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# In Vitro and In Vivo Effects of *Palmaria palmata* Derived Peptides on Glucose Metabolism

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## Abstract

Three synthetic peptides, ILAP, LLAP and MAGVDHI, derived from a *Palmaria palmata* protein hydrolysate were assessed for their antidiabetic potential in vitro and in vivo. In addition to inhibiting dipeptidyl peptidase-IV in a cell-based in situ assay all three peptides significantly increased the half-life of the incretin hormone glucagon-like peptide-1 (GLP-1). ILAP and LLAP mediated a significant increase ( $p < 0.001$ ) in insulin secretion from BRIN-BD11 cells compared to the glucose control, while MAGVDHI had no insulinotropic activity at an equimolar concentration ( $10^{-6}$  M). A significant increase in the concentration of cyclic adenosine monophosphate production in BRIN-BD11 cells mediated by ILAP ( $p < 0.001$ ) and LLAP ( $p < 0.01$ ) compared to the basal control, would indicate that insulin secretion may be mediated by membrane based activation. Furthermore, ILAP and LLAP acted as glucose-dependent insulinotropic polypeptide (GIP) secretagogues, stimulating a significant increase ( $p < 0.01$ ) in the concentration of GIP released from enteroendocrine STC-1 cells compared to the glucose control. When tested in vivo in healthy male NIH Swiss mice, ILAP and LLAP, mediated a significant increase ( $p < 0.01$ ) in plasma insulin and decrease ( $p < 0.05$ ) in blood glucose, respectively, compared to the control. MAGVDHI mediated a significant ( $p < 0.001$ ) sustained reduction in food intake in food deprived trained mice. These results demonstrate that the *Palmaria palmata* peptides studied herein have prospective antidiabetic activity and have the potential to act as agents that can be used alone or in combination with drugs, to aid in the prevention and management of Type 2 diabetes mellitus.

**Keywords** Bioactive peptides · Dipeptidyl peptidase-IV (DPP-IV) inhibition · Glucagon-like peptide-1 (GLP-1) · Glucose-dependent insulinotropic polypeptide (GIP) · Type 2 diabetes · *Palmaria palmata*

## Abbreviations

ACN	Acetonitrile	EDTA	Ethylenediaminetetraacetic acid
AMC	H-Gly-Pro-7-amino-4-methyl coumarin	ELISA	Enzyme-linked immunosorbent assay
AUC	Area under the curve	FBS	Fetal bovine serum
BW	Body weight	GIP	Glucose-dependent insulinotropic polypeptide
cAMP	Cyclic adenosine monophosphate	GLP-1	Glucagon-like peptide-1
DMEM	Dulbecco's modified Eagle's medium	HBSS	Hank's buffered saline solution
DPP-IV	Dipeptidyl peptidase-IV	ILAP	Isoleucine-leucine-alanine-proline
		IP	Intraperitoneal
		IPI	Isoleucine-proline-isoleucine
		KRBB	Krebs-Ringer bicarbonate buffer
		LDH	Lactate dehydrogenase
		LLAP	Leucine-leucine-alanine-proline
		MALDI-TOF MS	Matrix assisted laser desorption ionisation time of flight mass spectrometry
		MAGVDHI	Methionine-alanine-glycine-valine-aspartic acid-histidine-isoleucine

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m/z	Mass to charge ratio
PBS	Phosphate buffered saline
RP-HPLC	Reversed phase-high performance liquid chromatography
TFA	Trifluoroacetic acid
T2DM	Type 2 diabetes mellitus

## Introduction

Type 2 diabetes mellitus (T2DM) is a chronic metabolic condition characterised by a deficiency in the secretion and/or function of insulin (i.e., insulin resistance), resulting in high blood glucose levels (hyperglycaemia; NMIC 2017). With the prevalence of T2DM increasing worldwide, in particular in countries with increasing obesity incidences, effective interventions are required to prevent and manage the condition. Initial approaches to the management of T2DM includes lifestyle modification, particularly in relation to diet and exercise (NMIC 2017). However, these modifications are often insufficient to achieve satisfactory glycaemic control and pharmacological interventions are required. In recent years, a particular focus has been placed on the development of gut hormone-based therapies, which stimulate the biological activities and/or prolong the action of endogenous incretin hormones (Irwin and Flatt 2015). These include glucagon-like peptide-1 receptor (GLP-1R) agonists (incretin mimetics) and dipeptidyl peptidase (DPP)-IV inhibitors. Intestinal derived incretin peptides, GLP-1 and glucose-dependent insulinotropic polypeptide (GIP), play a significant role in postprandial insulin release (Green et al. 2004). However, GLP-1 and GIP are rapidly degraded by the aminopeptidase DPP-IV resulting in the loss of their insulinotropic potential in vivo (Deacon 2019). As peptidomimetics, GLP-1R agonists mimic the actions of the endogenous hormone GLP-1, where the structural modifications increase their stability and bioavailability for activation of the receptor, while DPP-IV inhibitors increase the half-life of circulating incretin hormones (Power et al. 2014; Irwin and Flatt 2015; Deacon 2019).

Although most synthetic GLP-1 mimetics and DPP-IV inhibitory agents are well tolerated, adverse side-effects, such as mild infections (common cold, urinary and upper respiratory tract infections), headaches, mild to moderate nausea, vomiting and diarrhoea have been reported (Krushner and Gorrell 2010; Lund et al. 2014). While safety concerns linking GLP-1 therapy with pancreatitis, pancreatic and thyroid cancer and glucagon-producing neuroendocrine tumours have been alleviated, pharmacovigilance around the use of GLP-1 drugs is still required, particularly in relation to patients with increased risk or a history of pancreatitis or thyroid cancer (Irwin and Flatt 2015). Furthermore, to date, the development of incretin mimetics has been directed

predominantly towards single molecules that modulate individual peptide receptor targets. However, future research aims to identify multi-agonist components which simultaneously modulate multiple receptor signalling pathways. This may be the identification of single components with multifunctional activities or the generation of designer hybrid peptides that can modulate multiple regulatory peptide hormone receptor pathways (Irwin and Flatt 2015; Brandt et al. 2018).

Due to the adverse side-effects posed by existing synthetic T2DM drugs the identification of natural agents that can be used alone or in combination with T2DM drugs, to aid in the prevention and management of T2DM has gained significant interest in recent years. Evidence-based research has shown that dietary proteins, protein hydrolysates, peptides and amino acids can beneficially regulate glycaemic control with the extent of the response varying vastly depending on the primary sequence of the peptides and amino acids generated during digestion (Newsholme et al. 2006; Promintzer and Krebs 2006; Oseguera-Toledonet et al. 2014).

Numerous food protein-derived peptides have been shown to exhibit DPP-IV inhibitory activity (Nongonierma and FitzGerald 2017). While food protein derived peptides may not be as potent as synthetic drugs they have the potential to be used in combination with such therapies thereby reducing drug dosage, or can be employed as part of dietary strategies to prevent or control the disorder. In a previous study three novel peptides (Ile-Leu-Ala-Pro, Leu-Leu-Ala-Pro and Met-Ala-Gly-Val-Asp-His-Ile), with promising DPP-IV inhibitory activity (IC<sub>50</sub> values in the range 43–159 µM) were identified within a protein hydrolysate generated from the red macroalgal species *Palmaria palmata* (Harnedy et al. 2015). All three peptides were shown to be resistant to degradation following simulated gastrointestinal digestion, which would suggest that these peptides may survive gastrointestinal transit and exert effects in vivo (Harnedy et al. 2015). The objective of the study herein was firstly to investigate the effect of these peptides on in situ DPP-IV inhibitory activity and in vitro insulinotropic, GLP-1 and GIP stimulatory and GLP-1 protective activity and secondly to investigate the effect of oral consumption of these peptides on blood glucose control in healthy mice following an oral glucose challenge.

## Materials and Methods

### Materials

The synthetic peptides Ile-Leu-Ala-Pro (ILAP), Leu-Leu-Ala-Pro (LLAP) and Met-Ala-Gly-Val-Asp-His-Ile (MAGVDHI) were obtained from Thermo Fisher Scientific (Ulm, Germany).

H-Gly-Pro-7-amino-4-methyl coumarin (AMC) and Diprotin-A (IPI: Ile-Pro-Ile) were obtained from Bachem Feinchemikalien (Bubendorf, Switzerland). GLP-1 (total) and GIP (total) ELISA assay kits were provided by Millipore (Hertfordshire, UK), lactate dehydrogenase (LDH) kit by Promega (Madison, WI, USA) and cAMP Parameter detection kit by R&D Systems (Abingdon, UK). Fetal bovine serum (FBS), Hank's buffered saline solution (HBSS 10×), penicillin–streptomycin (0.1 g/L), RPMI-1640 culture media, Dulbecco's modified Eagle's medium (DMEM) containing high glucose and trypsin/EDTA (10×) were obtained from Gibco Life Technologies Ltd. (Paisley, Strathclyde, UK). Radio-labelled sodium iodide ( $\text{Na}^{125}\text{I}$ , IMS 100 mCi/mL) was purchased from Perkin Elmer (Buckinghamshire, UK). Rat insulin standard was purchased from Novo Industria (Copenhagen, Denmark). All other reagents including human Caucasian colon adenocarcinoma (Caco-2) cells were supplied by Sigma Chemical Company Ltd. (Wicklow, Ireland).

### Cytotoxicity Assay

The cytotoxicity of the peptides ( $10^{-6}$  M) on cell culture lines was determined by the release of LDH in cell supernatants obtained from acute insulin, GLP-1 and GIP release experiments. LDH activity in the cell supernatants was determined using a CytoTox 96® non-radioactive cytotoxicity assay kit, where 50  $\mu\text{L}$  of cell supernatant was mixed with 50  $\mu\text{L}$  of LDH substrate solution. Following 30-min incubation in the dark at room temperature, 50  $\mu\text{L}$  of stop solution was added and the absorbance was determined at 595 nm.

### In Situ Quantification of DPP-IV Inhibitory Activity in Caco-2 Cells

The inhibition of DPP-IV in Caco-2 cells (passage 44–46) was determined according to the method described by Caron et al. (2017) with some modifications (Harnedy-Rothwell et al. 2020). The cells were grown in minimum essential Eagle's medium supplemented with 10% FBS, 100 U/mL penicillin, 100  $\mu\text{g}/\text{mL}$  streptomycin, 0.25  $\mu\text{g}/\text{mL}$  amphotericin B and 2 mM glutamine at 5%  $\text{CO}_2$  and 37 °C for 5 days. The cells were then trypsinised and seeded at a density of 20,000 cells/well in a 96-well optical black plate (Costar, Corning, NY, USA). The culture media was removed after 16 h contact and cells were washed with 100  $\mu\text{L}$  phosphate buffered saline (PBS, 0.01 M, pH 7.4). Fresh PBS (125  $\mu\text{L}$ ) was added to the well followed by 25  $\mu\text{L}$  of each test peptide solution (0.025 to 2.500 mM prepared in PBS) or PBS (no inhibition control). The DPP-IV inhibition reaction was initiated by the addition of 50  $\mu\text{L}$  of substrate (Gly-Pro-AMC) and the fluorescence was recorded every 2 min for 1 h using a plate reader (Biotek Synergy, HT,

USA) at 37 °C. Excitation and emission wavelengths were at 360 and 460 nm, respectively. DPP-IV inhibition was defined as the percentage of DPP-IV activity inhibited by a given concentration of peptide compared to the control. Activity was expressed as mean  $\text{IC}_{50}$  value (inhibitory concentration, which inhibited DPP-IV activity by 50%)  $\pm$  SD obtained from three independent replicates ( $n=3$ ).

### In Vitro Assessment of GLP-1 Protective Effects

The inhibition of GLP-1(7–36) amide hydrolysis by the peptides when incubated in the presence of DPP-IV was assessed using reversed phase-high performance liquid chromatography (RP-HPLC). In brief, 30  $\mu\text{L}$  of GLP-1(7–36) amide ( $10^{-6}$  M) was combined with either 30  $\mu\text{L}$  of peptide ( $10^{-6}$  M), Diprotin A ( $10^{-6}$  M) or distilled water (blank) and 430  $\mu\text{L}$  triethanolamine buffer (pH 7.4). Human DPP-IV (10  $\mu\text{L}$ , 5 mU) was added and incubated at 37 °C for 0, 2, 4, 8 and 24 h. The reaction was terminated by the addition of 50  $\mu\text{L}$  of 10% (w/v) trifluoroacetic acid (TFA).

Samples were analysed using a RP-HPLC (Thermo Finnigan Surveyor) equipped with a UV/VIS detector 4 port gradient inlet solvent proportioning pump, a Rheodyne 7125i injector with a 1 mL loop and multi-sampler (Thermo Finnigan) equipped with a 250  $\mu\text{L}$  reservoir and 100  $\mu\text{L}$  column loop. Samples were separated using a Aeris Peptide C18 RP column (250  $\times$  46 mm, 3.6  $\mu\text{m}$ ; Phenomenex (Macclesfield, Cheshire, UK) with mobile phase A and B consisting of 0.12% (v/v) TFA in HPLC grade  $\text{H}_2\text{O}$  and 0.10% (v/v) TFA in 70% (v/v) HPLC grade acetonitrile/water, respectively. The following gradient was used: 0–10 min: 0–30% B; 11–50 min: 30–70% B; 51–55 min: 70–100% B; 56–65 min: 100% B; 66 min: 0% B. The flow rate was set at 1 mL/min and the absorbance of the eluent was monitored at 214 nm. The eluted peaks (containing active and inactive GLP-1) were collected and analysed using matrix assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS; PerSeptive Biosystems Voyager-DE Biospectrometer, Hertfordshire, UK). A 10 mg/mL solution of  $\alpha$ -cyano-4-hydroxycinnamic acid (CHCA) was prepared in 80% (v/v) ethanol containing 0.1% (v/v) TFA, mixed thoroughly and centrifuged for 2 min at 2000 $\times g$ . A 10  $\mu\text{L}$  aliquot of the CHCA supernatant was mixed with 1.5  $\mu\text{L}$  of the collected HPLC peak and pipetted onto a predefined well in a 100-well stainless steel plate and allowed to dry. The plate was then inserted into the MALDI-TOF MS. Internal mass calibration of the instrument was preformed prior to sample analysis using Peptide Calibration Mix 2 (LaserBio Labs, Spohia-Antipolis, Cedex, France) which contained 4 individual peptides (Neurotensin, ACTH fragment 18–39, insulin bovine  $\beta$ -chain oxidised and bovine insulin with monoisotopic masses of 1672.9176, 2465.1989, 3494.6514 and

5730.6087 Da, respectively). All sample measurements were collected in linear positive ionisation mode using 50 laser shots/spectrum. The accelerating voltage was maintained at 20,000 V, the grid voltage and guide wire voltages were set at 93% and 0.05%, respectively, of the accelerating voltage. The nitrogen laser, set at 337 nm, was directed towards the densest area of the sample/matrix spot and the laser intensity adjusted to obtain the best spectral response. The mass/charge ratio ( $m/z$ ) was plotted against relative abundance.

### In Vitro Insulin Secretion

The effect of the peptides on in vitro insulin secretion was examined using rat pancreatic beta BRIN-BD11 cells, whose characteristics have been reported previously (McClenaghan et al. 1996). BRIN-BD11 cells were seeded into 24-well plates ( $1.5 \times 10^5$  cell/well) and allowed to attach overnight on incubation at 37 °C. Following 40 min of pre-incubation with 1.1 mmol/L glucose; 37 °C, the cells were incubated (20 min; 37 °C) in the presence of 5.6 mmol/L glucose with a range of test peptide concentrations ( $10^{-6}$ – $10^{-12}$  M). After 20 min of incubation, 900  $\mu$ L buffer was removed from each well and stored at  $-20$  °C before the determination of insulin by radioimmunoassay (Flatt and Bailey 1981).

### Acute Intracellular cAMP Production

Acute intracellular cAMP production was assessed with BRIN-BD11 cells. Cells were prepared as above and following overnight attachment the cells were washed with HBSS buffer before incubation (20 min, 37 °C) with the peptides ( $10^{-6}$  M) in the presence of 200  $\mu$ M 3-isobutyl-1-methylxanthine. The medium was then removed, the cells were lysed and the concentration of cAMP in the cell lysates was determined using a cAMP detection kit (R&D Systems, Abingdon, UK) according to the manufacturer's instructions.

### In Vitro GLP-1 Secretion

In vitro effects of the peptides on GLP-1 secretion from murine enteroendocrine GLUTag cells were determined as described previously (Ojo et al. 2013). In brief, cells were seeded into 24 well plates ( $1.5 \times 10^5$  cell/well) and incubated overnight at 37 °C. After a pre-incubation step (40 min, 37 °C), the cells were presented with the peptides ( $10^{-6}$  M) prepared in 2 mM glucose and incubated for 2 h at 37 °C. The supernatant was then removed and stored at  $-20$  °C prior to measurement of GLP-1 concentration using a Total

GLP-1 sandwich ELISA assay kit (Millipore, Hertfordshire, UK) according to the manufacturer's instructions.

### In Vitro GIP Secretion

In vitro effects of the peptides on GIP secretion were measured using the murine enteroendocrine STC-1 cells. STC-1 cells were cultured in DMEM media containing 4.5 g/L D-glucose, 17.5% FBS, 100 U/mL penicillin, 100 mg/L streptomycin and incubated in a 5% humidified air atmosphere at 37 °C. Cells were passaged at 80–90% confluence. Cells were then gently washed in 10 mL of HBSS prior to incubating with 3 mL of pre-warmed 1% trypsin/EDTA at 37 °C for 5–8 min. Detached cells were observed using a phase contrast microscope (Zeiss, Germany). Clusters of cells were gently broken by aspiration for single cell counting. Cells were centrifuged at 900 rpm for 5 min. Cell supernatant was then removed and cells were re-suspended in a known volume of pre-warmed DMEM media. A single aliquot of 100  $\mu$ L of re-suspended cells was mixed with 100  $\mu$ L of Trypan blue and was then transferred for counting to a Neubauer haemocytometer (Scientific Supplies Co., Middlesex, UK). Cells were seeded at a cell density of  $1.5 \times 10^5$  cell/well in 1 mL of DMEM containing 4.5 g/L D-glucose and left for 48 h to adhere. Media was removed and replaced with 1 mL of priming KRBB (11 mM NaCl, 4.7 mM KCl, 1.28 mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 1.2 mM  $\text{KH}_2\text{PO}_4$ , 1.2 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 10 mM  $\text{NaHCO}_3$ , 25 mM HEPES, 0.1% (w/v) bovine serum albumin, pH 7.4) supplemented with 1.1 mM glucose and incubated at 37 °C for 40 min. The KRBB was removed and replaced with KRBB containing 2 mM glucose and peptides ( $10^{-6}$  mol/L) and incubated for 2 h, after which 900  $\mu$ L of the supernatant was removed from each well and was stored at  $-20$  °C until analysed via ELISA. Analysis of total GIP was performed using a sandwich ELISA (Rat/Mouse, Millipore, Hertfordshire, UK) kit according to the manufacturer's instructions.

### Acute Glucose Lowering Effect of Peptides via Intraperitoneal Injection

Acute animal studies were carried out using male NIH Swiss mice (10 to 12 weeks old; Harlan Ltd., Blackthorne, UK) fed a standard rodent maintenance diet that contained 10% fat, 30% protein, and 60% carbohydrate with a total energy of 12.99 kJ/g; (Teklad Natural diet for rodents) (Envigo, Blackthorn, UK). Mice were housed in an air-conditioned room maintained within a temperature range of  $22 \pm 2$  °C with a 12 h light: 12 h dark cycle and had free access to drinking water and food. All animal experiments were conducted according to U.K. Home Office Regulations [U.K. Animals (Scientific Procedures) Act 1986] and EU Directive 2010/63EU for animal experiments, and were approved by

the Ulster University Animal Welfare and Ethical Review Board. All necessary steps were taken to prevent any potential animal suffering.

Plasma glucose and insulin responses were evaluated after intraperitoneal (i.p.) injection of glucose alone (18 mmol/kg body weight [BW]) or in combination with test peptides (25 nmol/kg BW) in 8 h fasted normal NIH mice. Food was withheld during experimentation, however, access to water was available. Prior to compound administration, a fasting blood sample was analysed for glucose concentration using a handheld glucometer (Bayer Contour, Leverkusen, Germany). Blood samples were collected at 0, 15, 30, 60, 90 and 120 min post administration from the tail vein of conscious mice and added into chilled fluoride/heparin microcentrifuge tubes (Sarstedt, Numbrecht, Germany). The blood samples were centrifuged at  $18,360\times g$  for 3 min (Universal 320, Hettich Zentrifugen, Germany). Blood glucose concentrations were measured using an Ascencia Contour blood glucose meter (Bayer Healthcare, Newbury, UK). Plasma insulin was determined using a modified dextran-coated charcoal RIA (Flatt and Bailey 1981).

### Investigating Reduction of Food Intake in 3 h Trained Feeding *HsdOla:T0* Mice

*Hsd:Ola:T0* mice (8 weeks old) were obtained from Envigo, Blackthorn, UK. Mice were housed in an air-conditioned room maintained within a temperature range of  $22 \pm 2$  °C with a 12 h light:12 h dark cycle. Drinking water and standard rodent diet was freely available. Mice were trained to eat for 3 h per day, by reducing food intake gradually from 24 to 10 h to 6 h and 3 h per day, over a period of 3 weeks (O'Harte et al. 2018). Groups of 21 h fasted mice ( $n=8$ )

were administered peptides (50 nmol/kg bw) within a saline vehicle (0.90% (w/v) NaCl) via intraperitoneal injection. Mice were only allowed access to food post-injection (at 10.00 am) and the food was weighed at 30 min intervals up until 180 min and food was then removed until the following day. Food intake was calculated and compared to the saline only (0.90% (w/v) NaCl) control group.

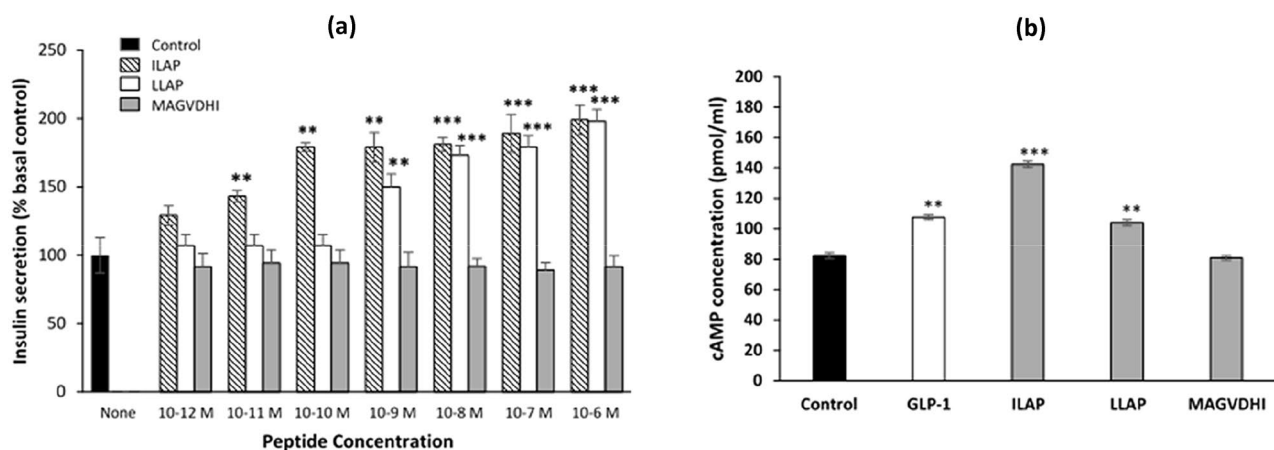
### Statistical Analysis

Statistical analyses were performed using the statistical software program SPSS (Version 22, IBM Inc., Chicago, IL, USA) and GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA, USA). Data was expressed as mean  $\pm$  SEM with values compared using one-way analysis of variance (ANOVA) followed by student's *t* test, Tukey's and Games–Howell post-hoc tests where applicable. Incremental area under the curve (AUC) for plasma glucose and insulin were calculated using GraphPad Prism.

## Results and Discussion

### Effect of *Palmaria palmata*-Derived Peptides on Insulin Release from BRIN-BD11 Cells In Vitro

The *Palmaria palmata*-derived peptides, ILAP and LLAP, were shown to stimulate the secretion of insulin from cultured pancreatic BRIN-BD11 cells in a dose-dependent manner from  $10^{-12}$  to  $10^{-6}$  M (Fig. 1a). While both peptides ( $10^{-6}$  M) were shown to mediate a significant increase ( $p < 0.001$ , 2.0-fold) in insulin secretion from BRIN-BD11 cells compared to the 5.6 mM glucose control, the peptide



**Fig. 1** Effects of *Palmaria palmata* peptides ILAP, LLAP and MAGVDHI at a concentration of  $10^{-6}$ – $10^{-12}$  M on insulin secretion from and **b**  $10^{-6}$  M on cyclic adenosine monophosphate (cAMP) production in BRIN-BD11 cells. Values are expressed as mean  $\pm$  SEM.

( $n=8$ ). \*\* $p < 0.01$  and \*\*\* $p < 0.001$  compared to buffer/media containing 5.6 mM glucose (black bar, control). Treatment bars represent cells cultured with 5.6 mM glucose and peptides

MAGVDHI had no insulinotropic activity at an equimolar concentration (Fig. 1a).

All three peptides had no cytotoxic effects at  $10^{-6}$  M (LDH assay, Supplementary Fig. S1). Significantly higher stimulatory activity was observed with 10 pM ILAP ( $p < 0.01$ ) compared to 1 nM LLAP ( $p < 0.01$ ), which would indicate that ILAP is more potent than LLAP. Further studies are required to assess the impact of presenting combination of peptides to the pancreatic cells in order to determine if synergistic effects on insulin secretion may take place.

To date, only peptides derived from casein and boarfish (*Capros aper*) proteins have been shown to stimulate the secretion of insulin from cultured pancreatic BRIN-BD11 cells (Drummond et al. 2018; Harnedy-Rothwell et al. 2020). Information in relation to the structure–activity relationship of food-derived insulinotropic peptides is non-existent, however, a similar trend to that seen with ILAP and LLAP herein was seen with the boarfish-derived peptides IPVDM and LPVDM, where peptides with isoleucine at position 1 had higher insulinotropic activity than the same peptides with Leu at position 1 (Harnedy-Rothwell et al. 2020). While the insulin secretory activity observed with ILAP and LLAP was lower than that mediated by Exendin-4, a GLP-1 paralogue, which was approved for treatment of T2DM in 2005, the activity exhibited by both peptides was similar to that observed with other promising peptides under investigation such as xenin, its enzymatically stable C-terminal octapeptide fragments, xenin 18–25 and xenin 18–25 Gln and stable hybrid peptide (DALa<sup>2</sup>) GIP/xenin-8-Gln (Irwin et al 2015; Martin et al 2016; Hasib et al. 2017). The significant increase in the concentration of cAMP released from BRIN-BD11 cells (Fig. 1b) when incubated in the presence of ILAP ( $p < 0.001$ ) and LLAP ( $p < 0.01$ ) compared to the basal control would suggest a downstream cascade effect which is likely to be due to membrane based activation, however, further work is needed to elucidate its mechanism of action on insulin secretion. In contrast, the concentration

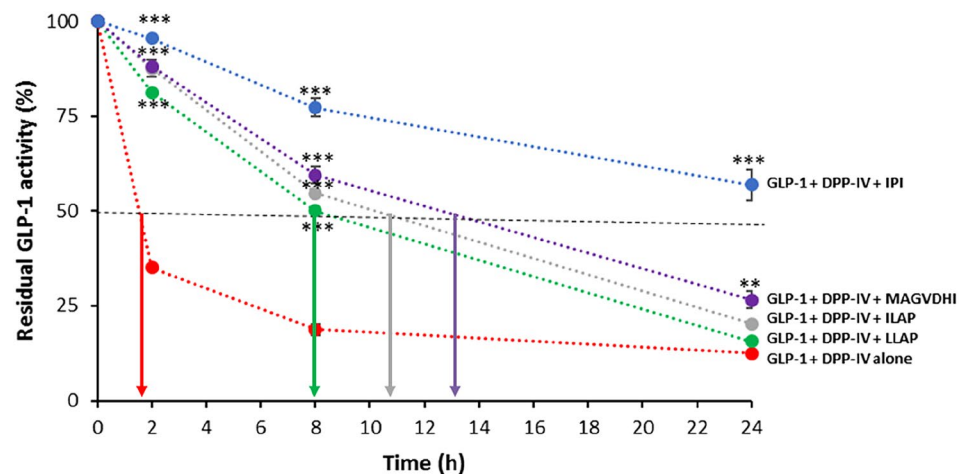
of cAMP detected on incubation of BRIN-BD11 cells with MAGVDHI was similar to that seen with the basal glucose control (Fig. 1b). This is in agreement with the results obtained previously showing the inability of MAGVDHI to stimulate the secretion of insulin from BRIN-BD11 cells beyond control values.

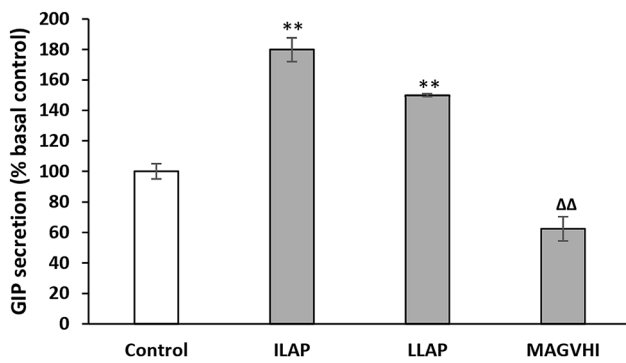
### Effect of *Palmaria palmata*-Derived Peptides on Release of the Incretin Hormones GLP-1 and GIP In Vitro

A defect in the postprandial insulin-secretory incretin response, mediated by the gut hormones GLP-1 and GIP, is a specific pathophysiological characteristic of T2DM. The main impairments of this include reduced postprandial GLP-1 secretion and less effective GIP incretin action during prolonged hyperglycaemia in diabetes (Holst et al. 2011). All three *Palmaria palmata*-derived peptides failed to stimulate the secretion of GLP-1 from murine enteroendocrine cells when assessed at 1  $\mu$ M (data not shown). However, the peptides which in a previous study were shown to mediate potent DPP-IV inhibitory activity in the conventional in vitro assay ( $IC_{50}$  values in the range 43–159  $\mu$ M), displayed good DPP-IV inhibitory activity in a cell-based in situ assay ( $IC_{50}$  values in the range 126–358  $\mu$ M, Harnedy et al. 2015). Furthermore, all three peptides mediated a protective effect for GLP-1 by inhibiting its degradation by DPP-IV (Fig. 2). The in vitro half-life of GLP-1 was increased from 1.75 h when incubated with DPP-IV alone to 8, 10.8 and 13 h, when co-incubated with LLAP, ILAP and MAGVDHI, respectively (Fig. 2).

In contrast to the results observed with GLP-1, ILAP and LLAP acted as GIP secretagogues, stimulating a significant increase in the concentration of GIP released from murine enteroendocrine STC-1 cells compared to the 2.0 mM glucose control ( $p < 0.01$ , Fig. 3). All three peptides had no cytotoxic effects in STC-1 cells at  $10^{-6}$  M (data not shown).

**Fig. 2** Protective effect of *Palmaria palmata* derived peptides ( $10^{-6}$  M) against dipeptidyl peptidase-IV (DPP-IV) degradation of active glucagon-like peptide (GLP)-1(7–36 amide). Values are mean  $\pm$  SEM ( $n = 3$ ). \*\* $p < 0.01$  and \*\*\* $p < 0.001$  compared to GLP-1 + DPP-IV alone. Diprotin A (IPI) was used as a positive control at ( $10^{-6}$  M). Arrows indicate the half-life (h)





**Fig. 3** Effect of *Palmaria palmata* peptides **a** ILAP, **b** LLAP and **c** MAGVDHI ( $10^{-6}$  M) on glucose-dependent insulinotropic polypeptide (GIP) release from STC-1 cells. Values are expressed as mean  $\pm$  SEM (n = 3). \*\*p < 0.01 compared to 2.0 mM glucose control.  $\Delta\Delta$ p < 0.01 compared to other peptides

To the best of our knowledge, this appears to be the first report of food protein-derived peptides stimulating the secretion of GIP from STC-1 cells. However, MAGVDHI failed to stimulate such a response. Although originally believed to be involved in inhibiting gastric acid secretion, the primary physiological role of GIP is now considered to be as a postprandial insulin secretagogue (Irwin and Flatt 2015). GIP exerts its insulin secretory action through agonistic effects on a specific G protein-coupled receptor family GIP receptor on the surface of pancreatic  $\beta$ -cells. This increases intracellular cAMP generation and subsequent influx of  $\text{Ca}^{2+}$  ions leading to exocytosis of insulin granules (Moffett et al. 2015). Furthermore, GIP is believed to act as a  $\beta$ -cell growth factor and to promote  $\beta$ -cell survival (Kubota et al. 1997; Ehses et al. 2003). Due to its promising biological activity at  $\beta$ -cell level there has been significant interest in the potential for GIP-based pharmaceuticals as antidiabetic agents, however, as with GLP-1 therapies, the pharmacokinetic profile of GIP is hindered due to the rapid degradation of this peptide in vivo by DPP-IV. More recently, the dual agonist GLP-1/GIP peptide appears to have a promising therapeutic profile (Frias et al. 2018) and is currently in stage 3 clinical trials. DPP-IV inhibitors are known to increase the circulating half-lives of both of these incretin hormones which also enhances their antidiabetic action.

The results of in vitro studies herein show that ILAP and LLAP stimulate the secretion of insulin and GIP from cultured pancreatic  $\beta$ -cells and murine STC-1 cells, respectively. This suggests that the peptides have multifunctional activities and may have the potential to stimulate the release of insulin from  $\beta$ -cells by multiple mechanisms. While both peptides failed to directly stimulate the secretion of GLP-1 from GLUTag cells, they do, however, protect the incretin hormone GLP-1 through inhibition of DPP-IV. In addition, GIP is thought to be the most potent of the two incretin

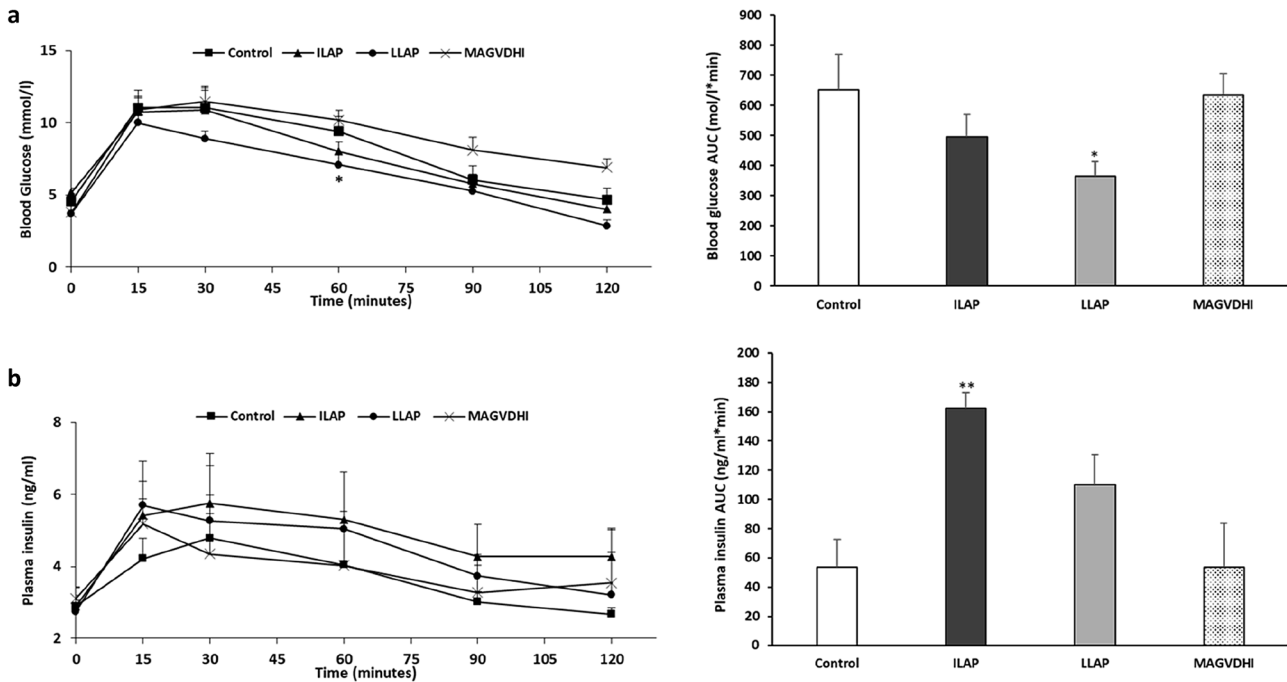
hormones (associated with 25–70% of the post meal insulin response) (Gasbjerg et al. 2020). In addition, GIP is thought to generate neuronal signals to more distal GLP-1 producing L-cells and promote its postprandial elevation where it too acts as a potent insulinotropic agent (Martin et al. 2020). In contrast, MAGVDHI only mediated DPP-IV inhibitory activity.

### Acute Glucose-Lowering and Insulinotropic Actions of *Palmaria palmata*-Derived Peptide in Lean Mice

The acute in vivo effects of *Palmaria palmata*-derived ILAP, LLAP and MAGVDHI on glucose homeostasis were studied in normal healthy NIH Swiss mice (Fig. 4). Over the time course of the study (0–120 min) a significant ( $p < 0.05$ ) reduction in plasma glucose concentration was observed with LLAP at 60 min only post injection compared to the glucose control. The plasma glucose area under the curve (AUC) for LLAP, however, was significantly lower than that observed with the control mice ( $p < 0.05$ , Fig. 4). In contrast, ILAP and MAGVDHI failed to stimulate a significant decrease in plasma glucose concentration at any of the time points post injection compared to mice receiving glucose alone (Fig. 4). While both ILAP and LLAP increased plasma insulin in mice receiving the peptides when compared with those that received glucose alone, a significant increase was observed only with ILAP ( $\text{AUC}_{0-120 \text{ min}}$ ,  $p < 0.01$ , Fig. 4). The plasma insulin  $\text{AUC}_{0-120 \text{ min}}$  for MAGVDHI was similar to that observed in the mice who receiving glucose alone indicating that it did not possess a glycaemic controlling potential.

At present there are no clear explanation for the effects observed with ILAP and LLAP. It may, for example, be that LLAP can somehow improve tissue insulin sensitivity, causing a glucose lowering effect without an apparent or obvious rise in circulating insulin. It is possible that ILAP mediates competing actions on insulin and glucagon and this might explain the significant rise in insulin which does not ultimately cause a reduction in circulating glucose. However, further detailed studies are required to elucidate the different responses observed herein.

It is unclear if the mechanism by which circulating insulin was elevated in mice injected with ILAP is either via DPP-IV inhibition and improvement in GLP-1 or GIP action upon the pancreas, stimulation of GIP, or direct pancreatic interaction and promotion of insulin secretion. Expanding on the knowledge that the peptide is capable of inhibiting DPP-IV, as displayed herein and previous studies, co-injection of the ILAP with GLP-1 further improved the glucose lowering effect versus the GLP-1 only injected group, when challenged via the same glucose concentration; however, improvement beyond GLP-1 only was marginal when analysed via AUC (data



**Fig. 4** Acute effect of *Palmaria palmata* peptides (ILAP, LLAP and MAGVDHI) on glucose tolerance in normal mice. Blood glucose (a) and plasma insulin (b) concentrations were measured before and after intraperitoneal injection of the peptide (25 nmol/kg body weight)

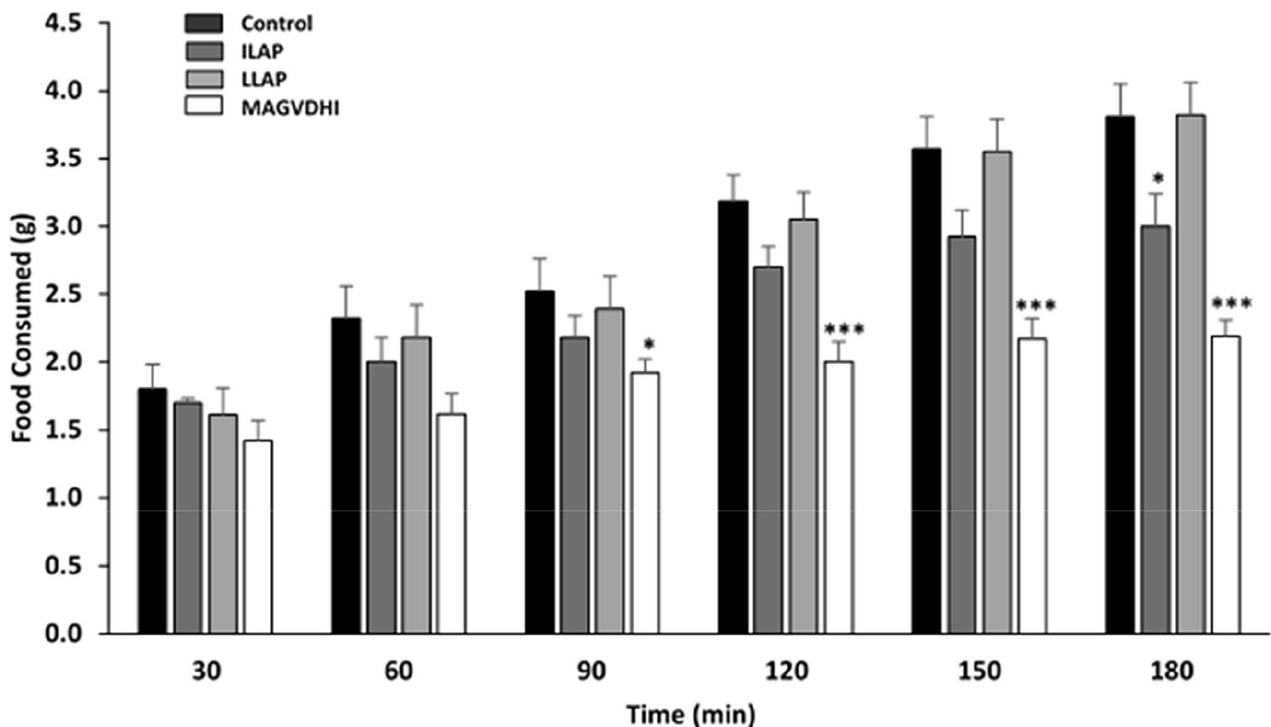
together with glucose (18 mmol/kg/body weight). Blood glucose and plasma insulin area under the curve (AUC) values for 0–120 min post-injection are also shown. Values are expressed as mean  $\pm$  SEM (n = 8). \*p < 0.05 and \*\*p < 0.01 compared to glucose alone

not shown). Thus, further work is required to determine the mechanism by which circulating insulin concentration was elevated in mice injected with ILAP.

The *Palmaria palmata*-derived peptides were also assessed for their ability to reduce food intake in the food restricted trained mice. Groups of trained mice which had access to food for 3 h per day were used to assess the effect upon food intake during food availability at 30 min intervals. LLAP had no effect upon food intake, whereas ILAP mediated a significant reduction in food intake but only at 180 min compared to the saline control (Fig. 5, p < 0.05). Interestingly, MAGVDHI resulted in a significant sustained reduction in food intake from 90 to 180 min with an overall food reduction across the entire group of mice averaging 45% versus the control group (Fig. 5). The in vitro studies herein show that MAGVDHI has no insulinotropic activity when incubated with BRIN-BD11 cells. This would indicate that the mechanism by which this peptide mediates the satiety effects observed in vivo is insulin independent and potentially via other satiety hormone secretion mechanisms. However, further studies are required to confirm this observation.

## Conclusion

This study shows that the food protein-derived peptides, ILAP and LLAP, stimulated the secretion of insulin and GIP from cultured pancreatic  $\beta$ -cells and enteroendocrine cells, respectively. Additionally, they exhibited DPP-IV inhibitory activity in situ and significantly increased the half-life of the incretin hormone GLP-1. While beneficial effects on different diabetic markers were observed in vivo with the two peptides in healthy lean mice following intraperitoneal injection further studies are required to assess the efficacy of the peptides following oral administration if they are to be employed as part of dietary strategies to prevent or control T2DM. Furthermore, in order to investigate the anti-diabetic potential of the *Palmaria palmata* derived peptides in vivo assessment in diabetic animal models is required in the first instance with subsequent studies in humans which are early stage/pre-diabetic or have been diagnosed with T2DM. In addition, MAGVDHI mediated a reduction in food intake in trained mice, however, further work is required to assess the effect of this peptide on appetite, energy intake and metabolic control during



**Fig. 5** Effect of *Palmaria palmata* peptides (ILAP, LLAP and MAGVDHI) on cumulative food intake in trained food restricted healthy mice following intraperitoneal injection (50 nmol/kg bw). Values are

expressed as mean  $\pm$  SEM. (n = 8). \*p < 0.05 and \*\*\*p < 0.001 compared to saline control

chronic mouse studies and to determine the mechanism by which it mediates its satiety effect. Multiple-acting co-agonist peptides represent novel therapeutic agents which may be beneficial in providing a mitigation strategy against the growing global health challenges of T2DM, obesity and related metabolic diseases (Irwin et al. 2015; Frias et al. 2018). While these natural peptides may not be as potent as existing synthetic mimetics they could be used in combination with synthetic drugs thus allowing for a reduction in conventional drug dosage, which in turn could lead to reduced side-effects and healthcare costs.

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**Author Contributions** PHR designed and performed experiments, collected and analyzed data, and wrote the manuscript. CMM, ALG and VD designed and performed experiments, collected and analyzed data, CM aided in performing an experiment. PA, EM, RF and FOH supervised the work and read and edited the manuscript.

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**Data Availability** The data that support the findings of this study are available from the corresponding author upon request.

**Code Availability** N/A.

## Declarations

**Conflict of interests** The authors declare that they have no competing interests.

**Consent to Participate** N/A.

**Consent for Publication** N/A.

**Ethical Approval** N/A.

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