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A novel peptide isolated from *Aphonopelma chalcodes* tarantula venom with benefits on pancreatic islet function and appetite control

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ABSTRACT

Proof-of-concept for therapeutic application of venom-derived compounds in diabetes is exemplified by the incretin mimetic, exenatide, originally extracted from the saliva of the venomous *Heloderma suspectum* lizard. In this regard, we have isolated and sequenced a novel 28 amino acid peptide named Δ-theraphotoxin-Ac1 (Δ-TRTX-AC1) from venom of the Mexican Blond tarantula spider *Aphonopelma chalcodes*, with potential therapeutic benefits for diabetes. Following confirmation of the structure and safety profile of the synthetic peptide, assessment of enzymatic stability and effects of Δ-TRTX-AC1 on *in vitro* beta-cell function were studied, alongside potential mechanisms. Glucose homeostatic and satiety actions of Δ-TRTX-AC1 alone, and in combination with exenatide, were then assessed in C57BL/6 mice. Synthetic Δ-TRTX-AC1 was shown to adopt a characteristic inhibitor cysteine knot (ICK)-like structure and was non-toxic to beta-cells. Δ-TRTX-AC1 evoked glucose-dependent insulin secretion from BRIN BD11 cells with bioactivity confirmed in murine islets. Insulin secretory potency was established to be dependent on K_{ATP} and Ca^{2+} channel beta-cell signalling. In addition, Δ-TRTX-AC1 enhanced beta-cell proliferation and provided significant protection against cytokine-induced apoptosis. When injected co-jointly with glucose in mice at a dose of 250 nmol/kg, Δ-TRTX-AC1 decreased blood-glucose levels and evoked a significant satiating effect. Moreover, whilst Δ-TRTX-AC1 did not enhance exenatide induced benefits on glucose homeostasis, the peptide significantly augmented exenatide mediated suppression of appetite. Together these data highlight the therapeutic potential of tarantula spider venom-derived peptides, such as Δ-TRTX-Ac1, for diabetes and related obesity.

1. Introduction

Type 2 diabetes mellitus (T2DM) is described as the biggest epidemic of the 21st Century, with a projected 454 million people likely to be diagnosed with the disease by 2045 [1]. A wide range of licensed pharmacological agents are available to help manage T2DM [2]. However, due to the degenerative nature of the disease and depletion of pancreatic beta-cell mass and function over time, treatment failure is unfortunately commonplace [3]. Polypharmacy is often required in T2DM, but even with this, control of blood glucose levels is T2DM is frequently inadequate [4]. Thus, an urgent need exists to develop safe and effective therapies to improve the quality of life of those people currently living with T2DM.

In this respect, the clinical approval of several compounds from the venom of snakes, lizards and leeches highlights the therapeutic potential of animal kingdom venom-derived drugs for human disease [5]. As such, crude venom contains a diverse mixture of molecules, that upon extraction, can yield individual targeted compounds that often possess inherent enzymatic stability and ultimately superior bioactivity [6]. The clinically approved incretin mimetic, exenatide, originating from the saliva of venomous *Heloderma suspectum* Gila monster lizard, represents proof-of-concept for this drug discovery pathway in T2DM [7].

Abbreviations: Δ-TRTX-AC1, Δ -theraphotoxin-Ac1; ANOVA, analysis of variance; Da, Daltons; K_{ATP} channel, ATP-sensitive potassium channel; LDH, lactate dehydrogenase; HPLC, high-performance liquid chromatography; IBMX, 3-isobutyl-1-methyl-xanthine; ICK, inhibitor cysteine knot; IFN-γ, interferon- γ; IL-1β, interleukin-1β; KCl, potassium chloride; Kv2.1 channel, voltage-gated potassium channel; KRBB, Krebs-ringer bicarbonate buffer; MALDI-ToF MS, matrix-assisted laser desorption/ionisation-time of flight mass spectrometry; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NaCl, sodium chloride; PBS, phosphate buffer solution; RFU, relative fluorescence units; RPMI, Roswell Park Memorial Institute; RIA, radioimmunoassay; T2DM, type 2 diabetes mellitus; TFA, trifluoracetic acid; TNF-α, tumour necrosis factor α; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling.

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Moreover, the extraction and characterisation of multiple bioactive peptides, with postulated benefits in diabetes, from venomous species such as cone snails, tarantulas, snakes and bees reinforce this concept [5].

Venom from the tarantula spider family contains millions of bioactive peptides, many of which are cysteine-rich, resulting in an inhibitor cysteine knot (ICK)-like structure that provides effective resistance against circulating enzymatic breakdown [8]. Tarantula venom-derived peptides with the ICK structural motifs, such as hanatoxin, grammotoxin and guangotoxin, have been isolated from the venom of the *Grammostola rosea* tarantula spider and shown to possess insulin secretory activity through regulation of pancreatic beta-cell ion channels [9,10]. However, issues relating to safety, specificity and overall effectiveness of these peptides has meant that they have not progressed further than *in vitro* testing for diabetes [11]. Following on from this, our current work with venom from the *Aphonopelma chalcodes* Mexican Blond tarantula spider has led to the isolation and identification of a novel 28 amino acid ICK peptide named Δ-theraphotoxin-Ac1 (Δ-TRTX-Ac1), with putative insulinotropic actions.

In the current study we initially synthesised synthetic C-terminally amidated or carboxylated Δ-TRTX-Ac1 in linear form and then confirmed ICK folding. We next progressed to examine toxicity profile and plasma stability followed by corroboration of *in vitro* and *ex vivo* insulinotropic actions, as well as determining putative mechanism of action at the level of the pancreatic beta-cell. In addition, the impact of Δ-TRTX-Ac1 peptides on beta-cell proliferation and protection against apoptosis was also investigated. Following substantiation of *in vitro* bioactivity, the impact of Δ-TRTX-Ac1 peptides, both alone and in combination with the incretin mimetic exenatide, on satiety and glucose homeostasis was examined in rodents. Our data support further investigation of novel tarantula venom-derived peptides, such as of Δ-TRTX-Ac1, as potential new and effective therapies for diabetes and/or obesity.

2. Materials and methods

2.1. Peptide extraction and identification

Lyophilised milked crude venom from the *A. chalcodes* tarantula was commercially acquired (Spider Pharm Yarnell, AZ) and subsequently fractionated by size exclusion and high-performance liquid chromatography (HLPC). Fig. 1 depicts HPLC fractionation of *A. chalcodes* crude venom. A fingerprint of the mass/charge ratio of HPLC test fractions was obtained using matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-ToF MS). Individual chromatographic fractions of *A. chalcodes* were then assessed for insulinotropic properties, in a similar manner as described below, with significant bioactivity observed around 31–32 min (Fig. 1). Subsequent MALDI-ToF mass spectrometry revealed the main peptide constituent to have an *m/z* of 2919 Da. Automated Edman degradation provided the amino acid sequence of this peptide as RCLPAGKPCAGVTQKIPCCGKCSRNKCT (Table 1). The novel peptide was named Δ-theraphotoxin-Ac1 (Δ-TRTX-AC1), consistent with standard peptide nomenclature guidelines.

2.2. Synthetic peptide synthesis

Both C-terminal amidated and carboxylated peptides were commercially synthesised based on the amino acid sequence of Δ-TRTX-Ac1 at greater than 95% purity (Synpeptide ltd., Shanghai, China). All peptides were further characterised in-house using RP-HPLC to confirm purity and MALDI-ToF MS to determine molecular weight, as described previously [12]. To corroborate presence of ICK structural motif, the expected mass of the carboxylated linear peptide, 2923.5 Da, and folded peptide, 2917.5 Da were compared, with the process repeated for the amidated linear and folded peptide, which have molecular masses of 2922.5 and 2916.5 Da, respectively.

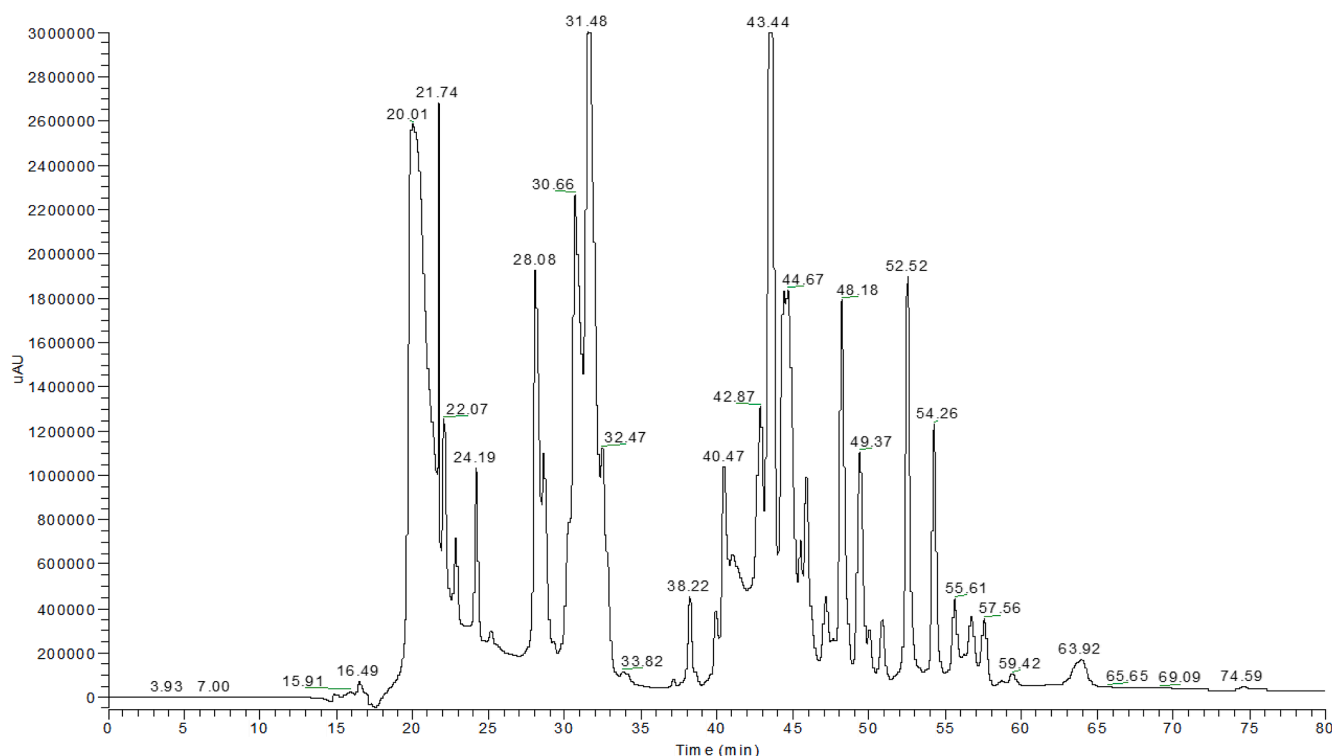

Fig. 1. HPLC fractionation of *Aphonopelma chalcodes* crude venom on a reversed-phase Jupiter C18 column (250 mm × 10 mm) using a gradient mobile phase of 100% Solvent A (0.05% TFA:99.95% water) to 100% Solvent B (80% acetonitrile:19.95% water:0.05% TFA) over 80 min with UV detection at 214 nm.

Table 1
Amino acid sequence, MS analysis and enzymatic stability of Δ-TRTX-Ac1 peptides.

Peptide	Amino acid sequence	Theoretical mass of linear peptide (Da)	Theoretical mass of ICK peptide (Da)	Experimental molecular mass (Da)	Percentage intact peptide remaining (%)			
					2 h	4 h	8 h	12 h
Δ-TRTX-Ac1-OH	R**C**LPAGKP**C**AGVTQKIP**CC**GK**C**SRNK**C**T-OH	2921.4	2915.4	2915.3	100 %	100 %	100 %	100 %
Δ-TRTX-Ac1-NH_2	R**C**LPAGKP**C**AGVTQKIP**CC**GK**C**SRNK**C**T-NH_2	2920.4	2914.4	2914.4	100 %	100 %	100 %	100 %

Amino acid sequence denoted using single letter amino acid notation. The cysteine residues (C) that form the ICK loop are highlighted in **bold underlined** text. Molecular mass of was determined using MALDI-ToF MS. Peptide stability was assessed as percentage intact peptide remaining following 0, 2, 4, 6 and 8 h incubation in murine plasma. Degradation products were separated using HPLC, analysed by MALDI-ToF MS, and percentage degradation calculated from peak areas.

2.3. *Plasma stability*

To determine the enzymatic stability of Δ-TRTX-Ac1-OH and Δ-TRTX-Ac1-NH_2, test peptides (1 mg/ml) were incubated at 37°C with 10 μl of overnight fasted C57BL/6 murine plasma in 380 μl triethanolamine-HCl (50 mmol/l, pH 7.8; Sigma-Aldrich, Poole, UK) for 0, 120, 240, 480 and 720 mins. Reactions were terminated by addition of 50 μl of 10% (v/v) TFA/water (TFA purchased from Sigma-Aldrich, Poole, UK). Degradation products were separated using HPLC with identification of peaks by MALDI-ToF MS as described previously [12].

2.4. *Beta-cell toxicity*

The lactate dehydrogenase (LDH) and MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, Poole, UK) assays were used to assess cytotoxic effects of Δ-TRTX-Ac1-OH and Δ-TRTX-Ac1-NH_2 in BRIN-BD11 beta-cells [13]. LDH activity was measured using the CytoTox 96® non-radioactive cytotoxicity kit (Promega, Madison, USA), using test samples generated from the insulin secretory assay described below. For the MTT assay, BRIN BD11 cells (10,000 cells per well) were grown in 96-well plates with RPMI-1640 media (Gibco Life Technologies ltd, Paisley, UK) for 24 h in the absence and presence of test peptides (10^{-8} -10^{-6} M). Cells were then supplemented with 20 μl MTT solution (5 mg/ml; Sigma Aldrich, Poole, UK) and incubated for 2 h at 37°C. Media was removed and formazan crystals dissolved using 100 μl DMSO (Sigma Aldrich, Poole, UK) with plate agitation for 10 min. Absorbance was read on a spectrophotometer at excitation and emission wavelengths of 570 nm and 630 nm, respectively.

2.5. *In vitro insulin secretion and mechanism of action*

The *in vitro* insulin secretory activity of test peptides was examined in BRIN-BD11 cells which were cultured and maintained as previously described [13]. For experimentation, BRIN-BD11 cells were seeded in 24-well plates at a density of 150,000 cells/well and allowed to attach overnight at 37°C. Following pre-incubation with Krebs–Ringer bicarbonate buffer (KRBB; Sigma-Aldrich, Poole, UK; pH 7.4) supplemented with 0.5% (w/v) BSA (Sigma-Aldrich, Poole, UK) and 1.1 mM glucose (40 min; 37 °C; BDH Chemicals, Poole, UK), cells were then incubated with test peptides (10^{-12} – 10^{-6} M) at 1.1-, 5.6-, 11.1- or 16.7-mM glucose, as appropriate, for 20 min. Aliquots (200 μl) of assay buffer were collected and then stored at −20°C prior to the assessment of insulin concentrations by an in-house radioimmunoassay (RIA). To corroborate insulin secretory activity of Δ-TRTX-Ac1 peptides, pancreatic islets were isolated from C57BL/6 mice by collagenase digestion (Sigma-Aldrich, Poole, UK), as described previously by Lacy and Kostianovsky [14] and insulin secretion was determined as outlined above, but over a 60 min test incubation period at 16.7 mM glucose.

For mechanism of action, the insulinotropic action of Δ-TRTX-Ac1 peptides was investigated in BRIN BD11 cells as above at 5.6 mM glucose, but in the absence or presence of established modulators of insulin secretion as described previously [15] including verapamil (50 μM, Ca^{2+} channel blocker; Sigma-Aldrich, Poole, UK), diazoxide (300 μM, K_{ATP} channel opener; Sigma-Aldrich, Poole, UK), IBMX (200 μM, phosphodiesterase inhibitor; Sigma-Aldrich, Poole, UK) or tolbutamide (200 μM, K_{ATP} channel blocker; Sigma-Aldrich, Poole, UK). In addition, BRIN-BD11 cells were also incubated with KCl (30 mM; cell membrane depolariser; Sigma-Aldrich, Poole, UK) at 16.7 mM glucose. Furthermore, the effects of Δ-TRTX-Ac1 peptides on BRIN BD11 beta-cell membrane potential and intracellular calcium $[Ca^{2+}]_i$ were also assessed using a FLIPR membrane or calcium assay kit (Molecular Devices, USA), as previously described [16]. Briefly, BRIN-BD11 cells (1 × 10^5) were incubated with KRBB containing 5.6 mM glucose and test peptides (10^{-6} M), with membrane potential or calcium mobilisation data collected and analysed using Softmax Pro software.

2.6. *Effects on proliferation and protection against apoptosis*

To assess beta-cell proliferative effects, BRIN-BD11 cells (40,000 cells per chamber slide) were incubated for 24 h in RPMI media with test peptides (10^{-8} and 10^{-6} M). The media was discarded, and cells were washed with phosphate buffer solution (PBS; Sigma-Aldrich, Poole UK) and fixed using 4% paraformaldehyde (BDH Chemicals, Poole, UK). Antigen retrieval was achieved using citrate buffer (90°C for 20 min; BDH Chemicals, Poole, UK) and subsequently cooled for 20 min. Cells were then blocked using 4% bovine serum albumin (BSA) for 45 mins, before being incubated with Ki-67 primary antibody (1:500; Abcam, ab15580), followed by Alexa Fluor® 488 secondary antibody (1:400, Invitrogen, A-11008). Chamber slides 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 Δ-TRTX-Ac1 peptides to protect against cytokine-induced apoptosis, BRIN BD11 cells were seeded as above. However, cells were also exposed to a cytokine-cocktail (IL-1β 100 U/mL, IFN-γ 20 U/mL, TNF-α 200 U/ml, all purchased from Sigma-Aldrich) in the presence or absence of test peptides (10^{-8} and 10^{-6} M) for 24 h. TUNEL staining (Roche Diagnostics ltd, UK) was employed to quantify beta-cell apoptosis, as previously described [12]. Apoptosis was expressed as percentage of total cells analysed. Approximately 150 cells were analysed per group.

2.7. *Acute in vivo experiments*

Acute *in vivo* studies were conducted in 12-week-old adult male C57BL/6 mice (Envigo ltd, UK). Mice were single-housed and kept in a temperature-controlled environment (22 ± 2°C) under a 12-hour light/dark cycle, with *ad libitum* access to drinking water and rodent maintenance diet (10% fat, 30% protein and 60% carbohydrate, percent of total energy 12.99 kJ/g; Trouw Nutrition, UK). All experiments were approved by Ulster University Animal Welfare and Ethics Review Body and conducted in accordance with the UK Animals (Scientific Procedures) Act 1986. To assess effects of Δ-TRTX-Ac1 peptides on satiety,

overnight (16 h) fasted mice (n = 7) were administered an intraperitoneal (i.p.) injection of saline vehicle (0.9% [w/v] NaCl), Δ-TRTX-Ac1-OH, Δ-TRTX-Ac1-NH_2 or exenatide (each at 2.5, 25 or 250 nmol/kg bw) and cumulative food consumption recorded at regular intervals over a 180 min observation period. For assessment of glucose homeostatic and insulin secretory benefits, blood glucose and plasma insulin concentrations were determined immediately prior to and 15, 30 and 60 min after i.p. injection of glucose alone (18 mmol/kg bw) or in combination with test peptides (each at 25, 75 or 250 nmol/kg bw) in 16 h fasted mice. To examine potential additive effects of Δ-TRTX-Ac1 peptides and exenatide on satiety or glucose homeostasis, mice were administered with Δ-TRTX-Ac1 peptide (25 nmol/kg bw) in combination with exenatide (0.25 or 2.5 nmol/kg bw) and experiments repeated as described above. Reduced doses of exenatide were employed for these experiments so as not to preclude additive effects of the peptides, given the prominent efficacy of exenatide in the chosen experimental systems [7].

2.8. Biochemical analyses

Blood samples were obtained from conscious mice via the cut tip on the tail vein and blood glucose was immediately measured using an Ascencia Contour blood glucose meter (Bayer Healthcare, Newbury, UK). Blood was also collected in chilled heparin/fluoride coated microcentrifuge tubes (Sarstedt, Numbrecht, Germany) and centrifuged for 15 min at 12,000 rpm using a Beckman microcentrifuge (Beckman Instruments, Galway, Ireland) to separate plasma. Plasma insulin was then measured by iRIA [17].

2.9. Statistical analyses

Statistical tests were conducted using GraphPad PRISM software (Version 5.0). Values are expressed as mean ± SEM. Comparative analyses between groups were performed using a one-way ANOVA with Bonferroni's post hoc test, a two-way ANOVA with Bonferroni's post hoc test or Student's unpaired *t*-test, as appropriate. Differences were deemed to be significant if $P < 0.05$.

3. Results

3.1. Peptide characterisation and plasma enzyme stability

MALDI-ToF analysis of synthetically manufactured Δ-TRTX-Ac1 peptides in KRBB buffer confirmed the presence of the ICK motif, with a mass of 6 Da lower than the linear peptide (Table 1). Both Δ-TRTX-Ac1-OH and Δ-TRTX-Ac1-NH_2 were fully resistant to murine plasma-mediated enzyme degradation up to and including a 12 h incubation period (Table 1).

3.2. Cell viability and in vitro insulinotropic actions

Neither Δ-TRTX-Ac1-OH nor Δ-TRTX-Ac1-NH_2 negatively affected BRIN-BD11 cell viability, either in terms of LDH production (Fig. 2A) or mitochondrial function (Fig. 2B). With respect to insulin secretory activity, Δ-TRTX-Ac1-OH and Δ-TRTX-Ac1-NH_2 evoked no notable change in insulin output from BRIN-BD11 cells at 1.1 mM glucose (Fig. 3A). However, at glucose concentrations of 5.6-, 11.1- and 16.7-mM glucose, the amidated peptide significantly ($P < 0.05$ - $P < 0.001$) increased insulin secretion (Fig. 3B-D). In addition, Δ-TRTX-Ac1-OH stimulated ($P < 0.05$ - $P < 0.001$) insulin secretion at 11.1- and 16.7-mM glucose when incubated at 10^{-6} M (Fig. 3C,D). Insulin secretory actions of the Δ-TRTX-Ac1 peptides were confirmed in isolated murine islets, with efficacy similar to that of exenatide, albeit non-significant for the Δ-TRTX-Ac1 peptides (Fig. 3E). In terms of related mechanisms, the insulinotropic action of both Δ-TRTX-Ac1 peptides was dependent on K_{ATP} and voltage gated Ca^{2+} channel activity, as no significant insulin release was detected when the peptides were incubated with verapamil or diazoxide (Fig. 3F). In addition, no additive insulinotropic benefits of the Δ-TRTX-Ac1 peptides was evident alongside the membrane depolarising agent KCl in BRIN BD11 cells (Fig. 3F). In contrast, both test peptides provoked an increase in insulin secretion in the presence of IBMX (Fig. 3F). In agreement with these observations, neither Δ-TRTX-Ac1 peptide affected BRIN BD11 beta-cell membrane depolarisation (Fig. 3G), but each peptide did evoke a small but significant ($P < 0.05$ - $P < 0.01$) positive effect on Ca^{2+} influx (Fig. 3H).

3.3. Beta-cell proliferation and protection against apoptosis

All test peptides, namely Δ-TRTX-Ac1-OH, Δ-TRTX-Ac1-NH_2 and exenatide, provoked significant ($P < 0.001$) beta-cell proliferative effects at 10^{-8} and 10^{-6} M in BRIN BD11 cells when compared to control cultures (Fig. 4A). As known, exenatide induced significant ($P < 0.001$) protective effects against cytokine induced beta-cell apoptosis at concentrations of 10^{-8} and 10^{-6} M (Fig. 4B). Both Δ-TRTX-Ac1-OH and Δ-TRTX-Ac1-NH_2 exhibited remarkably similar beta-cell protective actions (Fig. 4B).

3.4. Acute in vivo effects on food intake and glucose tolerance

When injected to overnight fasted mice at doses of 2.5, 25 or 250 nmol/kg, exenatide induced significant ($P < 0.001$) appetite suppressive actions at all observation time points during the 180-minute experimental period (Fig. 5A-C). In contrast, Δ-TRTX-Ac1-OH had no

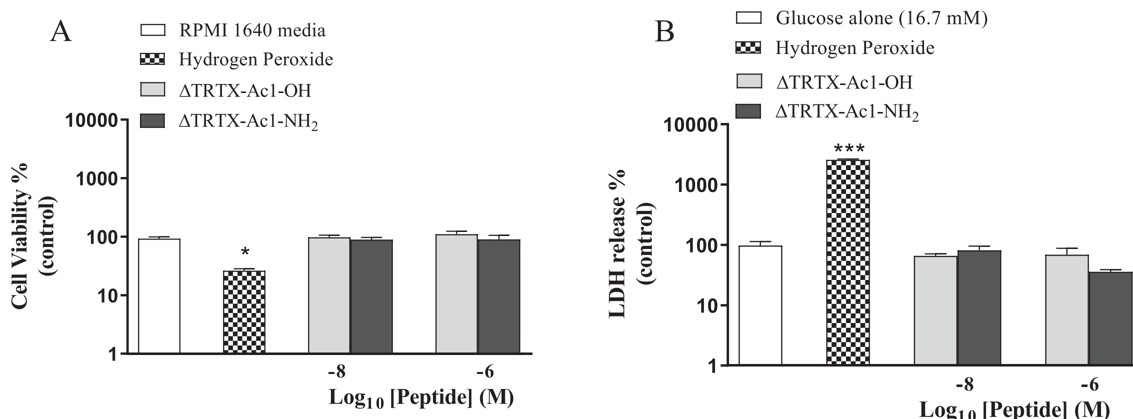

Fig. 2. (A) BRIN-BD11 cells (n = 3) were cultured (24 h) with test peptides (10^{-8} and 10^{-6} M) and cell viability assessed using MTT. (B) BRIN-BD11 cells (n = 8) were exposed to test peptides (10^{-8} and 10^{-6} M) for 4 h and LDH content assessed. All values are mean ± SEM. ***$P < 0.05$, ***$P < 0.001$ compared to respective control.

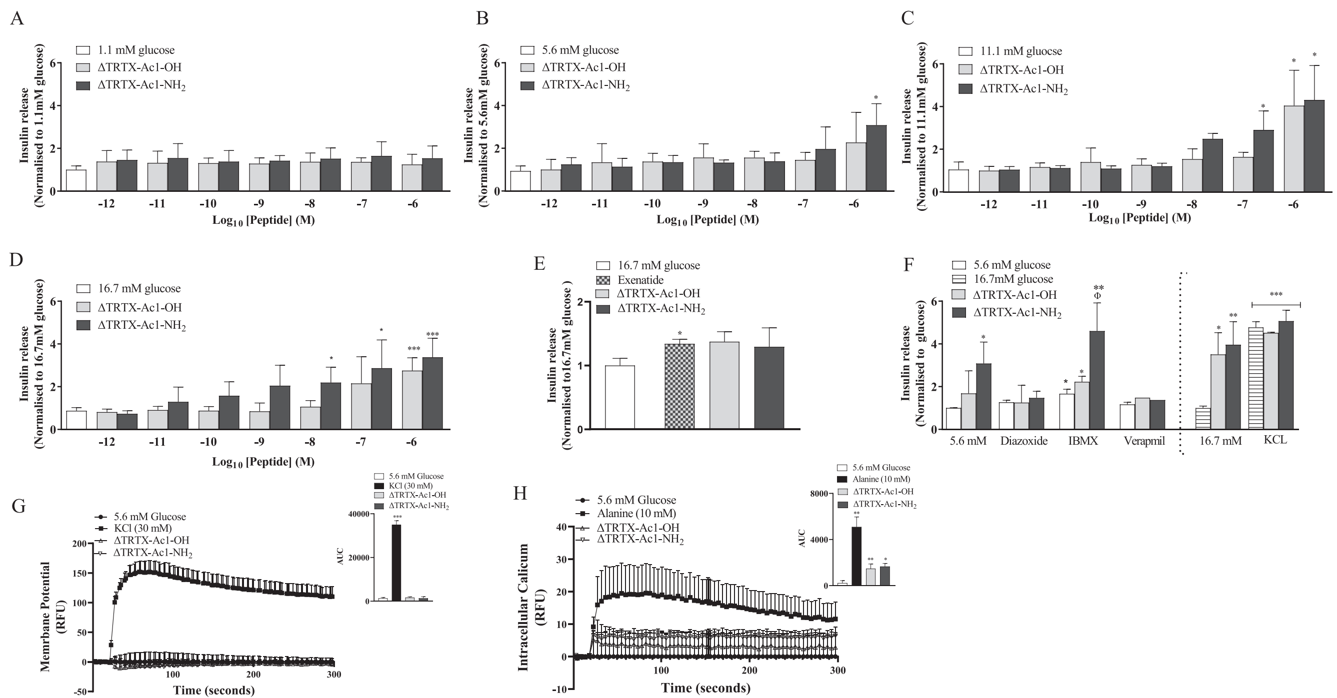

Fig. 3. BRIN-BD11 cells were incubated with (A) 1.1 (B) 5.6, (C) 11.1 or (D) 16.7 mM glucose supplemented with Δ-TRTX-Ac1-OH or Δ-TRTX-Ac1-NH_2 peptides (10^{-12} – 10^{-6} M) and insulin secretion determined. (E) Isolated murine islets were incubated with 16.7 mM glucose in the presence or absence of ΔTRTX-Ac1-OH and ΔTRTX-Ac1-NH_2 (10^{-6} M) and insulin secretion determined. (F) BRIN-BD11 cells were incubated with known insulin modulators in the presence or absence of Δ-TRTX-Ac1-OH or Δ-TRTX-Ac1-NH_2 peptides (10^{-6} M) and insulin secretion determined. (G) BRIN BD11 cells were incubated for 60 min with FLIPR membrane potential dye and test peptides (10^{-6} M) with RFU was recorded. (H) BRIN BD11 cells were incubated for 60 min with intracellular Ca^{2+} dye and test peptides (10^{-6} M) with RFU recorded. Values are mean ± SEM (n = 8). *P < 0.05 ***P < 0.001 **P < 0.01 compared to respective controls.

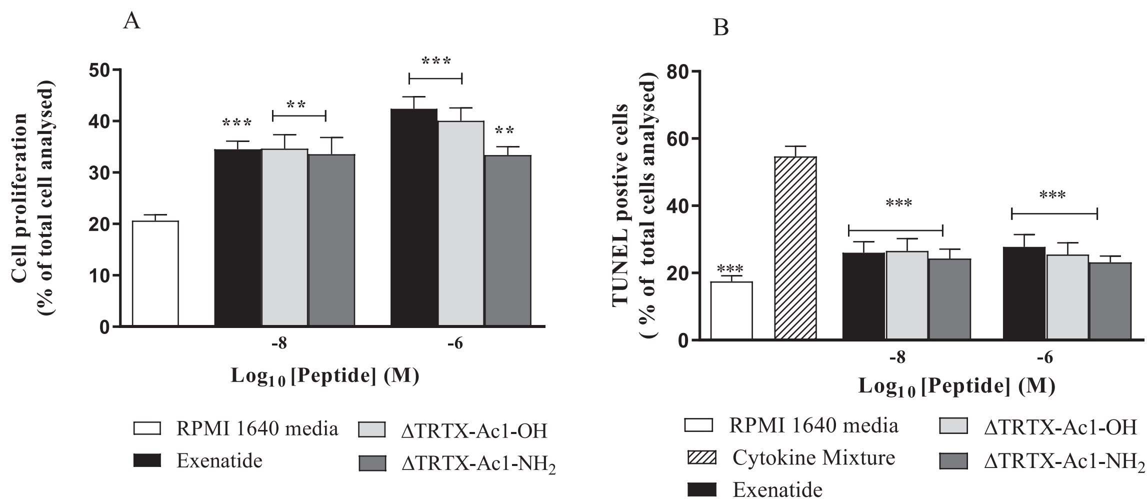

Fig. 4. Effects of ΔTRTX-Ac1-OH and ΔTRTX-Ac1-NH_2 on (A) beta-cell proliferation and (B) protection against cytokine-induced apoptosis. (A) BRIN-BD11 cells were cultured (24 h) with test peptides (10^{-8} and 10^{-6} M) and proliferation detected using Ki-76 staining. (B) BRIN-BD11 were cultured (24 h) with test peptides (10^{-8} and 10^{-6} M) in the presence of a cytokine cocktail and apoptosis detected using the TUNEL assay. All values are mean ± SEM (n = 3). **P < 0.01, ***P < 0.001 compared to (A) RPMI 1640 media alone or (B) cytokine cocktail.

significant satiety effects in this experimental system (Fig. 5A-C). However, at 2.5 nmol/kg, Δ-TRTX-Ac1-NH_2 reduced food intake at 180-min post-injection when compared to saline control mice (Fig. 5A). In agreement, Δ-TRTX-Ac1-NH_2 displayed significant (P < 0.05 - P < 0.01) appetite suppressive actions at doses of 25 and 250 nmol/kg at various observation points (Fig. 5B,C). Notably however, Δ-TRTX-Ac1-NH_2 was less effective than exenatide in this regard (Fig. 5A-C). Similar to effects on appetite, exenatide significantly (P < 0.05 - P < 0.001) improved glucose tolerance in mice at peptide doses of 25, 75 or 250 nmol/kg (Fig. 6A-C). Neither Δ-TRTX-Ac1-OH nor Δ-TRTX-Ac1-NH_2 affected glucose disposal when injected at 25 nmol/kg (Fig. 6A). However, at a dose of 75 nmol/kg, Δ-TRTX-Ac1-OH reduced (P < 0.05 - P < 0.001) individual blood glucose levels over the 60-minute observation period when compared to control mice (Fig. 6B), with both Δ-TRTX-Ac1 peptides decreasing (P < 0.05 - P < 0.001) overall 0–60 min glucose AUC values at 250 nmol/kg (Fig. 6C). Glucose homeostatic efficacy of Δ-TRTX-Ac1-OH and Δ-TRTX-Ac1-NH_2 was less impressive when compared to exenatide (Fig. 6A-C). As expected, exenatide mediated

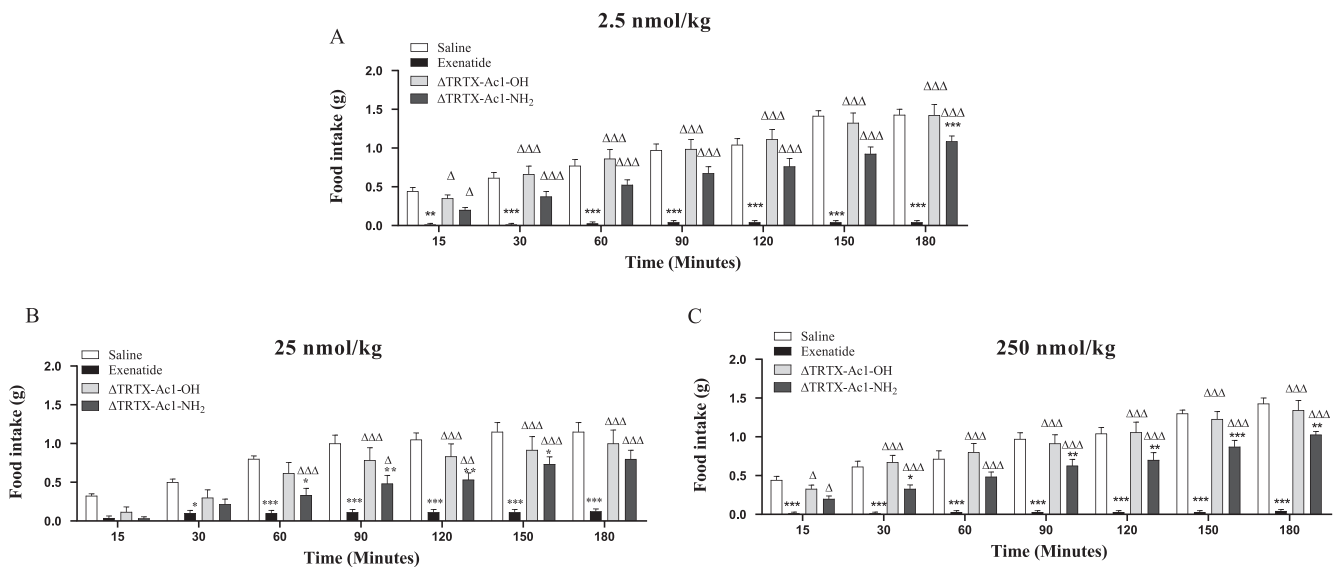

Fig. 5. Effects of Δ-TRTX-Ac1-OH, Δ-TRTX-Ac1-NH_2 and exenatide on food intake in mice. Test peptides were administered to overnight (16 h) mice at (A) 2.5, (B) 25 or (C) 250 nmol/kg, with saline vehicle (0.9% (w/v) NaCl) as control. Values represent mean ± SEM (n = 7) *P < 0.05, **P < 0.01, ***P < 0.001 compared to saline control. $^{\Delta}P < 0.05$, $^{\Delta\Delta\Delta}P < 0.001$ compared to exenatide.

Fig. 6. Effects of Δ-TRTX-Ac1-OH, Δ-TRTX-Ac1-NH_2 and exenatide on glucose tolerance and insulin secretion in mice. Blood glucose and plasma insulin concentrations were assessed following administration of test peptides at (A) 25, (B) 75 or (C) 250 nmol/kg in combination with glucose (18 mmol/kg bw) in overnight fasted (16 h) mice. Values are mean ± SEM (n = 7). *P < 0.05, **P < 0.01, ***P < 0.001 compared to glucose alone control. $^{\Delta\Delta\Delta}P < 0.001$ compared to exenatide.

benefits on glucose disposal were directly linked to elevated ($P < 0.05$ - $P < 0.01$) glucose-induced insulin secretion, whereas Δ-TRTX-Ac1 peptides had no obvious impact on plasma insulin concentrations apart from increased ($P < 0.05$ - $P < 0.01$) 0–60 min overall values at the 250 nmol/kg dose (Fig. 6A-C).

In terms of additive effects on glucose control or appetite suppression in mice, there was no obvious glucose homeostatic benefits of combined administration of 25 nmol/kg Δ-TRTX-Ac1 peptides with exenatide (Fig. 7A). However, Δ-TRTX-Ac1-NH_2 was able to significantly ($P < 0.05$ - $P < 0.001$) augment the appetite suppressive actions of 2.5 nmol/kg exenatide in overnight fasted mice (Fig. 7B).

4. Discussion

Tarantula spider venom represents an invaluable source of bioactive peptides that may have untapped therapeutic potential for various human diseases [5]. Many of these peptides are thought to modulate ion channel activity, raising the possibility of a positive impact on pancreatic beta-cell function. Thus, ion channels exert a critical role in regulating both electrical activity and signal transduction pathways within beta-cells, leading to subsequent insulin secretion [18]. Peptides previously isolated from tarantula venom have demonstrated insulin secretory activity, although some had promiscuous effects at several different ion channels [10], limiting their therapeutic potential. Nonetheless, clinical approval of the venomous lizard-derived insulinotropic agent, exenatide, highlights the feasibility of specific and safe peptides originating from animal venom to effectively manage human diabetes [7].

To capitalise on this largely unexploited drug discovery avenue for diabetes, we have isolated and sequenced a novel insulinotropic peptide from the venom of the Mexican Blond tarantula, *A. chalcodes*, named Δ-TRTX-Ac1. We employed both amidated and carboxylated versions of synthetic Δ-TRTX-Ac1, as C-terminal amidation of peptides is known to improve stability and potency [19]. However, both peptides remained fully intact when incubated in murine plasma, likely because of the presence of 6 cysteine amino acid residues, leading to formation of the classical ICK structure, prominent in other venom-derived peptides [20]. Thus, the ICK is well documented to impart excellent peptide enzymatic stability [8]. In this respect, we also investigated oral bioavailability and activity of the Δ-TRTX-Ac1 peptides in mice, but they were devoid of any obvious biological actions when administered via this route, even at suprapharmacological concentrations of 500 nmol/kg (data not shown). In contrast, relatively high doses of exenatide have been shown to exert some moderate bioactivity when administered orally to rodents [21], but further investigation and confirmation of this effect is required. Nonetheless, advances in peptide formulation are beginning to open avenues for oral delivery of small peptides [22], such as Δ-TRTX-Ac1. Moreover, semaglutide is a peptide-based drug that has recently gained clinical approval for diabetes in an oral formulation, albeit at an extremely high peptide dose [23].

Importantly, Δ-TRTX-Ac1-OH and Δ-TRTX-Ac1-NH_2 did not compromise beta-cell viability, whilst evoking clear glucose-dependent increases of insulin secretion from BRIN-BD11 beta-cells, which was also apparent in primary islets. On first look, the amidated peptide appeared to demonstrate slightly increased insulinotropic potency when

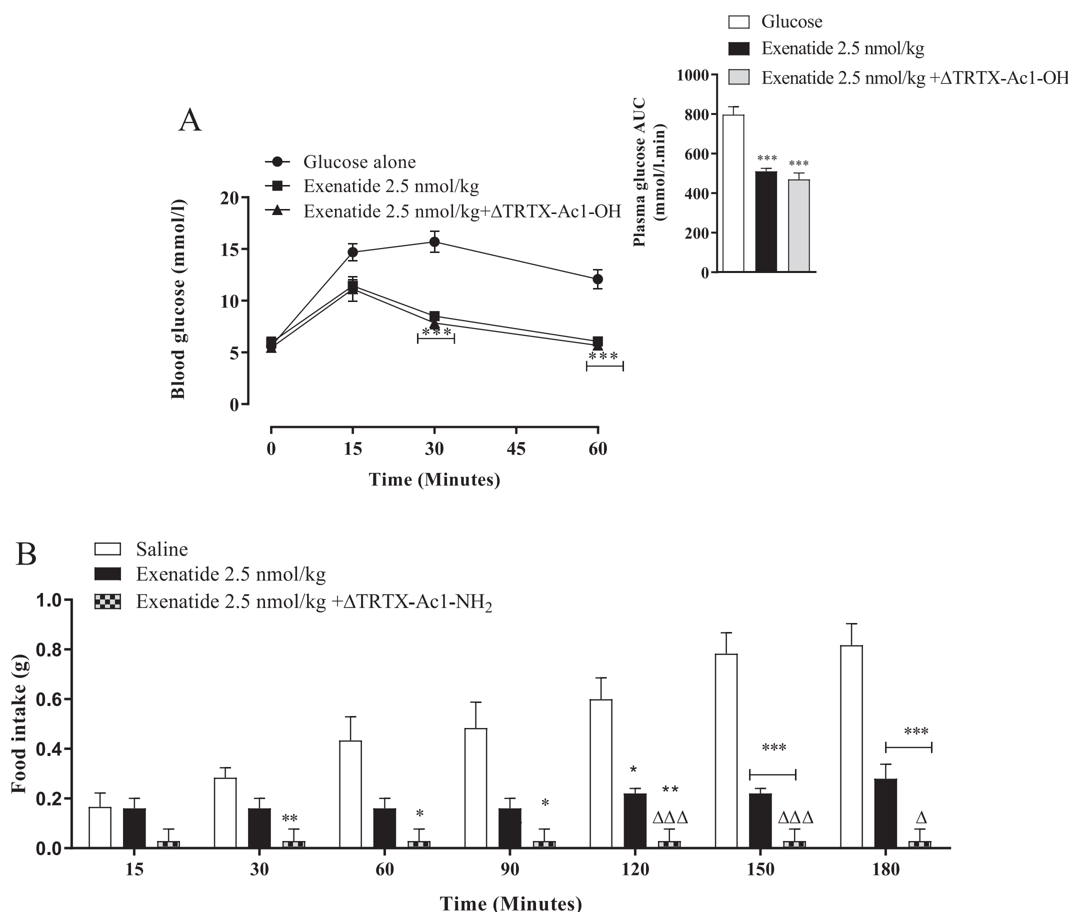

Fig. 7. Effects of Δ-TRTX-Ac1 peptides in combination with exenatide on (A) glucose homeostasis or (B) food intake in mice. (A) Fasted mice (16 h) were administered glucose control (18 mmol/kg) or together with Δ-TRTX-Ac1-OH (75 nmol/kg bw) alone and in combination with exenatide (2.5 nmol/kg). (B) Fasted mice (16 h) were administered saline vehicle (0.9% (w/v) NaCl) together with Δ-TRTX-Ac1-NH_2 (25 nmol/kg bw) alone or in combination with exenatide (2.5 nmol/kg). Values represent mean ± SEM (n = 7). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to glucose alone control. $^{\Delta}P < 0.05$, $^{\Delta\Delta\Delta}P < 0.001$ compared to exenatide.

compared to the carboxylated version in BRIN BD11 cells, but this was not evidenced in murine islets or the *in vivo* setting. Thus, bioactivity of Δ-TRTX-Ac1-OH and Δ-TRTX-Ac1-NH_2 at the level of the pancreatic beta-cell seems to be largely comparable. Certainly, glucose-dependent insulin secretion and avoidance of hypoglycaemic episodes was paramount in the prompt clinical approval of venom-derived exenatide for T2DM [24]. In terms of mechanism of insulinotropic actions of the Δ-TRTX-Ac1 peptides, insulin secretion is regulated by two major signaling pathways, namely the K_{ATP} channel dependent triggering pathway and K_{ATP} channel-independent augmentation pathway [25]. Our data suggest that activity of Δ-TRTX-Ac1 peptides were dependent on pathways downstream of the K_{ATP} channel and membrane depolarisation, which is supported by positive potentiation of insulin secretion when incubated alongside the phosphodiesterase inhibitor IBMX as well as only modest elevations of intracellular Ca^{2+} levels. Although we are unable to pinpoint the precise beta-cell target of Δ-TRTX-Ac1 peptides, insulinotropic actions in cell lines and isolated murine islets confirms activation of *bona fide* beta-cell signalling pathways that lead to insulin exocytosis. In this regard, it would also be interesting to assess the impact of Δ-TRTX-Ac1-OH and Δ-TRTX-Ac1-NH_2 on the expression of genes known to be involved in the modulation of beta-cell function [25].

Furthermore, Δ-TRTX-Ac1-OH and Δ-TRTX-Ac1-NH_2 imparted independent benefits on beta-cell growth and survival, highlighting potentially disease-modifying benefits on beta-cell mass that require further investigation. Interestingly, a low molecular weight compound with inhibitory activity at the Kv2.1 beta-cell channel [26], a similar target as for peptides isolated from the venoms of related tarantula species including *Grammostola rosea, Plesiophrictus guangxiensis* and *Chilobrachys jingzhao* [10,19,27], has been shown to exert benefits on the proliferation and survival of beta-cells [26]. Consequently, it seems likely that the growth and survival benefits of Δ-TRTX-Ac1-OH and Δ-TRTX-Ac1-NH_2 would be extended to all cells that express the cellular target for these peptides, including native beta-cells. In some disparity with our *in vitro* findings, Δ-TRTX-Ac1 peptides possessed less prominent insulinotropic actions in healthy mice, and benefits on glucose homeostasis were mild and observed only at elevated doses. Thus, Δ-TRTX-Ac1-OH did evoke some minor benefits on glucose disposal at 250 nmol/kg, but this was not partnered by similar effects of Δ-TRTX-Ac1-NH_2. Whether potential impact of these peptides on alpha-cell function and glucagon release could be important in this respect, as noted with exenatide [28], requires further detailed investigation. Interestingly, Δ-TRTX-Ac1-NH_2, but not Δ-TRTX-Ac1-OH, exerted notable and persistent appetite suppressive actions in mice. Importantly, there were no obvious signs of malaise following peptide injection in mice, such as piloerection, hunched posture, body shakes or twitching, indicating that satiety effects were not linked to toxicity or a detrimental impact on behaviour. These subtle differences in the *in vivo* bioactivity profile of Δ-TRTX-Ac1 peptides were not evident during our *in vitro* investigations. Whilst alterations in pharmacokinetic profile of the peptides could be one explanation, this seems unlikely. The slight C-terminal structural change between the peptides may alter receptor or channel binding kinetics, as well as potential passage through the blood brain barrier.

Given the likely differing modes of action of Δ-TRTX-Ac1 peptides and exenatide at a cellular level, we also examined potential additive benefits of these peptides on glucose homeostasis and appetite suppression in healthy mice. Unfortunately, at the peptide doses employed, there was no benefit of combined administration on glucose handling. GLP-1 receptor signalling exerts both direct pancreatic and indirect extrapancreatic glucose-lowering actions [29], with no apparent augmentation of these by Δ-TRTX-Ac1 peptides. However, exenatide also promotes well documented actions to reduce food intake [30], which were confirmed in the current setting. More notably, there was clear additive benefits of Δ-TRTX-Ac1-NH_2 with exenatide on satiety in overnight fasted mice. These combined positive actions could be highly favourable in obesity-driven forms of diabetes, such as T2DM. Indeed, the tarantula venom-derived peptide guangxitoxin has previously been reported to augment the antidiabetic benefits of GLP-1 receptor activation in diabetic *db/db* mice [31].

Overall, the positive effects of Δ-TRTX-Ac1 peptides on pancreatic beta-cell function and satiety are reminiscent of the actions of the incretin hormone, GLP-1 [32], where a venom-derived compound was the first clinically approved drug in this class. Additionally, observations of potential additive effects when co-administered with exenatide are encouraging. Thus, further consideration of the therapeutic potential of tarantula spider venom-derived peptides, such as Δ-TRTX-Ac1, for diabetes and related obesity is essential.

Data availability and sharing

The authors declare that the data supporting the findings of this study are available within the article. Any additional raw data supporting the conclusions of this article will be made available by the lead author, without undue reservation.

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CRediT authorship contribution statement

A. Coulter-Parkhill: Methodology, Validation, Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **SWM. Dobbin:** Methodology, Validation, Formal analysis, Investigation, Writing – review & editing. **N. Tanday:** Methodology, Validation, Formal analysis, Investigation, Writing – review & editing. **VA. Gault:** Conceptualization, Methodology, Validation, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition. **S. McClean:** Conceptualization, Methodology, Validation, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition. **N. Irwin:** Conceptualization, Methodology, Validation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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