Synthesis, Characterization and Pharmacodynamics of Vitamin-B$_{12}$-Conjugated Glucagon-Like Peptide-1

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The therapeutic potential of glucagon-like peptide-1 (7–36) amide (GLP-1) receptor (GLP-1R) targeting for the treatment of type 2 diabetes (T2D) has been validated extensively through basic and clinical research. [3] GLP-1, a major incretin, has a wide variety of physiological roles centered on the postprandial homeostasis of glucose, including the potentiation of insulin secretion and the suppression of glucagon secretion. [2] The insulinotropic functions of GLP-1 are glucose-dependent, making GLP-1 an ideal treatment for hyperglycemia with minimal risk of hypoglycemia. [3] Recently documented preclinical and clinical research has also established the role of GLP-1R agonists in increasing satiety, [4] promoting modest weight loss, [5] improving $\beta$-cell function, [6] and preserving $\beta$-cell mass by inhibiting apoptosis, [6] which might help to slow or reverse the progression of T2D. [7]

GLP-1 is secreted from enteroendocrine L cells in the ileum and colon in response to orally ingested fats and carbohydrates, and it enters the systemic circulation via intestinal capillaries draining into the hepatic portal vein. [2] GLP-1 is rapidly degraded ($t_{1/2} = 1$–2 min) by dipeptidyl peptidase IV (DPP-IV), and only 10–15% of secreted GLP-1 survives intact to the systemic circulation. [8,9] GLP-1R has been identified on pancreatic $\beta$ cells, in the hepatic portal vein, central nervous system, and on the vagal afferent fiber terminals of the gastrointestinal tract (GIT). [10]

A number of GLP-1-related therapeutics are currently available for the treatment of T2D, including a GLP-1 analogue, liraglutide, and a GLP-1R agonist, exenatide. [11] Both of these prescription therapeutics are administered by subcutaneous injections. [11] An orally deliverable GLP-1 therapeutic would have the potential to provide patients with a noninvasive therapy and mimic more closely the physiological effects of native GLP-1, specifically interactions with receptors in the intestines. [9]

Vitamin B$_{12}$ (B$_{12}$) is essential for the survival of all living organisms. It plays an important role in the normal functioning of the brain and nervous system, as well as the formation of red blood cells. [12] B$_{12}$ is only synthesized naturally by bacteria, so humans must acquire the vitamin through their diet. [12,13] Due to the low bioavailability of B$_{12}$, the body has an uptake mechanism, recently reviewed by Nexø et al., to ensure successful absorption of the vitamin from the diet. [13] Briefly, the pathway involves haptocorrin, a salivary enzyme that can protect and transport B$_{12}$ through the stomach and into the small intestine. The B$_{12}$ is then bound by intrinsic factor (IF) and proceeds down the small intestine where the IF–B$_{12}$ complex is recognized by the CUB$_{5,4}$ domain of cubilin-amnionless (cubam) receptor in the ileum. [13] The IF–B$_{12}$ receptor complex then undergoes transcytosis, releasing B$_{12}$ into the blood serum where it is bound to transcobalamin II (TCII) and transported to cells. [13]

The full potential of the B$_{12}$ uptake pathway as a delivery system has yet to be realized. [14] A number of limitations to this delivery strategy have been identified in recent years that have hindered development, including a limited uptake capacity (nmol per dose), [15] relatively slow delivery time (in the order of hours), [16] and questions regarding protection for the transported peptide. [16] The critical components to the success of this delivery strategy are 1) the uptake capacity of the B$_{12}$ pathway must meet the necessary increase in plasma peptide levels required to produce a physiological response; 2) questions regarding protection of the peptide must be addressed; and 3) B$_{12}$ conjugation must have minimal negative effect on the function of the peptide.

The B$_{12}$ uptake pathway could be the answer to overcoming the major hurdles to oral peptide delivery. Our previous reported work with B$_{12}$–insulin suggests there is a protective element to the B$_{12}$ uptake pathway for peptides. [17] We have also previously demonstrated the ability to use B$_{12}$ to orally deliver clinically relevant amounts of the appetite suppressant peptide YY$_{3-36}$. [18]

Although the IF-mediated endocytosis via cubam is the limiting step in the B$_{12}$ uptake pathway, it is important to note that cubam recycles, [19] and as such, multiple dosing (e.g., morning and evening) could be adopted to increase the quantity of peptide absorbed throughout the day. This recycling will also be important when considering dietary B$_{12}$ competition. [20]

To adapt the B$_{12}$ uptake pathway for the delivery of the insulinotropic peptide, GLP-1, the biological potency of GLP-1 (EC$_{50} = 3$ nm) [21] must not be greatly decreased by B$_{12}$ conjugation. [14] Herein, we report the synthesis, purification and characterization of a B$_{12}$–GLP-1 conjugate based on a GLP-1 analogue, namely K34R-GLP-1. The modification of lysine (K) with arginine (R) at position 34 has been shown to not effect GLP-1 activity and allows targeted conjugation to the K26 residue. [22] In vitro experimentation with the B$_{12}$–K34R-GLP-1 conjuga-
gate was utilized to determine the effect of B12 conjugation on the biological potency of GLP-1.

Unmodified GLP-1 has three amine groups (N terminus, K26 and K34) available for conjugation. For production of B12-K34R-GLP-1 (1), the peptide was prepared with modifications (amine acid substitution K34R, and fluorenly-9-methoxycarbonyl (FMOC) protection of the N-terminal amine to give FMOC-K34R-GLP-1) to ensure selective conjugation at K26 (see Figure 1). The FMOC protection was removed after purification to ensure biological activity would be maintained.[22]

Modification of the 5′-hydroxy group of B12 has been repeatedly shown to maintain binding with the transport proteins vital for oral B12 uptake,[15,18,23] and this site was again chosen for this work. Reaction of B12 with 1,1′-carbonyl-di-(1,2,4-triazole) (CDT) in dry dimethyl sulfoxide (DMSO) at room temperature for five minutes furnished an activated ester at the 5′-hydroxy group. A 2.5-fold excess of activated B12 was then added to a slowly stirring solution of FMOC-K34R-GLP-1 in DMSO with 0.6% triethylamine. The reaction proceeded for two hours before a 10-fold excess of 9-fluorenylmethyl succinimidyl carbonate (FMOC-OSu) was added to help with the purification of 1, as described below.

For the purification of B12-K34R-GLP-1 (1), the reaction was first extracted by diethyl ether addition and centrifugation. The isolated pellet was re-dissolved in 25/75 acetonitrile/water with 0.1% trifluoroacetic acid (TFA), and the target product (1) was isolated on a C18 analytical column using reversed-phase (RP)-HPLC. Unreacted B12 was readily separated from FMOC-K34R-GLP-1 in this manner. Purification of unconjugated FMOC-K34R-GLP-1 and B12-conjugated FMOC-K34R-GLP-1 proved challenging due to the similarity in their hydrophobicity. The addition of excess FMOC-OSu was used to aid in purification, as previously reported for our B12 insulin conjugate.[16] Figure 2 shows that the additional FMOC moiety increases the retention time of the unconjugated (hence, unprotected) FMOC-K34R-GLP-1, allowing for separation. Following HPLC purification, the protecting groups were removed with 10% piperidine (10 min) and dialyzed against water (5 L) overnight in dialysis tubing. A B12 immunoaffinity column was used to further establish the absence of unreacted GLP-1 in subsequent in vitro assays (for details, see the Experimental Section). While exhaustive and probably poorly scalable, this route was used to ensure no free K34R-GLP-1 remained in the sample, which could otherwise contribute to a positive result in subsequent in vitro assays.

B12-K34R-GLP-1 (1) was identified as the peak at a retention time of 20.7 minutes (see Figure 2) by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-ToF MS) after removal of the FMOC protection as described above. The peak in Figure 3 at 4682 m/z represents a 1:1 [1+–CN]− conjugate.

GLP-1 mediates its insulinotropic effects by binding to GLP-1R on pancreatic ß cells and stimulating cAMP formation, calcium mobilization and insulin secretion.[2,8,24] The effect of B12 conjugation on GLP-1 physiological function (cAMP signaling, calcium mobilization, and insulin secretion) was studied in HEK-293 cells stably expressing human GLP-1R (HEK-GLP-1R) and in isolated human pancreatic islets.

To determine the ability of the conjugate to perform as a cAMP elevating agent, HEK-GLP-1R cells were transfected with a reporter construct containing the coding sequence for the luciferase (Luc) enzyme and a promoter incorporating a cAMP responsive element (CRE) originally identified within the 5′ promoter region of the rat insulin 1 gene (RIP1-CRE). [25] This cAMP-responsive Luc reporter (RIP1-CRE-Luc) can be used to determine the effect of agonist for G protein-coupled receptors positively linked to cAMP production, such as GLP-1R. The level of Luc activity was determined in whole-cell lysates by Luc-catalyzed oxidation of luciferin, which generated photons detected by a photomultiplier tube. After exposure to 1 for four hours, Luc activity was determined and shown to be concentration-dependent with an EC50 value of 4.4 nm (Figure 4). These findings were comparable to those found for the K34R-GLP-1 control with an EC50 value of 4.1 nm. These EC50 values are consistent with those reported for GLP-1 in the literature (~3 nm). [21] Figure 4 also shows that B12 did not produce any response in this assay. The EC50 value recorded for 1 suggests
that B_{12} has little negative effect on the binding of GLP-1R by GLP-1.

We subsequently sought to determine whether an actual physiologically relevant response could be achieved with 1. The stimulatory effects of GLP-1 on pancreatic islet insulin secretion have been attributed to the capability of GLP-1 to increase levels of cytosolic [Ca^{2+}]_{i} in β cells. In HEK cells, GLP-1 produces a rise in cytosolic [Ca^{2+}]_{i} by facilitating the action of P2Y purinergic receptor agonists to mobilize Ca^{2+} from intracellular Ca^{2+} stores. The ability of 1 to facilitate an increase in cytosolic [Ca^{2+}]_{i} was therefore assessed in HEK-GLP-1R cells that express endogenous P2Y purinergic receptors using a Fura-2 assay.[26] Figure 5 demonstrates that both forms of GLP-1 potentiated the action of ADP to mobilize intracellular [Ca^{2+}]_{i} in a dose-dependent manner, with native GLP-1 only slightly more potent than 1.

To further document the physiologically relevant action of 1, insulin secretion was assayed under standard conditions of static incubation using human islets.[27] Human pancreatic islets are a critical tool for diabetes researchers because they provide invaluable information about the cellular biology of the pancreas and the pathology of diabetes. Furthermore, the uniqueness of human pancreatic biology is an invaluable tool for the development of therapeutics for the treatment of diabetes.

Human islets were equilibrated in Krebs-ringer buffer (KRB) containing glucose (2.8 mM), followed by a subsequent elevation of the glucose concentration to 16.7 mM with or without the added test compound. The rise in glucose concentration causes glucose stimulated insulin secretion (GSIS) in healthy insulin-producing islets, and the addition of functional GLP-1 should potentiate this effect. For three batches of human islets from three donors, the average GSIS measured in response to 16.7 mM glucose was 2.2-fold in the absence of 1 (Figure 6). GSIS was potentiated an additional 45% by 15 nM 1 (total 3.2-fold stimulation). The effect of 1 was similar to that measured when islets were instead treated with 15 nM K34R-GLP-1 (total 3.3-fold stimulation).

We have reported herein the synthesis, purification and in vitro characterization of a B_{12}-K34R-GLP-1 conjugate. Experimen-
Experimental Section

General: Chemicals and solvents were purchased from Sigma–Alrich or Fluka and were used without further purification. Glucagon-like peptide (7–36) amide with a K34R amino acid substitution containing 16.7 mM glucose (G) and no test compound (2nd bar), 1 mucosal buffer (2nd bar), 1 denoted here as B12–GLP-1; 3rd bar), or K34R–GLP-1 (denoted here as GLP-1; 4th bar). In the absence of added glucose, peptide produced a 2.2-fold stimulation of insulin secretion. When 1 was included with 16.7 mM glucose, a 3.2-fold stimulation was measured, which is comparable to that measured for K34R-GLP-1 (3.3-fold increase).

Figure 6. Static incubation assays using human pancreatic islets. Human islets (40–50 per insert) were exposed to Krebs-ringer buffer (KR B) containing 2.8 mM glucose for 30 min producing an average basal insulin secretion of 21.4 ± 3 ng mL⁻¹·30 min (1st bar). The islets were then exposed to KR B containing 16.7 mM glucose (G) and no test compound (2nd bar), 1 denoted here as B12–GLP-1; 3rd bar), or K34R–GLP-1 (denoted here as GLP-1; 4th bar). In the absence of added glucose, peptide produced a 2.2-fold stimulation of insulin secretion. When 1 was included with 16.7 mM glucose, a 3.2-fold stimulation was measured, which is comparable to that measured for K34R-GLP-1 (3.3-fold increase).

Experimental Section

General: Chemicals and solvents were purchased from Sigma–Alrich or Fluka and were used without further purification. Gluca- gon-like peptide (7–36) amide with a K34R amino acid substitution and N-terminal FMOC protection (FMOC-K34R-GLP-1) was pur- chased from Pierce. H2O was distilled and deionized to 18.2 M of 21.4

Luciferase assay: HEK-GLP-1R cells were plated in a 96-well plate at a density of 50,000–65,000 cells per well and incubated overnight at 37 °C. The next day, the cells were transfected with RIP1-CRE-Luc using Lipofectamine 2000 according to the manufacturer’s instruc- tions. The cells (HEK-GLP-1R-IP1-CRE-Luc) were cultured in DMEM containing 10% FBS overnight and then exposed to serum-free culture medium containing 1% bovine serum albumin (BSA) and the test substance for 4 h. Cells were then lysed and assayed for luci- ferase-catalyzed photoemissions using a luciferase assay kit and lumino- meter.

Fura-2 assay: The experiment was performed using a monolayer of Fura-2-loaded HEK-GLP-1R cells grown on rat-tail-collagen-coated Costar 3904 plate. Fura-2 was loaded in a standard extracellular so- lution (SES) containing: 138 mM NaCl, 5.6 mM KCl, 2.6 mM CaCl2, 1.2 mM MgCl2, 10 mM HEPES (pH 7.4) and 11.1 mM glucose. The SES was also supplemented with 20 μL per mL of FBS, 1 μL per mL of Pluronic F-127, and 1 μM Fura-2AM. Spectrofluorimetry was performed using excitation light at 355/9 and 375/9 nm (center/band- pass) delivered using a 455 nm dichroic mirror. Emitted light was de- tected at 505/15 nm, and the ratio of emission light intensities due to excitation at 355 and 375 nm was calculated. Insulin secre- tion was determined by static incubation.

Insulin secretion: After overnight culture in CMRL-1066 medium, human islets were transferred to MilliCell polycarbonate film (PCF) culture plate filter inserts at a density of 40 islets per insert. The in-
serts were mounted within individual wells of a 24-well cell culture plate, and each well of the plate was filled with 1 mL of Krebs-ringer buffer (KRB) containing 24 mM aq NaHCO$_3$ and the indicated concentration of glucose (pH 7.4). Culture plates containing islets were placed within a cell culture incubator gassed with 95% air and 5% CO$_2$ at 37°C. Prior to the start of an experiment, islets were exposed to KRB with 2.8 mM glucose for 30 min. The inserts were then transferred between adjacent wells on the plate to measure basal and stimulated insulin secretion. For each test solution, the duration of exposure was 30 min with 16.7 mM glucose. A 200 μL fraction of each solution was then collected and assayed for insulin content using an ultrasensitive insulin ELISA kit, after appropriate dilution of samples.

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