 1C). When xenin peptides were co-incubated with GIP in BRIN-BD11 cells, both xenin-6 and Ψ-xenin-6 significantly ($p < 0.05$ to $p < 0.001$) augmented the insulinotropic actions of GIP (Fig. 1D). In this regard, Ψ-xenin-6 exhibited superior ($p < 0.01$ to $p < 0.001$) GIP-potentiating efficacy than xenin-6 at all concentrations examined (Fig. 1D). Under glucotoxic culture conditions, the insulin secretory responses of GIP, xenin-6 and Ψ-xenin-6 were significantly ($p < 0.05$ to $p < 0.01$) impaired (Fig. 1E). Interestingly, when xenin-6 was co-incubated with GIP, the insulinotropic response was not significantly different in BRIN BD11 cells cultured under normal or glucotoxic culture conditions (Fig. 1E). In contrast, although co-incubation with Ψ-xenin-6 and GIP under glucotoxic conditions augmented insulin secretion to a higher degree than co-incubation with xenin-6 and GIP, insulin secretion was reduced ($p < 0.01$) when compared to control cultures (Fig. 1E). None of the xenin peptides compromised BRIN-BD11 cell plasma membrane integrity, as displayed by lack of effects on lactate dehydrogenase levels following a 20-minute incubation at the highest (10^{-6} M) peptide concentration used in normal cells (Fig. 1F).

3.3 Glucagon secretory actions

As expected, arginine (10 mM) and native GIP (10^{-6} M) augmented ($p < 0.001$) glucagon secretion from α-TC1.9 cells at 1.4 mM glucose levels (Fig. 2A). Neither xenin-6 nor Ψ-xenin-6 (both at 10^{-6} M) altered glucagon secretion (Fig. 2A). However, xenin-6 partially reversed the glucagonotropic actions of GIP, whereas Ψ-xenin-6 did not (Fig. 2A). The observations on glucagon secretion following incubation of arginine, GIP, xenin-6 and Ψ-xenin-6 in α-TC1.9 cells were paralleled in isolated murine islets (Fig. 2B). In addition, whilst the ability of xenin-

6 to reverse GIP-induced elevations of glucagon secretion was again apparent, this failed to reach significance in mouse islets (Fig. 2B).

3.4 Beta-cell proliferation and protection against apoptosis

Similar to GLP-1, Ψ-xenin-6 significantly ($p < 0.001$) augmented BRIN-BD11 and 1.1B4 beta-cell proliferation at both 10^{-8} and 10^{-6} M (Fig. 3A,B). Xenin-25 only displayed beta-cell proliferative effects in BRIN-BD11 cells at a concentration of 10^{-8} M (Fig. 3A). Native xenin-6 displayed no proliferative actions in 1.1B4 beta-cells (Fig. 3A,B), with a slight ($p < 0.05$) increase noted at 10^{-6} M in BRIN-BD11 cells (Fig. 3A). In relation to protection against cytokine-induced apoptosis, all test peptides reversed the detrimental DNA damaging effects of co-incubation with cytokines in BRIN-BD11 cells, but only at the highest concentration employed (Fig. 3C). In 1.1B4 beta-cells, Ψ-xenin-6 offered full protection against cytokine-induced apoptosis at both 10^{-8} and 10^{-6} M, with benefits of GLP-1, xenin-25 and xenin-6 only observed at 10^{-6} M (Fig. 3D). In 1.1B4 cells Ψ-xenin-6 (10^{-6} M) reduced ($p < 0.05$) cytokine-induced elevations of TUNEL staining to below control levels (Fig. 3D).

3.5 Acute in vivo food intake

At a dose of 25 nmol/kg, xenin-25 and Ψ-xenin-6 induced a significant ($p < 0.05$) reduction in food intake at 180 min post-injection in overnight fasted mice, when compared to saline controls (Fig. 4A). Interestingly, at a dose of 250 nmol/kg, only Ψ-xenin-6 exhibited appetite suppressive actions, evident at both 30 ($p < 0.01$) and 180 ($p < 0.05$) min post-injection (Fig. 4B). Native xenin-6 had no effect on feeding at either of the doses employed (Fig. 4A,B).

3.6 Acute glucose lowering and insulin releasing effects in mice

Administration xenin-6 or Ψ-xenin-6 in combination with glucose resulted in significantly (p < 0.05 to p < 0.001) decreased individual and overall 0-105 min AUC plasma glucose levels when compared to glucose alone control (Fig. 5A,B). Native GIP did decrease (p < 0.05) glucose levels at 30 min post-injection (Fig. 5A), but this did not translate to significant reductions in AUC values (Fig. 5B). When xenin-6 and Ψ-xenin-6 were administered in combination with GIP, there was a significant (p < 0.01 to p < 0.001) reduction in glucose levels at 15 and 30 min post-injection when compared to GIP alone (Fig. 5A). Reductions in overall AUC values when compared to GIP alone were only observed when GIP was combined with Ψ-xenin-6 (p < 0.05), but not xenin-6 (Fig. 5B). All treatments elevated (p < 0.05 to p < 0.01) corresponding 0-105 min AUC plasma insulin concentrations (Fig. 5D). However, only Ψ-xenin-6 significantly (p < 0.01) increased individual plasma insulin levels, although this was only apparent at 105 min post-injection (Fig. 5C). There was no obvious augmentation of the insulinotropic actions of GIP by either xenin-6 or Ψ-xenin-6 (Fig. 5C,D).

3.7 Persistent glucose lowering and insulin releasing effects in mice

Administration of either xenin-6 or Ψ-xenin-6 2 h prior to glucose load, resulted in significantly reduced individual (p < 0.05 to p < 0.01) and AUC overall (p < 0.001) blood glucose levels when compared to saline control mice (Fig. 6A,B). Comparable observations were made when the peptides were injected 4 h before the glucose challenge (Fig. 6C,D), although Ψ-xenin-6 was significantly (p < 0.05) more effective than xenin-6 (Fig. 6D). When administered 8 h prior to the glucose load, both xenin-6 and Ψ-xenin-6 reduced (p < 0.05 to p < 0.01) individual glucose levels at 30 min post-injection, but only Ψ-xenin-6 significantly (p < 0.01) decreased overall AUC glucose values (Fig. 6E,F). Both peptides failed to elicit any significant glucose-lowering actions when delivered 12 h prior to a glucose challenge (Fig. 6G,H).

4. Discussion

Previous reports have established that chemical manipulation of the C-terminal hexapeptide of xenin-25, namely xenin-6, through introduction of a reduced pseudopeptide bond between Lys[20] and Arg[21], yields an enzymatically stabilised peptide with enhanced biological activity (Feurle et al., 2003). Similar enhanced metabolic stability and bioactivity has been observed following introduction of a reduced pseudopeptide bond in neurotensin (Lugrin et al., 1991), a peptide closely related to xenin (Feurle et al., 2002). Such effects are in complete harmony with previous studies from our laboratory, demonstrating enhanced stability and biological activity of an octapeptide C-terminal fragment of xenin-25 following rationally introduced amino-acid modifications (Parthsarathy et al., 2016; Martin et al., 2016).

Consistent with this knowledge, in the present study xenin-25, xenin-6 and especially Ψ-xenin-6 evoked clear increases of insulin secretion from pancreatic BRIN-BD11 cells (Feurle et al., 1992; Taylor et al., 2010). Indeed, both xenin hexapeptides had comparable, or even enhanced, efficacy when compared to the parent peptide. Notably, Ψ-xenin-6 was significantly more efficacious than xenin-6 at 5.6 mM glucose, expect at 10^{-8} M, which is interesting and may require further study. The insulinotropic potency of the xenin peptides appeared to be somewhat reduced at higher glucose concentrations in BRIN BD11 cells, and this may be due to difficulties in assessing additive peptide effects in the face of increased insulin output by elevated glucose. Nonetheless, these observations corroborate that the C-terminal hexapeptide of xenin-25 is capable of activating xenin related beta-cell signalling pathways. Indeed, insulin secretory studies using functional isolated mouse islets fully confirmed this concept. GIP-potentiating actions of xenin-25 are recognised at the level of the beta-cell (Wice et al., 2010; 2012 Martin et al., 2012; Parthsarathy et al., 2016), which were reproduced by xenin-6, and to a significantly greater magnitude by Ψ-xenin-6. In BRIN BD11 cells cultured under glucotoxic conditions to mimic beta-cell stress encountered in diabetes

(Pathak et al., 2014), as would be expected, there was an impairment of the insulin-releasing action of GIP and xenin-6 peptides. GIP-augmenting actions of the truncated xenin peptides were still observed under glucotoxic culture, especially in the case of xenin-6. Nonetheless, the more effective of the two compounds in terms of GIP-potentiation was Ψ-xenin-6, albeit with notably less efficacy than in normal beta-cells. In addition, Ψ-xenin-6 was the only peptide examined that significantly enhanced the glucose-lowering action of GIP in mice. Whilst the exact mechanism of xenin-induced GIP potentiation remains to be elucidated (Clemens et al., 1997; Wice et al., 2010; Mazella et al., 2012), it does substantiate the idea that Ψ-xenin-6 possess enhanced biological potency over xenin-6 (Feurle et al., 2003). It is also encouraging to note that the reduced pseudopeptide bond present within Ψ-xenin-6 had no detrimental impact in terms of beta-cell cytotoxicity.

Based on previous observations of glucagonotropic effects of a C-terminal octapeptide fragment of xenin-25 (Silvestre et al., 2003), and evidence that GIP induces glucagon secretion under fasting glycaemic levels (Christensen et al., 2011), we sought to further examine this concept with Ψ-xenin-6. Unlike GIP, both xenin-6 and Ψ-xenin-6 were devoid of glucagonotropic actions in αTC1.9 cells and isolated rodent islets. This does slightly contrast with previous studies using xenin-8 in rodent islets, although in situ perfusion as opposed to static incubations was employed for this earlier study (Silvestre et al., 2003). In addition, the most prominent secretory effect of xenin-8 was noted to be potentiation of arginine- and carbachol-induced glucagon elevations (Silvestre et al., 2003). Given this, and the notable interactions between xenin and GIP (Martin et al., 2012; Wice et al., 2012; Chowdhury et al., 2013; Hasib et al., 2016), the impact of co-incubation with the xenin-6 fragment peptides and GIP was considered. Interestingly, xenin-6, but not Ψ-xenin-6, had a strong tendency to reverse GIP-mediated increases in glucagon release. This is interesting, as both augmentation and blockade of glucagon receptor signalling has been advocated as being beneficial in diabetes

(Pathak et al., 2015; McShane et al., 2016). The difference in biological activity between the two xenin C-terminal hexapeptides is intriguing and presumably not related to the enhanced stability of Ψ-xenin-6 noted here and elsewhere (Feurle et al., 2002). It could also relate to structure/function complexities between the peptides, which would require more in-depth study. However, the overall impact of xenin on GIP-induced alterations of glucagon secretion is of significant interest, since GIP infusion has been shown to prevent insulin-induced hypoglycaemia in humans (Christensen, 2016). Indeed, this may represent an important homeostatic metabolic mechanism of GIP (Christensen et al., 2011), where xenin may also play a key role, and could have particular relevance in the diabetes setting.

Further to this, previous studies with xenin-25 have clearly highlighted potential benefits at the level of the pancreatic beta-cell. As well as enhancing insulin release and potentiating GIP-induced insulin release (Taylor et al., 2010; Wice et al., 2010; Martin et al., 2012; 2014), the parent xenin peptide has been shown to promote pancreatic beta-cell growth and survival (Khan et al., 2017). Promisingly, Ψ-xenin-6 had similar, or even enhanced, beta-cell proliferative and survival beneficial effects in both rodent BRIN-BD11 and human 1.1B4 beta-cells. This is encouraging given that type 2 diabetes is a disease characterised by beta-cell loss (Halban et al., 2014), although the exact mechanisms of these xenin-induced beneficial effects still needs to be elucidated. Similar to previous reports (Mohan et al., 2018; 2019), baseline apoptosis rates were somewhat elevated in both beta-cell lines, representing a particularly challenging environment. As such, it may have been interesting to assess beta-cell survival benefits of Ψ-xenin-6 and xenin-6 under less severe beta-cell insults. However, the aforementioned positive effects of Ψ-xenin-6 on glucose homeostasis, insulin release and GIP potentiation, coupled with an *in vivo* duration of approximately 8 hours in mice, further promote its potential therapeutic value for diabetes. The observation of enhanced glucose homeostatic actions of combined GIP and Ψ-xenin-6 administration in mice, despite no

obvious augmentation of insulin secretion, likely reflects enhancement of the notable extrapancreatic glucose-lowering effects of GIP and xenin (Irwin and Flatt, 2015; Craig et al., 2018). Furthermore, xenin has been suggested to supress food intake (Craig et al., 2018) and delay gastric emptying rate (Kim and Mizuno, 2010), which would also be complementary in the type 2 diabetes setting (Al-Goblan et al., 2014). In our hands, Ψ-xenin-6 induced small, but significant, appetite suppressive effects in mice at doses of 25 and 250 nmol/kg, whereas xenin-6 was ineffective. Notably, physiological circulating concentrations of xenin are not well defined, and local tissue production of xenin has been documented outside of the gut (Khan et al., 2017). In addition, previous studies demonstrating prominent efficacy of xenin peptides to inhibit feeding have largely employed intracerebroventricular administration (Alexiou et al., 1998; Cooke et al., 2009; Leckstrom et al., 2009; Bhavya et al., 2017; Kerbel et al., 2018), as opposed to peripheral application in the present study. This may suggest that efficient passage through the blood-brain barrier is critical for xenin-based peptides to positively modulate energy balance.

In conclusion, these data substantiate the notion that Ψ-xenin-6 is a stable, long-acting xenin analogue that retains the full biological action profile of the parent peptide. Benefits of Ψ-xenin-6 on pancreatic islet cell function and survival, appetite suppression and GIP-potentiation, coupled with a prolonged half-life, emphasise prospective therapeutic potential of this peptide for diabetes. Future studies should therefore evaluate the preclinical utility of Ψ-xenin-6 in appropriate models of diabetes, both alone and in combination with established antidiabetic drugs.

Author Contributions

SLC contributed to conduct/data collection, analysis and writing of the manuscript. VAG, SMcC, GH and NI contributed to study design, analysis and writing of the manuscript. All authors approved the final version of the manuscript. NI and VAG are named on patents filed by the Ulster University for exploitation of incretin-based drugs and other peptide therapeutics.

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Table 1. Amino acid sequence, theoretical and experimental masses as well as murine plasma half-lives of xenin peptides

Peptide	Amino acid sequence	Theoretical mass (Da)	Experimental mass (Da)	*In vitro* half-life (murine plasma)
Xenin-6	H-LYS-ARG-PRO-TRP-ILE-LEU-OH	812.2	812.0	< 2 h
Ψ-xenin-6	H-LYS-ψ-ARG-PRO-TRP-ILE-LEU-OH	798.1	798.5	> 6 h

Amino acid sequence of peptides using three letter amino acid nomenclature. Peptide masses were calculated using MALDI-MS, as previously described (Gault et al., 2003). *In vitro* stability of peptides was evaluated using 18-h fasted murine plasma. Xenin-6 and Ψ-xenin-6 (50 μg) were incubated with mouse plasma (50 μl) in 50 mM triethanolamine–HCl for 0, 2, 4 and 6 h and degradation profiles followed by HPLC, with MALDI-TOF MS analyses of collected HPLC peaks.

Figure legends

Fig. 1. Effects of Ψ-xenin-6 on insulin release from BRIN-BD11 cells (A,B,D,<u>E</u>) and isolated mouse islets (C) as well as LDH accumulation (F) in BRIN-BD11 cells. (A,B,D) BRIN-BD11 were incubated (20 min) with a range of concentrations (10^{-12} to 10^{-6} M) of xenin-6 and Ψ-xenin-6 alone (A,B) or in combination with GIP (D) in the presence of (A,D) 5.6 or (B) 16.7 mM glucose. (C) Insulin release was measured in isolated mouse islets incubated (60 min) with of xenin-6 and Ψ-xenin-6 (10^{-8} and 10^{-6} M) at 16.7 mM glucose. <u>(E) BRIN BD11 cells were cultured under glucotoxic (22.2 mM glucose) culture conditions for 48 h and then incubated (20 min) with (10^{-6} M) GIP, xenin-6 and Ψ-xenin-6 alone or in combination, as appropriate, in the presence of 5.6 mM glucose</u>. (A-E) Insulin was measured using a RIA. (F) LDH accumulation was assessed in BRIN-BD11 cells following a 20 min incubation with 10^{-6} M xenin-25, xenin-6 or Ψ-xenin-6. Values represent means ± SEM (A,B,D n=8; C,E and n=4). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to respective glucose controls. $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$ compared to (A,B) respective xenin-25 control or (E) same concentration of respective peptide treatment under normal culture conditions. $^{\pi}p < 0.05$, $^{\pi\pi}p < 0.01$, $^{\pi\pi\pi}p < 0.001$ compared to xenin-6. $^{\Phi}p < 0.05$, $^{\Phi\Phi}p < 0.01$, $^{\Phi\Phi\Phi}p < 0.001$ compared to respective GIP control.

Fig. 2. Effect of Ψ-xenin-6 on glucagon secretion from (A) αTC 1.9 cells and (B) isolated mouse islets. The effects of xenin-6 and Ψ-xenin-6 alone (10^{-6} M) or in combination with GIP (10^{-6} M) on glucagon secretion from (A) αTC 1.9 cells (120 min incubation) and (B) isolated mouse islets (60 min incubation) was assessed at 1.4 mM glucose. Glucagon was measured using an ELISA. Values represent means ± SEM (n=4). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to 1.4 mM glucose control. $^{\Delta}p < 0.05$ compared to respective GIP control.

Fig. 3. Effect of Ψ-xenin-6 on proliferation and protection against apoptosis in rodent BRIN-BD11 and human 1.1B4 beta cells. (A) BRIN-BD11 and (B) 1.1B4 beta-cells were incubated overnight (18 hours) with GLP-1, xenin-25, xenin-6 or Ψ-xenin-6 (each at 10^{-8} and 10^{-6} M). Proliferation was measured using Ki-67 immunocytochemistry. (C,D) TUNEL positive apoptotic cells were assessed following 2 h exposure to a cytokine cocktail (IL-1β 100 U/mL, IFN-γ 20 U/mL, TNF-α 200 U/mL) with or without co-culture in the presence of GLP-1, xenin-25, xenin-6 or Ψ-xenin-6 (each at 10^{-8} and 10^{-6} M) in (C) BRIN-BD11 and (D) 1.1B4 beta-cells. Values represent means ± SEM (n=4). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to respective media control.

Fig. 4. Effects of Ψ-xenin-6 on cumulative food intake in 18 hour fasted mice. Cumulative food intake was measured in 18 h fasted mice at 30, 60, 90, 120, 150, 180 min after i.p. injection of saline vehicle (0.9% w/v NaCl), xenin-25, xenin-6 or Ψ-xenin-6 at (A) 25 and (B) 250 nmol/kg bw. Values represent means ± SEM (n=8). *$p < 0.05$, **$p < 0.01$ compared to respective saline control.

Fig. 5. Effects of xenin-6 and Ψ-xenin-6 alone as well as in combination with GIP on plasma glucose and insulin concentrations in mice. (A) Blood glucose and (C) plasma insulin concentrations were measured immediately before and 15, 30, 60 and 105 min after i.p. injection of glucose alone (18 mmol/kg bw) or in combination with GIP, xenin-6 or Ψ-xenin-6, as well as combined injection of GIP and xenin-6 or Ψ-xenin-6 (each peptide injected at 25 nmol/kg bw) in 4 h fasted mice. (B,D) Blood glucose and plasma insulin area under the curve (AUC) values for 0-105 min post injection are also shown. Values represent mean ± SEM for 6 mice. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to glucose alone. $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$, $^{\Delta\Delta\Delta}p < 0.001$ compared to GIP control.

Fig. 6. Persistent glucose lowering effects of Ψ-xenin-6 in mice. (A,C,E,G) Blood glucose concentrations were measured immediately before and 15, 30, 60 and 105 min following an i.p. glucose load (18 mmol/kg bw) in 4 h fasted mice injected with saline vehicle (0.9% w/v NaCl), xenin-6 or Ψ-xenin-6 (each at 25 nmol/kg bw) 2 (A), 4 (C), 8 (E) or 12 h (G) previously. (B,D,F,H) Blood glucose AUC values for 0-105 min post injection are also shown. Values represent mean ± SEM for 6 mice. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to glucose alone. $^{\Lambda}p < 0.05$ compared to xenin-6.

Figure 1

Figure 2
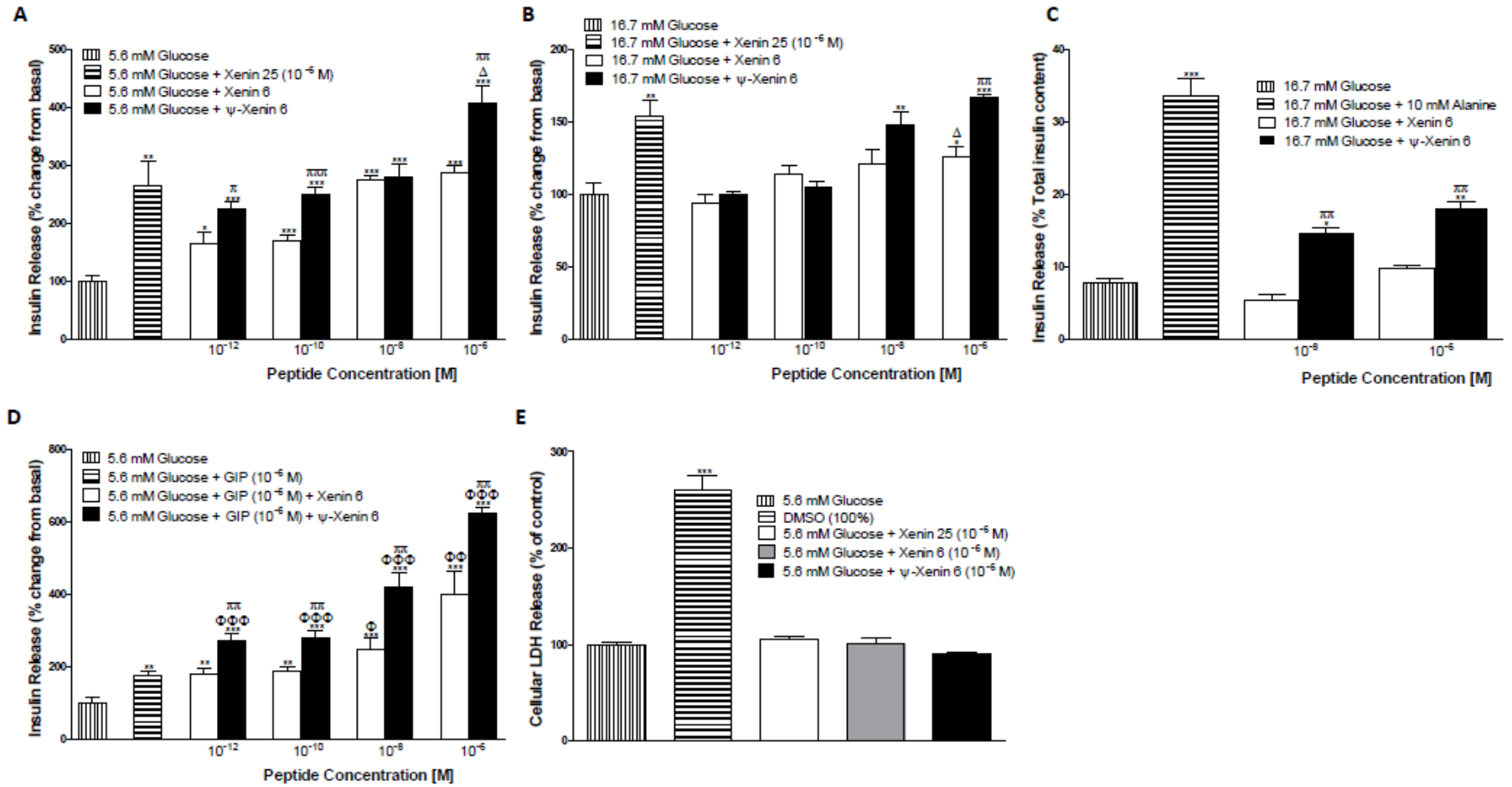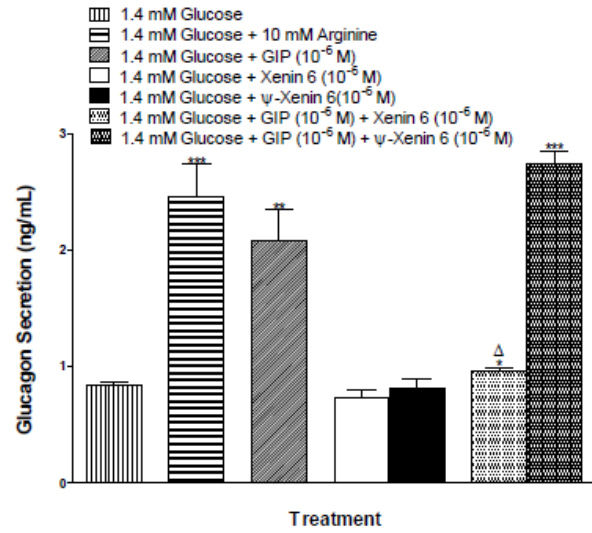
A

Legend:
- 1.4 mM Glucose
- 1.4 mM Glucose + 10 mM Arginine
- 1.4 mM Glucose + GIP (10^{-6} M)
- 1.4 mM Glucose + Xenin 6 (10^{-6} M)
- 1.4 mM Glucose + ψ-Xenin 6 (10^{-6} M)
- 1.4 mM Glucose + GIP (10^{-6} M) + Xenin 6 (10^{-6} M)
- 1.4 mM Glucose + GIP (10^{-6} M) + ψ-Xenin 6 (10^{-6} M)

Y-axis: Glucagon Secretion (ng/mL)
X-axis: Treatment

B

Legend:
- 1.4 mM Glucose
- 1.4 mM Glucose + 10 mM Arginine
- 1.4 mM Glucose + GIP (10^{-6} M)
- 1.4 mM Glucose + Xenin 6 (10^{-6} M)
- 1.4 mM Glucose + ψ-Xenin 6 (10^{-6} M)
- 1.4 mM Glucose + GIP (10^{-6} M) + Xenin 6 (10^{-6} M)
- 1.4 mM Glucose + GIP (10^{-6} M) + ψ-Xenin 6 (10^{-6} M)

Y-axis: Glucagon Secretion (% of islet content)
X-axis: Treatment

Figure 3

Figure 4

A

B

Figure 5

Figure 6