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# Novel dual incretin agonist peptide with antidiabetic and neuroprotective potential

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ABSTRACT

Glucose-dependent insulinotropic hormone (GIP) and glucagon-like peptide-1 (GLP-1) are incretin hormones that exert an array of beneficial actions on metabolism and cognitive function. GLP-1-based therapeutics have been highly successful in terms of obesity and diabetes management, however GIP therapies have found no clinical utility to date. In the present study we describe, for the first time, the therapeutic effectiveness of a novel GIP/GLP-1 hybrid peptide based on the amino acid sequences of GIP, GLP-1 and the clinically approved GLP-1 mimetic, exendin-4. The hybrid peptide, *N*-ac(D-Ala$^2$)GIP/GLP-1-exe, was enzymatically stable for up to 12 h when incubated with DPP-4. *N*-ac(D-Ala$^2$)GIP/GLP-1-exe significantly ($P < 0.001$) stimulated insulin secretion from BRIN-BD11 cells and isolated mouse islets, and evoked dose-dependent increases ($P < 0.001$) in cAMP production in both GIP-R and GLP-1-R transfected cells. In mice, injection of the hybrid in combination with glucose significantly ($P < 0.001$) reduced glucose and increased insulin concentrations, with metabolic actions evident ($P < 0.05$) 8 h post-injection. Twice-daily injection of *N*-ac(D-Ala$^2$)GIP/GLP-1-exe to high fat fed (HFF) mice for 28 days significantly ($P < 0.05$–$P < 0.001$) reduced body weight, $HbA_{1c}$, circulating glucose and insulin concentrations. Furthermore, both oral and i.p. glucose tolerance were improved ($P < 0.001$) and insulin sensitivity enhanced. The hybrid peptide also increased ($P < 0.05$–$P < 0.001$) beta cell number, islet area, pancreatic insulin content and islet insulin secretory responsiveness in HFF mice. Finally, *N*-ac(D-Ala$^2$)GIP/GLP-1-exe treated mice exhibited improved ($P < 0.01$) recognition memory which was accompanied by enhanced ($P < 0.05$–$P < 0.001$) hippocampal neurogenesis, synapse formation and reduced neuronal oxidative stress. These data demonstrate for the first time the beneficial actions of the novel GIP/GLP-1 hybrid, *N*-ac(D-Ala$^2$)GIP/GLP-1-exe, on glucose homeostasis and memory function in diabetes.

## 1. Introduction

Glucose dependent insulinotropic hormone (GIP) and glucagon like peptide 1 (GLP-1) are enteroendocrine incretin hormones released from the gut in response to feeding that act as important regulators of post prandial glycaemic control [1]. Incretin hormones are known to impart several direct beneficial pancreatic effects, including stimulation of insulin secretion and insulin gene expression, promoting beta-cell survival, improving beta cell glucose sensitivity and decreasing glucagon secretion [2]. In addition, incretin hormones also possess numerous extrapancreatic actions that help reduce blood glucose levels [1]. As might therefore be expected, this has led to the clinical introduction of several long-acting, protease resistant, GLP-1 analogues for the treatment of type 2 diabetes mellitus (T2DM) including; exenatide, liraglutide, dulaglutide, lixisenatide and albiglutide in the UK [3].

Similar attempts to create clinically viable GIP therapeutics have not been successful, and this is largely due to the reported ineffectiveness of GIP in T2DM [4]. Potential origins for this are believed to be associated with reduced activity or desensitisation of the GIP-receptor (GIP-R) in T2DM [5,6]. However, preclinical and clinical studies demonstrate that amelioration of hyperglycaemia with insulin [7], phlorizin [8], sulfonylureas [9], xenin [10] or SGLT-2 inhibitors [11], as well as weight loss [12], can restore GIP efficacy in T2DM. Further to this, co-administration of GLP-1 with GIP has also been shown to significantly augment GIP receptor activity [13]. It follows that design of a single hybrid peptide based on the amino acid structures of GIP and GLP-1, capable of simultaneous activation of both incretin receptors, could have real therapeutic promise for T2DM. Indeed, the therapeutic benefits of employing unimolecular dual acting hybrid peptides, as opposed to separate injection of parent incretin peptides, have gained much encouragement of late for T2DM [1]

To generate such a compound, we have created a novel, rationally

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designed, GIP/GLP-1 hybrid peptide, *N*-ac(D-Ala$^2$)GIP/GLP-1-exe, derived from parent amino acid sequences of well characterised GIP and GLP-1 receptor agonists. As such, the hybrid peptide was created through capping the N-terminal of the molecule with and acetyl adduct [14] and then linking of the first 15 amino acid residues of (D-Ala$^2$)GIP (1–42) to residues 16–35 of GLP(7–36)amide [15]. This amino acid series was further fused to the last 11C-terminal residues of (D-Ala$^2$)GIP (1–42) and finally to the 10 amino acid C-terminal extension of exendin-4 [16]. Thus, *N*-ac(D-Ala$^2$)GIP/GLP-1-exe is a 56 amino acid hybrid peptide that includes amino acid motifs to permit binding to GIP [14,15] and GLP-1 receptors [17], with hypothesised resistance to DPP-4 cleavage [14,15]. In addition, *N*-ac(D-Ala$^2$)GIP/GLP-1-exe also contains the characteristic Trp-cage from exendin-4 known to prolong pharmacodynamic profile of peptides through enhanced metabolic stability and reduced clearance [16].

Initially *in vitro* DPP-4 resistance, insulin secretion and *in vivo* glucose-lowering and insulinotropic actions of *N*-ac(D-Ala$^2$)GIP/GLP-1-exe were examined. In addition, cAMP production capabilities of *N*-ac(D-Ala$^2$)GIP/GLP-1-exe were evaluated in cells transfected with either the GIP, GLP-1 or glucagon (GcG) receptor. We then progressed to a 28-day twice-daily injection regime with *N*-ac(D-Ala$^2$)GIP/GLP-1-exe in high fat-fed (HFF) mice to examine effects of chronic treatment on body weight, food intake, $HbA_{1c}$, metabolic control, lipid profile and pancreatic histology. In addition, given that diabetes is linked to the development of cognitive impairment and neurodegeneration [18], and knowledge that both GIP and GLP-1 receptor activation can improve cognitive function [19–22], we assessed the impact of *N*-ac(D-Ala$^2$)GIP/GLP-1-exe on behaviour, recognition memory as well as hippocampal neurogenesis and oxidative stress in HFF mice. Taken together, the data highlight the promise of a novel GIP/GLP-1 hybrid, such as *N*-ac(D-Ala$^2$)GIP/GLP-1-exe, for improved metabolic control and memory performance in an established rodent model of T2DM.

## 2. Materials and methods

### 2.1. Peptides

Table 1 displays the amino acid sequence of *N*-ac(D-Ala$^2$)GIP/GLP-1-exe as well as parent peptides (D-Ala$^2$)GIP(1–42), GLP-1(7–36)amide and exendin-4. All peptides were purchased from GL Biochem Ltd. (Shanghai, China) at > 95% purity and further characterised in-house using MALDI-TOF MS, as described previously [23].

### 2.2. DPP-4 degradation

Peptides were incubated at 37 °C in 50 mmol/l TEA-HCl (pH 7.8; Sigma-Aldrich, UK) with purified porcine DPP-4 (5 mU; Sigma-Aldrich, UK) for 0, 2 and 12 h. Degradation profiles were obtained using RP-HPLC analysis and peak area data used to calculate the percentage of intact peptide remaining at time points during the incubation, as described previously [24].

### 2.3. In vitro insulin secretion

Effects of *N*-ac(D-Ala$^2$)GIP/GLP-1-exe and related peptides on *in vitro* insulin secretion were examined using BRIN-BD11 cells, whose characteristics have been reported previously [25]. Briefly, BRIN-BD11 cells were seeded (150,000 cells/well) into 24-well plates (Nunc, Roskilde, Denmark) and allowed to attach overnight at 37 °C. Following a 40 min pre-incubation (1.1 mmol/l glucose; 37 °C), cells were incubated (20 min; 37 °C) in the presence of 5.6, 11.1 or 16.7 mmol/l glucose with a range of test peptide concentrations ($10^{-12}$–$10^{-6}$ mol/l). After 20 min of incubation, buffer was removed from each well, and aliquots (200 µl) were stored at −20 °C prior to determination of insulin by radioimmunoassay [26]. Studies in isolated mouse islets (NIH Swiss mice, Envigo Ltd., Blackthorn, UK, 12–14 weeks old) were performed identical to above but with a 60 min incubation period, following standard collagenase-based islet isolation [27].

### 2.4. In vitro cAMP production

Effects of *N*-ac(D-Ala$^2$)GIP/GLP-1-exe and related peptides on cAMP production were assessed in Chinese hamster lung cells transfected with either the human GIP- or GLP-1 receptor, as well as human embryonic kidney (HEK293) cells transfected with the human glucagon receptor [28]. Cells were seeded (200,000 cells/well) into 96-well plates (Nunc) and washed with Hanks' balanced salt solution (HBSS) buffer before incubation with test peptides ($10^{-10}$–$10^{-6}$ mol/l) in the presence of 200 µmol/l 3-isobutyl-1-methylxanthine for 20 min at 37 °C. After incubation, medium was removed, and the cells lysed before measurement of cAMP using Parameter cAMP assay (R&D Systems, Abingdon, UK) according to the manufacturer's instructions. All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich, UK.

### 2.5. Animals

For acute and long-term *in vivo* studies, NIH Swiss mice (aged 10–14 weeks) were purchased from Envigo Ltd. (Blackthorn, UK). All animals were housed separately in an air-conditioned room (22 ± 2 °C) with artificially controlled 12:12 h dark/light cycles (08:00–20:00 h) and had free access to water and normal rodent chow diet, containing 10% fat, 30% protein and 60% carbohydrate (percent of total energy of 12.99 kJ/g; Trouw Nutrition, Cheshire, UK). Prior to commencement of long-term experiments, groups of mice were kept on high fat diet comprising 45% fat (Product Code 824053; Special Diet Services, Witham, UK; total energy 26.15 kJ/g) for 13 weeks. All experiments were performed according to UK Home Office Regulations (UK Animals Scientific Procedures Act 1986) under appropriate project and personal licenses and approved by the Ulster University Animal Welfare and Ethical Review Body (AWERB).

### 2.6. Acute actions on blood glucose and plasma insulin in vivo

Blood glucose and plasma insulin concentrations were measured in normal mice that received glucose alone (18 mmol/kg bw; *ip*) or in combination with (D-Ala$^2$)GIP, exendin-4 and *N*-ac(D-Ala$^2$)GIP/GLP-1-exe (each at 25 nmol/kg bw; *ip*). In a separate series of experiments, persistent actions of peptides were assessed in normal mice. As such, blood glucose and plasma insulin responses were evaluated after intraperitoneal injection of glucose alone (18 mmol/kg of body weight) 4, 8 or 12 h after an intraperitoneal injection of saline vehicle (0.9% (w/v) NaCl) or test peptides (each at 25 nmol/kg bw; *ip*).

**Table 1**
Amino acid sequences of novel *N*-ac(D-Ala$^2$)GIP/GLP-1-exe hybrid peptide and parent peptides.

| | |
|---|---|
| *N*-ac(D-Ala$^2$)GIP/GLP-1-exe | Acetyl-Y-(DA)-E-G-T-F-I-S-D-Y-S-I-A-M-D-V-S-S-Y-L-E-G-Q-A-A-K-E-F-I-A-W-L-V-K-G-K-K-N-D-W-K-H-N-I-T-Q-G-P-S-S-G-A-P-P-P-S-$NH_2$ |
| (D-Ala$^2$)GIP(1–42) | Y-A-E-G-T-F-I-S-D-Y-S-I-A-M-D-K-I-H-Q-Q-D-F-V-N-W-L-L-A-Q-K-G-K-K-N-D-W-K-H-N-I-T-Q-$NH_2$ |
| GLP-1(7–36) | H-A-E-G-T-F-T-S-D-V-S-S-Y-L-E-G-Q-A-A-K-E-F-I-A-W-L-V-K-G-R– $NH_2$ |
| Exendin-4 | H-G-E-G-T-F-T-S-D-L-S-K-Q-M-E-E-E-A-V-R-L-F-I-E-W-L-K-N-G-G-P-S-S-G-A-P-P-P-S-$NH_2$ |