Stereochemical inversion as a route to improved biophysical properties of therapeutic peptides exemplified by glucagon

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Peptides and small proteins are attractive therapeutic candidates due to their inherent selectivity and limited off-target effects. Unfortunately, their potential is often hindered by unfavorable physicochemical properties. This is particularly true in the case of glucagon, a peptide indispensable in the treatment of life-threatening hypoglycemia. Glucagon displays extremely low solubility in physiological buffers and suffers chemical degradation when the pH is adjusted in either direction. Here we systematically examine site-specific stereochemical inversion as a means to enhance aqueous solubility and stability, yet not diminish bio-potency or pharmacodynamics. We report several analogs that maintain full biological activity with substantially increased aqueous solubility, and resistance to fibrillation. We conclude that d-amino acids offer an attractive option for biophysical optimization of therapeutic peptides.
Nature’s preference for proteinogenic L-amino acids and the molecular basis for what has been termed “the asymmetry of life” have been the subject of notable commentary, and much speculation. While uncommon, inverse stereochemistry has been observed in native sequences as a result of posttranslational modification. Single or multiple D-amino acid substitution can increase the duration of action by inhibiting proteolytic processing, while in other instances stereo-inversion lessened propensity to physical aggregation. Physical aggregation of a membrane-active peptide induced by beta-sheet forming intermediates was inhibited by a site-specific change in stereochemistry. The common determinant in the formation of higher-order physical aggregates was a propensity toward the formation of beta-sheet intermediates. However, the separate question of whether stereochemistry appreciably changes aqueous solubility has not been directly addressed. The presence of D-amino residues has been shown to decrease peptide hydrophobicity relative to the all-L-sequence based upon shorter reversed-phase high-performance liquid chromatography (RP-HPLC) retention times. The present report explores the question of whether stereochemical modification could be employed as a means to enhance aqueous solubility and biophysical stability using native glucagon as a model peptide.

Glucagon is a 29-amino acid pancreatic hormone whose primary physiological role is the mobilization of hepatic glucose through stimulation of glycogenolysis, and gluconeogenesis required for glucose homeostasis. Yet, despite a half-century of work, diabetic patients continue to rely on a primitive emergency treatment that consists of lyophilized glucagon requiring reconstitution in dilute hydrochloric acid prior to injection. This approach is necessitated by the inherently low aqueous solubility of glucagon in physiological solution, its strong propensity for fibrillation and chemical degradation. In pursuit of a more optimal medicinal alternative we have explored the refinement of the native structure. Herein, we report our results demonstrating that single-point stereochemical inversion can be employed to dramatically enhance the solubility of glucagon in physiological buffers, and significantly inhibit physical aggregation while maintaining near-native biological properties.

Results

Peptide synthesis. Glucagon analogs were prepared as their C-terminal acids by automated solid-phase synthesis and their analytical characterization is reported in Supplementary Table 1.

Glucagon analogs’ solubility at physiological conditions. The maximum aqueous solubility of D-amino acid modified glucagon analogs was evaluated in phosphate-buffered saline (PBS), pH 7.4 at 1 h (Table 1 and Fig. 1a). D-substitutions at the N- and C-terminal regions of the sequence (1–7 and 28–29) did not appreciably affect solubility in contrast to substitutions in the middle of the peptide, particularly at positions 11, 12, 17 and 28 which exhibited solubility of more than 3 mg/mL. The retention of maximal aqueous solubility in PBS was determined after incubation at 4 °C for 2 weeks (Supplementary Figure 1). There was a fractional loss in solubility with extended storage at refrigerated temperature. The reduction in maximal solubility was consistently across the full set of analogs, with certain exceptions that displayed greater response to low temperature. Importantly, a dozen of the analogs retained solubility that exceeds the therapeutically relevant concentration of 1 mg/mL, and far in excess of the native hormone.

Analysis of amyloidal aggregation. The study of the propensity of the glucagon analogs to form physical aggregates was performed in 0.1 N hydrochloric acid at a concentration of 1 mg/mL. The peptides were agitated with a stir bar at 37 °C for 4 h and the formation of the amyloid-like fibrils was assessed by Thioflavin-T (ThT) fluorescence. The intensity of the fluorescence for each peptide is presented in Fig. 1b. Modifications at the first 7 residues of the N terminus (analogos 1–7) had only a modest effect on fibrillation, while those at position 8, 12, 17 and 28 exhibited a 2–9-fold increase in ThT fluorescence relative to that of native glucagon. In contrast, stereochemical inversion at positions 9, 11, 14, 16, 19, 20, 21, 23 and 27 appeared to suppress fibrillation, as indicated by an absence of measurable ThT fluorescence.

In vitro bioactivity characterization. The in vitro potency of D-amino acid substituted analogs was evaluated in a cell-based assay utilizing engineered cells that overexpress the glucagon receptor.

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**Table 1 Solubility, bioactivity and percent helicity of glucagon analogs**

| Peptide | Amino acid | Solubility in PBS pH 7.4 (mg/mL) | EC50 (pM) | EC50 (NM) | % Helicity |
|---------|------------|---------------------------------|----------|-----------|------------|
| 1       | ω-His1     | 0.26 | 46.4 ± 5.6 | 26.3 ± 6.2 |
| 2       | ω-Ser2     | 0.24 | 16.7 ± 3.4 | 5.5 ± 1.7 |
| 3       | ω-Gln3     | 0.32 | 33.8 ± 8.9 | 39.7 ± 7.7 |
| 4       | G4(native) | 0.21 | 28.7 ± 9.1 | 11.4 ± 0.4 | 26.1 |
| 5       | ω-Thr5     | 0.18 | >1000       | >1000     |
| 6       | ω-Phe6     | 0.30 | >1000       | >1000     |
| 7       | ω-Thr7     | 0.47 | 136 ± 43    | >1000     |
| 8       | ω-Ser8     | 1.51 | >1000       | >1000     |
| 9       | ω-Asp9     | 0.26 | 280 ± 49    | 608 ± 142 |
| 10      | ω-Tyr10    | 1.22 | 35.6 ± 10.4 | 71.0 ± 28.0 |
| 11      | ω-Ser11    | 0.66 | 57.9 ± 14.9 | 27.2 ± 9.5 |
| 12      | ω-Lys12    | 1.76 | 34.7 ± 9.5  | 101 ± 47  | 19.4 |
| 13      | ω-Tyr13    | 1.68 | 216 ± 67    | 103 ± 14  | 20.2 |
| 14      | ω-Leu14    | 0.87 | 248 ± 92    | 7.0 ± 2.2 | 18.4 |
| 15      | ω-Asp15    | 1.53 | 78.7 ± 22.1 | 10.2 ± 4.1 | 17.5 |
| 16      | ω-Ser16    | 2.06 | 314 ± 10.3  | 4.9 ± 1.3 | 18.2 |
| 17      | ω-Arg17    | 4.52 | 20.3 ± 9.4  | 11.7 ± 3.1 | 23.5 |
| 18      | ω-Arg18    | 2.14 | 491 ± 9.9   | 0.5 ± 0.6 | 22 |
| 19      | ω-Ala19    | 2.01 | >1000       | >1000     |
| 20      | ω-Gln20    | 1.03 | 37.5 ± 12.4 | 8.6 ± 2.4 | 19.6 |
| 21      | ω-Asp21    | 1.88 | 26.6 ± 11.2 | 11.1 ± 2.8 | 17.6 |
| 22      | ω-Phe22    | 3.24 | 150 ± 70    | >1000     |
| 23      | ω-Val23    | 4.00 | 66.3 ± 26.9 | 10.5 ± 4.9 | 14.5 |
| 24      | ω-Gln24    | 1.73 | 26.6 ± 10.4 | 0.5 ± 0.2 |

EC50 of GLP-1 standard on GLP-IR = 0.0334 ± 0.013 (SD) nM
SD standard deviation.
(GCGR), as well in a similar assay with cells overexpressing the GLP-1 receptor (GLP-1R) (Table 1). Relative potency of the analogs at the glucagon and GLP-1 receptors are reported in Table 1 with standard deviations and presented as percent of native hormone (4) in Fig. 1c, and as dose-dependent stimulation of cyclic adenosine monophosphate (cAMP) in Supplementary Figure 2 and Supplementary Figure 3. D-amino acid substituted glucagon analogs (with exception of D-Tyr10 and D-Gln20) elicited a full agonist response which varied from 0.01 to 170% of native glucagon potency. The most significant drop in activity was observed for analogs 5–9, 13, 14, 19, 22 and 25–27. In contrast to our previously reported alanine scan40, the first three analogs tolerated the stereochemical inversion (8% for Ala1 vs. 60% for d-His1; 35% for Ala2 vs. 173% for d-Ser2 and 9% for Ala3 vs. 80% for d-Gln3). Loss of glucagon receptor activity for the analogs 5–9 is consistent with the activity loss observed in a comparable set of site-specific alanine mutants. Analogos 10–12 and 15–18 exhibited in vitro activity within 40% of that of native glucagon, while analogs 20, 21 and 28–29 were comparable to the native hormone (4). The substantial drop in activity of D-Ala19 is consistent with the previously observed low activity of glucagon hormone (219, 231 and 208% respectively). Bioactivity at the closely related GLP-1R was investigated given the homology of the two ligands, and the counter-regulatory effect of GLP-1 to lower blood glucose. Most analogs showed substantial decrease in potency at the GLP-1 receptor when compared to the native hormone 4, which serves to maintain a large margin for selective glucagon agonism (Table 1, Fig. 1c and Supplementary Figure 3). Analogos with high glucagon receptor activity (>60% of native hormone) which includes analogs 1–3, 10, 12, 16, 17, 20, 21 and 24 stimulated the GLP-1 receptor at 20% or less than that of native hormone, 4. Interestingly, analogs 18, 28 and 29 exhibited reduced selectivity. These analogs while full agonists of high potency at the glucagon receptor were proportionally twice as active at the GLP-1 receptor in comparison to native hormone (219, 231 and 208% respectively).

Helicity of the peptide analyzed by circular dichroism. The effect of n-amino acid substitution in the central portion of the glucagon sequence (residues 12–23) was assessed by circular dichroism (CD) in 50 mM Tris buffer pH 7.4, with 20% TFE (Supplementary Figure 4). The fraction of helicity (f) calculated upon the mean residual ellipticity at λ = 222 nm indicated that under these conditions native glucagon (4) possesses ~26% helical structure (Table 1)47. Introduction of D-residues decreased helicity in a relative sense by 25–33%, to an absolute range of

![Fig. 1](https://example.com/fig1.png)  
**Fig. 1** Summary of physical and in vitro biological properties of the glucagon analogs.  
**a** Solubility in PBS of the glucagon analogs with single D-amino acid substitution with standard deviation.  
**b** Amyloid-like fibril formation in 0.1 N HCl as measured by Thioflavin-T fluorescence with standard deviation.  
**c** Percent in vitro potency of glucagon analogs relative to native hormone at the glucagon (red) and GLP-1 (green) receptors as determined by comparative EC50 values, and standard deviations reported in Table 1.
Glucagon analogs 17 and 18 retained native helical content, while analog 23 exhibited nearly a 50% decrease to <15% fractional helicity.

**Chemical stability.** Select glucagon analogs (16, 20, 21 and 23) as determined by the most optimal physical and biological properties (Fig. 1) were subjected to a prolonged stability study in 50 mM sodium phosphate buffer at pH 7. Chemical stability was assessed after 8 and 16 days of incubation at 37 °C and 95% humidity, by comparative liquid chromatography–mass spectrometry (LC-MS). Samples were prepared at 0.5 mg/mL and stability was evaluated by change in concentration (ultraviolet (UV) absorbance after centrifugation). Except for peptide 23, each analog displayed robust stability in each method of assessment. No change in analytical chromatography peak intensity, total area of the main peak or change in peak mass ion was observed for 16 and 20 (Fig. 2, Supplementary Figure 5, 6 and 7). Peptide 23 displayed reduced aqueous solubility when assessed after 8 days without change in chemical integrity as determined by LC-MS. The UV concentration measurement indicated a lower than intended 0.5 mg/mL concentration of native glucagon 4, which reflects the limits of native hormone solubility at room temperature. All analogs studied, including the native hormone, maintained biological activity after 16 days of incubation at 37 °C.

**In vivo rat study.** Blood glucose elevation was measured in lean male Sprague-Dawley rats. Native glucagon 4 and analogs 16, 20 and 21 were subcutaneously administered at 30 nmol/kg dose and blood glucose was monitored over the subsequent 2 h via tail vein laceration (Fig. 3 and Supplementary Figure 8). Administration of each peptide resulted in a statistically significant increase in blood glucose relative to the vehicle control. The pharmacodynamic response was characteristic of native glucagon with an initial rapid increase in glucose peaking after 20–30 min before returning to initial level within 2 h of injection. Peptides 16, 20 and 21 demonstrated a comparable level of in vivo performance relative to the native hormone 4. It is also conceivable that analogs of lesser in vitro potency might equally perform in vivo, but this was not assessed. The time action and magnitude in glucose elevation of analogs 16, 20 and 21 proved equal to glucagon when tested at lower administered doses.

**Solubility of analogs of inverted stereochemistry.** To further explore the specificity in the inverted stereochemistry we prepared a glucagon of full d-amino acid sequence (30), and two analogs where just one of the amino acids were of L-stereochemistry; l-Arg17 (31) and l-Val23 (32). These peptides as expected were without biological effect, but the primary purpose was to explore whether the improved biophysical properties could be inversely introduced by a comparable single inversion in

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**Fig. 2** Assessment of the chemical stability. LC-MS and in vitro bioactivity assay assessment of chemical stability of selected glucagon dS16 (16) and dQ21 (21) analogs incubated in 50 mM sodium phosphate, pH 7, at 37 °C (0.5 mg/mL) (T = 0 is blue, T = 8 days is red, T = 16 days is green)
stereochemistry. As shown in Fig. 4, the peptide of full d-amino acid sequence (30) was of comparably low aqueous solubility to native glucagon (4) (solubility for 30 was 0.22 mg/mL). The single L-amino acid substitution within peptide 30 at residues 17 (31) or 23 (32) resulted in an enhanced solubility that mirrored the effect of inverse change in stereochemistry at these two residues in native hormone (solubility of 5.28 mg/mL for analog 31 and 4.18 mg/mL for analog 32).

**Structural basis for improved solubility.** The pursuit of a non-aggregating glucagon analog with increased solubility in physiological buffers with near-native in vivo pharmacodynamics has emerged in the last decade as a priority for achieving enhanced safety in treatment of insulin-dependent diabetes. The optimization of physiological hormones for medicinal purposes is best accomplished with minimal change to the native sequence. This lessens the chance for off-target adverse events, particularly development of a neutralizing immunological response. Specific to glucagon, such a development could constitute a life-threatening limitation to continued insulin therapy. The reversal of stereochemistry represents a subtle modification to native sequence and the approach has been commonly used to enhance proteolytic stability. In this study we report that the site-specific introduction of d-amino acids can significantly change physical and biological properties of glucagon. It remains to be determined in human testing whether such change is devoid of off-target adverse effects. This would include assessment of immunogenicity which is difficult to test in a preclinical setting, but we have observed no change in rodent pharmacology with repeat dose administration.

Without precedence in peptide structure–activity studies, we observe that d-amino acids when individually introduced at various positions substantially increased glucagon solubility in physiological buffer (Fig. 1a). More than half of the analogs achieved aqueous solubility that exceeded the medicinal target of 1 mg/mL, and one-quarter of them exceeded 2 mg/mL. As previously reported by Chen et al., incorporation of d-amino acids in L-amino acid composed peptides decreased their apparent hydrophobicity, as measured by reverse-phase high-performance chromatography (capacity factor, k'). (Supplementary Table 1). While increased hydrophilicity generally associate with enhanced solubility, we observed no definitive correlation between k' and solubility for these glucagon analogs (Supplementary Figure 9a). Similarly, no correlation of hydrophilicity was detected with propensity to aggregation (Supplementary Figure 9b), or amyloid-like fibrillation with solubility (Supplementary Figure 10). The analogs with substitutions at positions 9, 11, 16, 19, 20, 21 and 23 demonstrate substantially decreased amyloid-like fibril formation, while in contrast stereochemical inversion at positions 8, 12, 17 and 28 appears to have opposite effect by inducing fibrillation as indicated by increased Thioflavin-T fluorescence (Fig. 1b). The in vitro bioactivity of the d-amino acid analogs varies with the position and nearly half of them are greater than 50% relative to human glucagon (Fig. 1c), and several are comparable or even enhanced (2, 17, 24, 29). There are at last three analogs (16, 20 and 21) that achieved the stated objective of enhanced aqueous solubility and resistance to fibrillation, while maintaining appreciable in vitro potency. When assessed for in vivo performance, all of these analogs demonstrated a native hormone-like increase in blood glucose, as assessed by the time to onset, the magnitude of the glucose response and the speed to disappearance of the effect (Fig. 3).

The structural basis for the improved physical properties was investigated by CD. There is no apparent linear correlation between the degree of helicity in the analog set and aqueous solubility, bioactivity or propensity to fibrillation (Fig. 5 and Supplementary Figure 11). The dramatic increase in solubility of certain analogs in the purported central helix, such as peptides 17, 22 and 23, is associated with less fractional helicity than the native hormone (Fig. 5a). Additionally, the same three peptides vary in potency, where two (22 and 23) are less than one-half of glucagon (4) and one is enhanced (17) (Fig. 5b). In contrast, a
peptide such as 23 displays approximately one-half the helicity and potency of native hormone. The measure of peptide helicity does not provide sufficient explanation for molecular and structural basis of aqueous solubility or in vitro bioactivity.

Recently, Zhang et al.\textsuperscript{53} reported the structure of a glucagon analog bound to its receptor. It presents helical structure as assessed by size-exclusion chromatography (SEC)–analog bound to its receptor. It shows that substitution with single D-amino acids can dramatically increase solubility at physiological conditions, and in several instances with high biological activity. It seems plausible that maintaining monomeric character at increased concentration would be the basis for the enhanced aqueous solubility. This proved difficult to confirm experimentally as we did not observe by any physical measure the appearance of higher molecular aggregates in the native hormone as peptide concentration was increased. Specifically, the native hormone (4) and select analogs (16 and 17), as assessed by size-exclusion chromatography (SEC)-HPLC (2–3 mg/mL at pH 3), exhibit comparable chromatographic profile (Supplementary Figure 12). We remain committed to determining the molecular basis for the improved aqueous solubility, but until the mechanism is discovered the pragmatic virtue justifies a broader application of D-substitutions in peptide optimization.

**Discussion**

The ability to achieve dramatic improvement in peptide physical properties with only a subtle change in primary structure has important medicinal ramifications. Stereochemical inversion is a well-recognized tool in therapeutic peptide optimization for resisting proteolysis, and to a lesser extent diminishing physical aggregation. We report the additional virtue of enhanced aqueous solubility in concert with enhanced physical character, coupled with high in vitro and in vivo potency. These specific glucagon analogs or the integration of such site-specific stereochemical inversion with other subtle changes in structure could constitute an optimal candidate for the treatment of hypoglycemia.

**Methods**

**Peptide synthesis.** Peptides were prepared by Fmoc/tBu solid-phase methodology on a Symphony automated peptide synthesizer (Gyros Protein Technology, Tustin, CA) starting with pre-loaded Fmoc-Thr(Bu)-Wang resin (Apepta, Louisville, KY) and employing repetitive DIC/6-Cl-HOBt activation. The side chain protecting group scheme consisted of Arg(Pbf); Asp(OtBu); Asn(Trt); Gln(Trt); His (Trt); Lys(Boc); Ser(tBu); Thr(Bu); Tyr(tBu); and Trp(Boc). Both L- and D-amino acids were purchased from Aapptec (Louisville, KY) or Peptide International (Louisville, KY). DIC from Fmoc-Lys(COH) or HOBt from 4-carboxy-2-nitrobenzoic acid were cleaved from the resin and deprotected by treatment with trifluoroacetic acid (TFA) containing 2.5% triisopropylsilane (TIS), 2.5% water, 2.5% phenol, 0.5% TFA, 90% CH\textsubscript{3}CN, 10% water (B) as elution buffers. Purified peptides were analyzed and characterized by LC-MS (1200 Infinity-6120 Quadrupole LC-MS, Agilent) on a Kinex C8 (3.5 μm, Phenomenex) column with 0.05% TFA/H\textsubscript{2}O (A) and 0.05% TFA, 90% CH\textsubscript{3}CN, 10% water (B) as elution buffers. Peptide concentration was assessed based on UV absorbance at λ = 280 nm measured on a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). Extinction coefficient at λ = 280 nm was calculated using on-line Peptide Property Calculator (Innovagen, PepCalc.com).

**Solubility.** To 1.5 (± 0.5) mg of peptide in an Eppendorf tube, 200–800 μL of PBS buffer pH 7.4 at room temperature was added. The volume of the buffer was kept below the minimum necessary to fully dissolve the peptides. Solutions remained cloudy with undissolved material until samples were vortexed and sonicated for 5 min. After equilibration at room temperature for 1 h, they were centrifuged at 10,000 rpm for 10 min. The concentration of the solubilized peptide in the supernatant was assessed based on UV absorbance at λ = 280 nm, and the calculated extinction coefficient. UV absorbance was measured three times per sample. Maximum solubility was confirmed by the presence of undissolved peptide pellet in all tubes. Undisturbed aliquots were then equilibrated at 4 °C for 48 h and 2 weeks. Concentration was evaluated after equilibrating samples once more to room temperature for 1 h, followed by centrifugation as described above. Data presented represent the average and standard deviation as determined in a minimum of two separate experiments, and a minimum six individual concentration checks.

**Aggregation.** Approximately 1 mg of each peptide was dissolved in 250 μL of 0.1 N hydrochloric acid. Solutions were vortexed and sonicated for 3–5 min, and then filtered through a 0.22 μm syringe filter. UV absorbance was measured at λ = 280 nm and the concentration was calculated as described above. All samples were diluted to equal concentration of 1 mg/mL with 0.1 N HCl and incubated at 37 °C for 48 h, with agitation by magnetic stir bar at 500 rpm. Fibrillation was measured according to a modified Thioflavin-T fluorescence assay protocol (http://www.assay-protocol.com/biochemistry/protein-fibrils/thioflavin-t-spectroscopic-assay.html). Then, 8 mg of ThT was dissolved in 10 mL of PBS (pH 7.4). The solution was filtered through a 0.22 μm syringe filter and stored at 4 °C in the dark. Prior to the experiment, 300 μL of ThT stock solution was diluted in 15 mL of the phosphate buffer. Then, 30 μL of investigated peptide solution at a concentration of 1 mg/mL was added to 300 μL of working solution of ThT in PBS. The solution was incubated for 5–10 min. The fluorescence intensity was measured on a Perkin-Elmer LS50B Luminescence Spectrometer (Perkin-Elmer, Waltham, MA) using the following experimental parameters: 300 μL of peptide–ThT solution in sub-micro quartz cuvette (path length = 10 mm; size 12.5 mm × 12.5 mm × 4 mm; window 2 mm × 8 mm; Z = 15 mm) (Sterna Cells, Atascadero, CA); excitation λ = 450 nm (slitwidth = 5 nm); emission λ = 482 nm ( slitwidth for 10 nm) and integration time 60 s, accumulated for 3 min. Fluorescence of sample was assayed in triplicate, and the results reported as an average with standard deviation. Freshly prepared insulin (1 mg/mL) was used as a standard for non-
aggregated sample, while forcefully fibrillated insulin (1 mg/mL) was used as standard for samples containing amyloid-like fibrils (data not shown).

**Glucagon and GLP-1 receptor-mediated cAMP assay.** The glucagon (or GLP-1)-induced cAMP production was measured in HEK293 cells overexpressing the glucagon (or GLP-1)-receptor with a luciferase reporter gene linked to a cAMP-responsive element. The cells were serum deprived for 16 h and then incubated with serial dilutions of glucagon for 5 h at 37 °C, 5% CO2 in 96 well polystyrene-coated “Bioscat” plates (BD Biosciences, San Jose, CA). Samples were prepared in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% bovine serum albumin (from Roche). At the end of the incubation period, 50 µL SteadyLight plus luminescence substrate reagent (Perkin-Elmer, Waltham, MA) was added to each well. The plate was briefly shaken, incubated for 10 min in the dark and light output was measured on a Beckman DTX-880 multimode detector (Beckman Coulter, Brea, CA). Effective 50% concentrations (EC50) were calculated using Origin software. EC50 value and standard deviation presented from minimum three independent experiments.

**Circular dichroism.** CD spectra were measured at 25 °C using a J-715 spectro-polarimeter (Jasco, Tokyo, Japan) in a quartz cell of 10 mm path length. Spectra were the average of 10 assessments over the λ = 190–370 nm range at 0.1 nm interval. Samples (0.25 mg/mL) were prepared in 20% TFE, 50 mM Tris buffer, pH 7.4, and the exact concentration was assessed with UV absorbance at λ = 280 nm, as described above. The results are presented as the mean residue molar ellipticity (θ) in deg cm²/dmol. The fraction of helicity (f) was calculated according to the Rohl and Baldwin.47

**Stability at pH 7.0 and 37 °C.** A total of 3.5 mg of lyophilized peptide was dissolved in 400 µL of 0.1 N HCl. Solutions were vortexed and sonicated for 3–5 min, then filtered through a 0.22 µm syringe filter. UV absorbance was measured at λ = 280 nm and concentration was calculated as described above. Aliquots were prepared by adding the appropriate amount of peptide solution in HCl and additional 0.1 N HCl to 68 µL total volume of acid into 532 µL volume of sodium phosphate with 150 mM sodium chloride buffer at pH 7.5, all resulting in 600 µL of 0.5 mg/mL solution at pH 7.0 ± 0.1. Tightly closed Eppendorf tubes were secured with parafilm film cover and kept in a tissue culture sterile incubator with ~95% humidity. At the time of start, and 8 and 16 days thereafter, 50 µL of aliquots were withdrawn and stored at 80 °C. An additional 100 µL of each sample was kept for LC-MS analysis. The sample was prepared in a 1:1 ratio of sample solution and 1% TFA in water. All samples were stored at –80 °C until simultaneously analyzed. LC-MS analysis was conducted as described above on a Konikochi C8 column with 5% B to 70% B in a 15 min gradient and 2.5 min post-injection isocratic delay (as described above). Two injections per sample were performed. Aliquots from different time points (10 mM solutions in DMEM) were prepared assuming constant 0.5 mg/mL concentration of each peptide during duration of experiment. Samples were bioanalyzed as previously described in cells overexpressing the glucagon receptor.

Remaining solution of the incubated sample was centrifuged at 10,000 rpm for 10 min. Remaining concentration of peptide in the supernatant was assessed by taking UV absorbance, as described above. The in vitro bioassay was repeated and the EC50 obtained for incubation samples was calculated based on peptide concentration in solution, as obtained from UV measurement of the centrifuged samples.

**Rat in vivo studies.** All studies were approved and performed according to the guidelines of the Institutional Animal Care and Use Committee of the University of Cincinnati. Lean Sprague-Dawley rats (Harlan, IN; body weight 323 ± 19 g) were housed in a 12:12 h light–dark cycle (8 am to 8 pm lights on) at 22 °C and constant humidity with free access to standard chow (Teklad LM-485) and water, except as noted. The food was removed at the onset of the light phase, 3 h prior to the administration of the compounds. All peptide analogs were formulated in 50 mM Tris buffer pH 8 (vehicle) at 50 mM concentration and administered by subcutaneous injection at 30 nmol/kg dose. The blood glucose level was measured at the intervals indicated using a handheld glucometer (Freestyle, Abbott). The statistical analysis of the results obtained in the in vivo experiments was performed using Prism 6.0h (GraphPad Software, CA) applying one-way analysis of variance (ANOVA) followed by Dunnett’s test, using the vehicle group as control.

**Data availability**

The authors declare that all data supporting the findings of this study are available within the article and its Supplementary Information files or from the corresponding authors upon reasonable request.

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Author contributions
P.A.M. designed, performed peptide synthesis, analytical characterization and other experimental work presented herein except in vivo studies. D.P.-T. performed and analyzed in vivo experiments. R.D.D. supervised all studies. P.A.M., J.P.M. and R.D.D. co-wrote the manuscript with select contributions from D.P.-T.

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