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Corrination mitigates peptide aggregation as exemplified for Glucagon

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ABSTRACT

Pharmaceutical development of glucagon for use in acute hypoglycemia has proved challenging, due in large part to poor solubility, poor stability and aggregate formation. Herein, we describe highly soluble, low aggregating, glucagon conjugates generated through use of the commercially available vitamin B_{12} precursor dicyanocobinamide ('corrination'), which retain full stimulatory action at the human glucagon receptor. The modified glucagon analogs were tested in a chemical stability assay in 50 mM phosphate buffer and the percentage of original concentration retained was determined after two weeks of incubation at 37 ° C. Aggregate formation assays were also performed after 48 h of agitation at 37 °C using a thioflavin (ThT) fluorescence-based assay. All corrinated compounds retained original concentration to a higher degree than glucagon controls and showed markedly decreased aggregation compared to their respective noncorrinated analogues. Based on the statistically significant increase in chemical stability coupled with the notably decreased tendency to form aggregates, analogues **2** and its corrinated conjugate **5** were used for a functional assay study performed after agitation at 37 °C for 24-hr after which agonism was measured at the human glucagon receptor using a cAMP FRET assay. Corrinated **5** exhibited a 6.6-fold increased potency relative to glucagon, which was shown to have a 165-fold reduction in potency. The relative potency of **5** was also improved compared to that of **2** with EC_{50} values of 5.5 nM and 9.6 nM for **5** and **2**, respectively. In conclusion, corrination of peptides mitigates aggregation, presenting a compound with prolonged stability and agonism as demonstrated for glucagon.

1. Introduction

Alterations to the chemical structure of a peptide, either into the primary structure, via 'stapling' or via conjugation for example, have proven effective in improving peptide bioactivity, solubility and/or stability (to proteolysis or aggregation, for example).

Glucagon (Gcg) is a 29-amino acid pancreatic hormone derived from proglucagon [1,2]. In the fasting state between meals when blood glucose levels begin to drop, Gcg is released from alpha-cells located in the pancreatic islets. Gcg then acts as a homeostatic circulating hormone to stimulate hepatic glucose release, thereby ensuring that the blood glucose concentration does not drop to dangerously low levels. Recently, it is also appreciated that pharmacological doses of synthetic Gcg receptor agonists increase whole-body energy expenditure, thereby indicating a potential role for such therapeutic agents as weight loss remedies [3–5]. Gcg acts at the glucagon receptor (GcgR), a G_s-coupled G-protein coupled receptor found primarily in liver, adipose, heart, and kidney tissues [6,7]. Gcg has a short half-life (< 5 min) [8] and is highly prone to aggregation [8,9], a reason pharmaceutical development of such to treat acute hypoglycemia has proven challenging. Over the past 70 years, there have been only two FDA-approved formulations of standard Gcg: GlucaGen HypoKit® from Novo Nordisk and Glucagon Emergency Kit® from Eli Lilly [10]. Both Gcg products are supplied as lyophilized powder and need to be reconstituted with water or dilute hydrochloric acid [10,11] prior to injection. Dissolved Gcg has a propensity to fibrillate and form aggregates [10,11] and thus, must be used immediately after reconstitution, making it inadequate for prolonged use. Due to this complex process, several industrial groups are developing a stable liquid form of Gcg such as Gvoke® from Xeris Pharmaceuticals. The Gvoke HypoPen™ is a single-use auto-injector in which Gcg is dissolved in dimethyl sulfoxide (DMSO) [11] for the treatment of severe hypoglycemia. Although DMSO prevents aggregation and

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fibrillation of the drug, it causes side effects such as irritative and burning skin reactions [10] at the injection site. Since it is delivered subcutaneously, other side effects include nausea and vomiting [12]. Therefore, there is a need to develop a method to improve both the physical and chemical stability of peptides that are often unstable in aqueous solutions such as Gcg.

The Doyle group has recently coined the term 'corrination' [13,14] to describe the (well established) [15–20] conjugate modification of a compound/peptide/protein with a corrin ring containing compound, such as vitamin B_{12} (B_{12}) [19] or dicyanocobinamide (Cbi) [13] (Fig. 1). Herein, we describe the corrination of Gcg at two sites (K17 or K30; Table 1) on the peptide backbone with the highly water-soluble Cbi (400 mg/mL H_2O) [14], using copper catalysed azide-alkyne cycloaddition (CuAAC) [21]. The presence of single D-amino acids has been shown to decrease peptide hydrophobicity and increase aqueous solubility. As Di Marchi *et al.* [9] reported, D-substitution of Gcg at position 17 exhibited increased solubility with high Gcg receptor activity. Hence, we incorporated D-arginine (D-Arg) at position 17 of Gcg (Table 1). The effects of corrination, and corrination position, on Gcg solubility, stability, aggregation, and function over time, at the GcgR is described.

2. Materials and methods

Cbi was produced from commercial B_{12} (cyanocobalamin) starting material using a microwave reaction as described by Gryko *et al.* [22]. Briefly, a 10 mL microwave reaction vessel was charged with B_{12} (100.8 mg, 0.074 mmol) and NaCN (14.2 mg, 0.29 mmol) in 5 mL of EtOH producing a red coloured solution. The reaction vial was sealed, and microwave heated to 120 °C for 10 min at 300 W. After reaction, the now deep purple solution was diluted with H_2O (total volume 25 mL), flash frozen in liquid N_2 and lyophilized. Cbi was purified on an Isolera One Biotage system using a normal phase flash chromatography cartridge (Biotage® HP Sil SNAP, 25 g) with an isocratic method of MeOH in EtOAc (2:1 v:v) at a flow rate of 5 mL/min, tracked at 360 nm. The isolated product was precipitated with Et_2O, flash frozen as before and lyophilized. Purity was confirmed by RP-HPLC on a C_{18} column.

In preparation for conjugation to Gcg, a linker moiety in the form of 1-amino-5-hexyne (AH) was reacted with the Cbi hydroxyl group to prepare Cbi with an available alkyne reactive group (**1**). **1** was prepared by combining Cbi (42.5 mg, 0.041 mmol) with 1,1'-carbonyl-di-(1,2,4-triazole) (CDT) (167.3 mg, 1.019 mmol) in 5 mL of *N*-methyl-2-pyrrolidone (NMP) and stirring for one hour under argon at 40 °C, at which time AH (123 μL, 98 mg, 1.012 mmol) and triethylamine (TEA) (50 μL) were added to the solution. After an additional hour, a second equivalent of AH and TEA were added, and the reaction was allowed to stir overnight at 40 °C. **1** was purified by RP-HPLC on a C_8 column.

Table 1
Peptide sequence and coding for Gcg peptides. Coding is as follows: **X** = ε-azidolysine (site of Cbi conjugation); **r** = D-arginine; **Z** = Cbi-lysine.

Peptide Sequence	Coding	
HSQGTFTSDYSKYLDSRRAQDFVQWLMNT	Glucagon	**Gcg**
HSQGTFTSDYSKYLDS**r**RAQDFVQWLMNT	Glucr17	**Gcg17**
HSQGTFTSDYSKYLDS**X**RAQDFVQWLMNT	GlucK17	**2**
HSQGTFTSDYSKYLDSRRAQDFVQWLMNT**X**	GlucK30	**3**
HSQGTFTSDYSKYLDS**r**RAQDFVQWLMNT**X**	Glucr17K30	**4**
HSQGTFTSDYSKYLDS**Z**RAQDFVQWLMNT	Cbi-**2**	**5**
HSQGTFTSDYSKYLDSRRAQDFVQWLMNT**Z**	Cbi-**3**	**6**
HSQGTFTSDYSKYLDS**r**RAQDFVQWLMNT**Z**	Cbi-**4**	**7**

Azido-modified Gcg analogues (Table 1) were designed to test the hypothesis that corrination of Gcg could improve stability over time, measured as reduced aggregation and maintained functional agonism at the GcgR, relative to Gcg (**2–4**).

The synthesis **2-4** (Table 1) was accomplished by Fmoc/tBu solid-phase synthesis on a CEM Liberty Blue synthesizer using non-preloaded resin and repetitive DIC/Oxyma activation. Upon completion the peptides are cleaved from resin and deprotected using a cocktail containing 95 % TFA, 2.5 % TIPS, 2.5 % H_2O at 40 °C for 40 min using a Liberty Blue Razor. **2-4** were precipitated from solution using cold ET_2O and centrifuged at 4000 rpm for 10 min. The supernatant was discarded and peptide pellet resolubilized in water and lyophilized in vacuo prior to purification (Figs. S3-7). To help stabilize the peptides and increase their bioavailability, all peptides were made with an amidated C-terminal end.

Corrination of 2–4 was facilitated by CuAAC (Scheme 1). CuI (6.5 mg, 0.034 mmol) was added to a solution of tris[(1-benzyl-1 H-1,2,3-triazol-4-yl)methyl]amine (TBTA) (22 mg, 0.042 mmol) in 4:1 DMF: water and allowed to stir for approximately 30 min at room temperature producing a faint yellow colour. 1 (15.3 mg, 0.013 mmol) and 2–4 (5 mg, 0.0014 mmol) were then dissolved in 4:1 DMF: H_2O, producing a final reaction volume of 2 mL with the reaction gently stirred at room temperature overnight to produce 5–7, which were purified by dialysis using a Slide-a-Lyzer cassette.

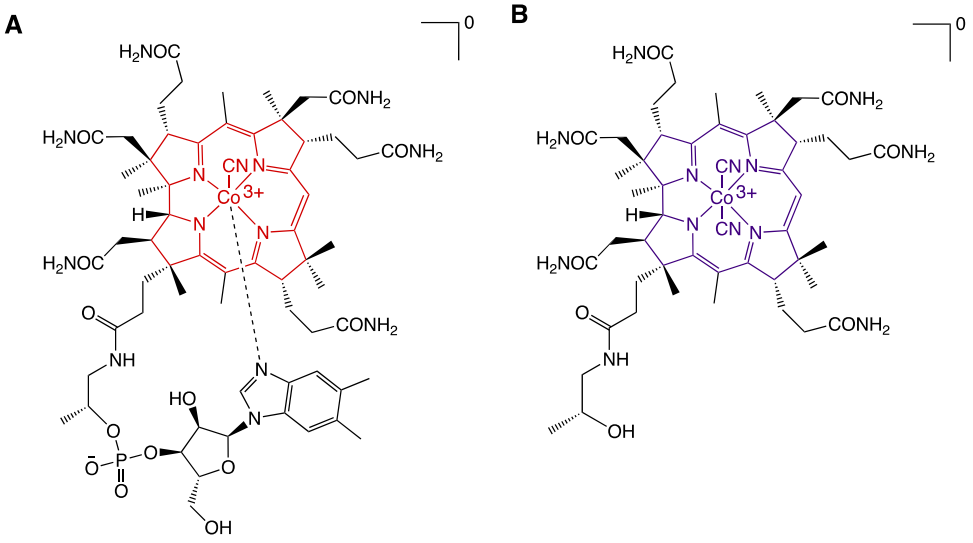
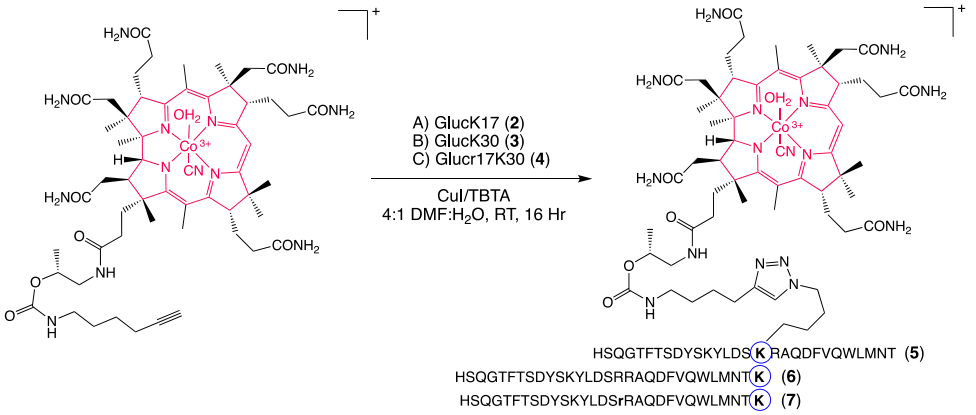


Fig. 1. The structures of (A) vitamin B_{12} and (B) dicyanocobinamide (Cbi) with the tetrapyrrole 'corrin' ring shown in red or purple (reflecting compound natural colour in the solid state), respectively.



Scheme 1. Synthesis of corrinated Gcg conjugates (**5-7**) using copper mediated alkyne-azide 'click' chemistry [21].

3. Results

3.1. Conjugate syntheses

Cbi was produced via microwave-assisted reaction of B12 with sodium cyanide in EtOH as previously described [22]. Two signals are observed for Cbi due to the presence of isomers as fully investigated by Zelder *et al.* [23] with 90 % purity (Fig. S1). MALDI-TOF-MS expected $m/z = 1042$, observed $m/z = [M\text{-}2CN+H]^+ = 991$; ^1H NMR (400 MHz, D_2O): δ 5.891 (s, 1 H), 3.907 (m, *J*=5.95 Hz, 1 H), 3.839 (d, *J*=8.23, 1 H), 3.748 (d, *J*=10.44, 1 H), 3.654 (q, *J*=7.11, 2 H), 3.572 (q, *J*=7.08 Hz, 1 H), 3.404 (dd, *J*=7.00,4.78, 1 H), 3.312–3.151 (3 H, overlapped), 2.914–2.857 (m, 1 H), 2.748–2.712 (m, 4 H), 2.600–2.380 (6 H, overlapped), 2.316–2.231 (14 H, overlapped), 2.148–2.069 (m, 4 H), 2.030–1.943 (m, 1 H), 1.915 (s, 3 H), 1.891–1.744 (m, 3 H), 1.683 (s, 3 H), 1.532 (s, 3 H), 1.489 (s, 3 H), 1.430 (s, 3 H), 1.312 (s, 3 H), 1.184 (t, *J*=7.12 Hz, 7 H), 1.151 (d, *J*=6.36 Hz, 3 H); UV–vis (H_2O): λ_{max} 277, 313, 368, 540, 580 nm (Fig. S1). Yield was 65 % based on B_{12}. Cbi was activated with CDT in NMP under argon and reacted with excess AH and the product was purified by RP-HPLC on a C_8 column (Fig. S2) to produce **1** at 80 % yield and 90 % purity, with unreacted Cbi reclaimed during purification. MALDI-TOF-MS expected $m/z = 1157$, observed $m/z = [M\text{-}H_2O]^+$: 1139; ^1H NMR (400 MHz, D_2O): characteristic signals of β- (6.43) and α- (6.36) aquo-isomers; UV–vis (H_2O): λ_{max} 355, 405, 495, 528 nm (Fig. S2). Coupling of **1** with **2-4** to produce conjugates **5-7** was achieved via CuAAC [21], and all conjugates were produced to at least 95 % purity (Figs. S8-10).

3.2. Chemical stability

Given Gcg's poor solubility at physiological solutions, we aimed to increase this solubility through a combination of D-amino acids substitutions and corrination attempts. The solubility of all peptides was recorded at 1 hr, 48 hr, and at 2-week time points (Table S1). Peptides were dissolved in 1X PBS buffer (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl) (pH 7.4) solution lower than the volume needed to fully dissolve the peptide to ensure maximal solubility. Solutions were centrifuged at 3000 rpm for 5 min to pellet any undissolved solid and solubility of the supernatant was then measured by UV absorption at 280 nm on a NanoDrop OneC UV-Vis Spectrophotometer. All analogues had only a fractional loss of solubility as tracked by over the 2-week mark at 4 °C and the reduction of maximal solubility was consistent across the board.

2–7 were tested in a chemical stability study in 50 mM sodium phosphate buffer at a pH of 7 and incubated at 37 °C. Samples were prepared at 150 μM. All peptides and peptide conjugates were assessed for evidence of aggregation utilizing MALDI-TOF MS over a 2-week time span.

After 2 weeks, a final sample was assayed to reveal how much of the original [150 μM] remained in solution (Fig. 2). Corrination helped to increase the stability of **2** (as seen by **5**) and all corrinated compounds (**5-7**) retained original concentration to a higher degree than Gcg, and comparable with the non-corrinated analogues. It is important to note that analogues containing the D-Arg substitution (Gcg17, **4**, **7**) trend towards the highest chemical stability as previously reported by Di Marchi et al. [9]. Gcg17 was significantly different from all analogues except **4** and **7**.

With obvious improvements in chemical stability relative to Gcg, the impact of corrination (**5-7**) on aggregation and functional agonism at GcgR was assayed next.

3.3. Physical stability

Aggregate formation assays were performed in triplicate in 0.1 M HCl at a concentration of 25 μM. Analogues were continually agitated

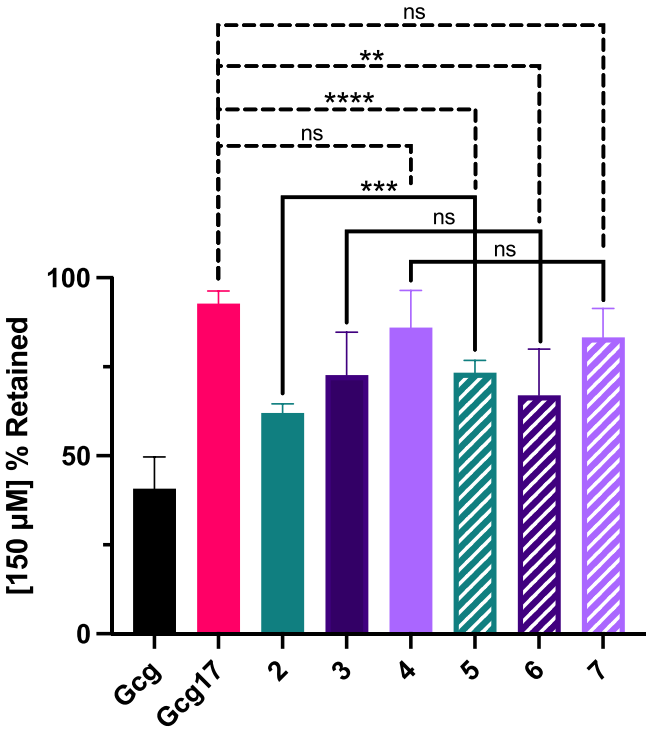


Fig. 2. Assessment of the chemical stability (tracked as % retained soluble Gcg and Gcg analogues after two weeks at 37 °C starting as 150 μM of peptide in phosphate buffer pH 7). **p < 0.01, ***p < 0.001, ****p < 0.0001.

with a stir bar at 37 °C over 48 h prior to conducting a thioflavin-T (ThT) fluorescence-based assay with Aβ40 as a positive control and dopamine as a negative control (Fig. 3).

Corrinated **5-7** showed markedly decreased aggregation compared to Gcg and Gcg analogues **2-4**. Fluorescence data of Cbi and all three Cbi compounds were assessed at an excitation of 440 nm and an emission of 484 nm in which no background fluorescence response was detected (Fig. S11). This ensured the fluorescence capabilities of Cbi did not interfere or quench the ThT assay.

3.4. Functional assay

Based on the statistically significant increase in chemical stability coupled with the markedly decreased tendency to form aggregates due to Cbi conjugation, **2** and **5** were used for a functional assay study to investigate the effect of corrination on GcgR agonism (Fig. 4A_1-C_3). Gcg, **2**, and **5** activities at the GcgR was measured in a FRET assay using suspensions of HEK293 cells in which GcgR stimulation leads to increased levels of intracellular cAMP. This assay utilizes HEK293-hGcgR-H188 clone 10 cells that stably co-express the human GcgR (hGcgR) and the genetically encoded biosensor H188 [25]. When levels of intracellular cAMP rise, this biosensor exhibits reduced FRET that is measurable as an increase of the 485/535 nm FRET emission ratio [26]. The primary goal was to determine whether **2** and **5** substitutions preserved the *in-solution* stability and agonist properties of these peptides relative to Gcg, itself. If so, the characteristic loss of GcgR agonist action that is reported for solubilized Gcg[9] would be reduced. Importantly, the FRET assay we used for this analysis allowed live-cell, real-time assessment of the kinetics of agonist-stimulated cAMP production, while also providing an estimate of agonist efficacy and potency in a 96-well format.

GcgR agonist action was monitored using 25 μM stock solutions diluted in a standard extracellular saline solution (SES). Each peptide was tested on day-1 immediately after solubilization and serial dilution (0.03 – 30 nM) in SES. For comparison, the same 25 μM stock solution was incubated for 24-hr at 37°C in a cell culture incubator, after which serial dilutions were performed on day-2. The FRET assay was then repeated using the same protocol as for day-1. Initially, the dose-response (DR) relationship for peptide-stimulated cAMP production was determined on day-1 for Gcg (Fig. 4A_1), **2** (Fig. 4 B_1) and **5** (Fig. 4C_1). These DR relationships were then compared to those obtained for Gcg (Fig. 4A_2), **2** (Fig. 4B_2) and **5** (Fig. 4 C_2) on day-2 after the 24-hr incubation at 37°C. Day-1 and day-2 data sets were then processed by linear regression analysis so that Hill plot overlays could be created for each of the peptides tested (Fig. 4 A_3, Fig. 4B_3, Fig. 4C_3).

Gcg agonist action to raise levels of cAMP was markedly diminished after a 24-hr incubation in SES at 37°C. This was measurable as reduced agonist efficacy, as evidence by a reduced maximal % change of FRET ratio (%ΔFRET) when testing a 30 nM saturating concentration of Gcg (*c.f.,* Fig. 4 A_1 Fig. 4A_2). Simultaneously, Hill plot analysis revealed that Gcg agonist potency was also reduced. The EC_{50} value obtained on day-1 was 0.22 nM, in comparison to a value of 36.3 nM on day-2 after a 24-hr incubation at 37°C (Fig. 4 A_3). Thus, the Gcg DR relationship was right-shifted by 165-fold after incubation for 24-hr at 37°C (Table 2).

2 acted with similar efficacy as compared to Gcg when it was tested after solubilization on day-1 (*c.f., A_1, B_1*). It also exhibited similar potency so that the EC_{50} value for **2** measured on day-1 was 0.17 nM, whereas that of Gcg was 0.22 nM (*c.f., A_3, B_3*). Notably, measurements of agonist efficacy after a 24-hr incubation at 37°C revealed that **2** retained its ability to stimulate a maximal response (B_2). However, its potency was reduced so that a 56-fold right-shift of the DR relationship was measured (B_3). Thus, EC_{50} values for assays run on day-1 and day-2 were 0.17 nM and 9.6 nM, respectively (Table 2).

A novel consequence of Cbi-conjugation was also measurable in these FRET assays. For 5, a 24-hr incubation at 37°C led to a 5-fold right-shift of the DR. Thus, the right-shift measured for 5 was 11-fold less than that measured for 2 (*c.f., B_3, C_3*). Furthermore, the relative potency of 5 was improved compared to that of 2 after a 24-hr incubation at 37°C. EC_{50} values measured on day-2 were 5.5 nM and 9.6 nM for 5 and 2, respectively (*c.f., B_3, C_3*). Remarkably, when comparing data obtained in assays using peptide solutions equilibrated for 24-hr at 37°C, 5 exhibited a 6.6-fold increased potency relative to Gcg (*c.f., A_3, C_3*). Still, it is important to note that 5 exhibited 23-fold less potency in comparison to Gcg when tested immediately after solubilization on day-1. (*c.f., A_3, C_3*).

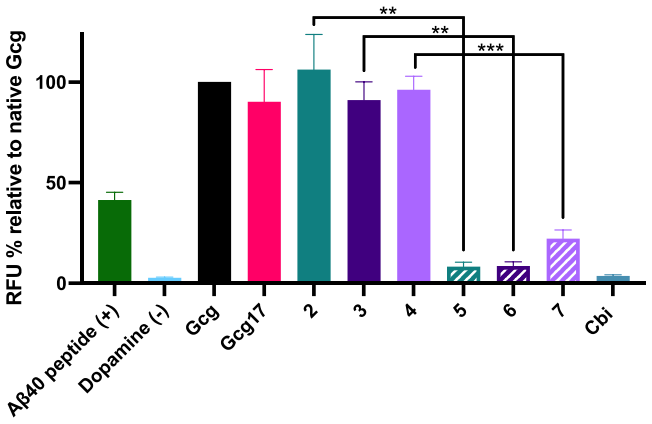


Fig. 3. Aggregate formation assays in 0.1 M HCl using the ThT fluorescence assay [24]. **p < 0.01, ***p < 0.001.

4. Discussion

The development of peptide therapeutics is frequently limited by both chemical/physical properties and their stabilities. Glucagon as a therapeutic is problematic due to its propensity to aggregate and form amyloid fibrils. Its formation of aggregates and fibrils leads to a loss in activity and can lead to toxicity and immunogenicity. Herein we successfully synthesized eight glucagon analogues (Table 1) with the intention of improving both chemical and physical properties.

In a chemical stability assay, it was found that the use of D-isomer amino acids improved the solubility of the amidated native sequence, consistent with previously reported results. To prepare the peptide for 'corrination', we site selectively substituted ε-azidolysine at positions 17 and 30, sites acceptable to sequence modification and conjugation. These sequence modifications were shown to have improved solubility and chemical stability compared to that of Gcg (Table 2 and Fig. 2). The ε-azidolysine modified sequences (**2-4**) were subsequently conjugated to Cbi to produce three new constructs (**5-7**). The corrinated compounds were shown to have reduced solubility compared to that of their non-corrinated counterparts but had improved properties relative to that of native sequence (Table S2 and Fig. 2). All compounds with D-Arg (Gcg17, **4**, **7**) had the highest % concentration retained with Gcg17 leading. Gcg17 was statistically different from all analogues except **4** and **7**, which suggest the use of D-amino acids considerably contributed to the increased chemical stability in terms of solubility preserved.

While glucagon is limited by its solubility and chemical stability, it is further limited in action by its tendency to form amyloid fibrils. To investigate the physical properties of our modified Gcg constructs a thioflavin T (ThT) assay was performed to monitor the formation of fibrils. While the utilization of D-isomer amino acids and ε-azidolysine substitution in Gcg sequence (**2-4**) were shown to have a positive effect on chemical stability, these modifications had little to no effect on physical stability and aggregate formation. However, the corrination of Gcg (**5-7**) lead to near elimination of aggregate formation (Fig. 3).

While improving the physical and chemical properties of Gcg is of utmost importance; the modifications performed on the sequence must maintain function. In a real-time in vitro assay Gcg, **2**, and **5** were monitored for their functional activity. It was shown that the

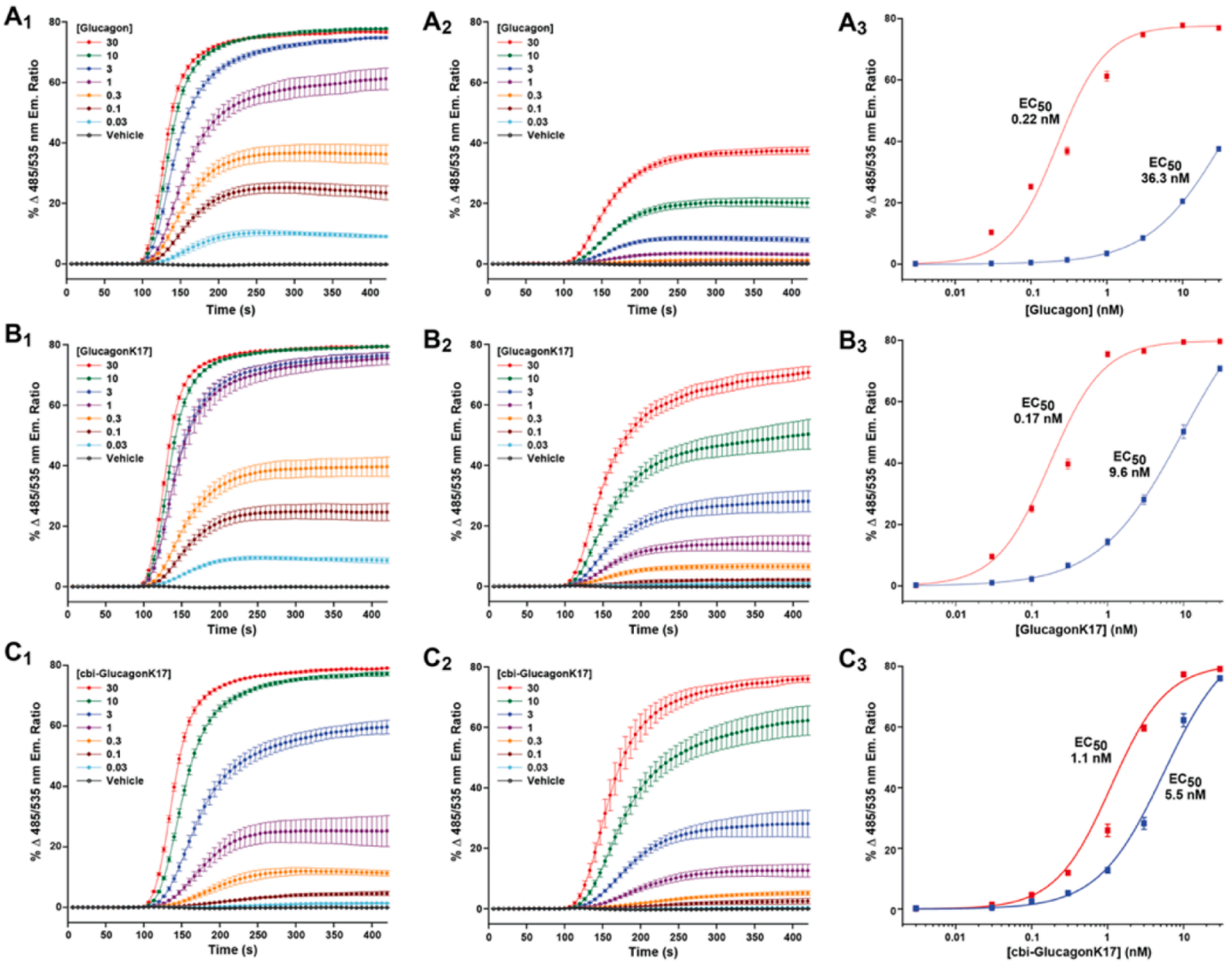


Fig. 4. Differential GcgR agonist properties of synthetic Gcg, **2**, or **5**, as revealed in FRET assays that monitor intracellular levels of cAMP. (A_1-A_3) The Gcg concentration-response relationship was determined under conditions in which 0.03 – 30 nM of the peptide was tested immediately after reconstitution in SES buffer (A_1), or after a 24-hr incubation of the reconstituted peptide in SES maintained at 37°C (A_2). Linear regression analysis (A_3) was used to determine EC_{50} values. (B_1-B_3) Same experimental design as in A_1-A_3 except that **2** was tested. (C_1-C_3) Same experimental design as in A_1-A_3 except that **5** was tested. For these live-cell FRET assays using HEK293-hGcgR-H188 clone 10 cells, the percent increase of the 485/535 nm FRET emission ratio (%ΔFRET) signifies increased levels of cAMP, as detected by the H188 biosensor. Standard error bars indicate the mean ± s.d. %ΔFRET measured in $N = 12$ wells of a 96-well plate. Summary shown in Table 2. See also general methods in the supplementary materials file.

Table 2
Summary of functional stability after 24-hr at 37°C as tracked by FRET assays in SES buffer measuring cAMP elevation upon agonism of the GcgR.

Peptide	Freshly prepared		24-hr @37°C		
	Max. Resp. 30 nM	EC_{50} (nM)	Max. Resp. 30 nM	EC_{50} (nM)	Fold Change
Gcg	Δ78 %	0.22	Δ38 %	36.3	165-fold
2	Δ79 %	0.17	Δ72 %	9.6	56-fold
5	Δ79 %	1.1	Δ77 %	5.5	5-fold

ε-azidolysine modified sequence **2** maintained equipotent activity in comparison to unmodified Gcg when both peptides were freshly solubilized. In contrast, corrination to yield **5** led to reduced potency, while retaining full efficacy as judged by the maximal response.

Following a 24-hour incubation, Gcg, **2** and **5** were assayed again to determine how chemical and physical stability affects function. Gcg was shown to have a 165-fold reduction in potency, while **2** had a 56-fold reduction in potency (Table 1). In contrast, **5** had a marginal 5-reduction in potency while retaining full efficacy (**Figure 4C$_3$**; Table 2).

5. Conclusion

Our data indicates that the corrination of Gcg not only improves solubility and mitigates aggregation, but it also preserves GcgR agonism after 24-hr incubation at 37°C to a higher degree than that of native Gcg. Thus, use of Cbi conjugation with unstable peptides in aqueous solution, such as Gcg and amylin, offers a compound with prolonged stability and agonism.

CRediT authorship contribution statement

Chepurny Oleg G: Data curation, Formal analysis, Methodology, Validation. **Cham Nancy:** Formal analysis, Investigation, Validation, Writing – original draft. **Doyle Robert:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Project administration,

Supervision, Writing – original draft. **Holz George G:** Methodology, Writing – review & editing. **Tinsley Ian C:** Conceptualization, Methodology. **Liles Amber:** Data curation, Investigation, Validation. **Opp Morgan L:** Data curation, Formal analysis, Investigation.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.peptides.2023.171134.

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