Original article

MOLECULAR METABOLISM

A glucagon analog chemically stabilized for immediate treatment of life-threatening hypoglycemia*

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ABSTRACT

For more than half a century glucagon has been used as a critical care medicine in the treatment of life-threatening hypoglycemia. It is commercially supplied as a lyophilized powder intended to be solubilized in dilute aqueous hydrochloric acid immediately prior to administration. We have envisioned a "ready-to-use" glucagon as a drug of more immediate and likely use. Through a series of iterative changes in the native sequence we have identified glucagon analogs of appreciably enhanced aqueous solubility at physiological pH, and of chemical stability suitable for routine medicinal use. The superior biophysical properties were achieved in part through adjustment of the isoelectric point by use of a C-terminal Asp-Glu dipeptide. The native glutamines at positions 3, 20 and 24 as well as the methionine at 27 were substituted with amino acids of enhanced chemical stability, as directed by a full alanine scan of the native hormone. Of utmost additional importance was the dramatically enhanced stability of the peptide when Ser16 was substituted with alpha,aminoisobutyric acid (Aib), a substitution that stabilizes peptide secondary structure. The collective set of changes yield glucagon analogs of comparable *in vitro* and *in vivo* biological character to native hormone but with biophysical properties much more suitable for clinical use.

Keywords Glucagon; Hypoglycemia; Insulin-dependent diabetes; Peptide synthesis

1. INTRODUCTION

Glucagon is a twenty-nine amino acid peptide hormone secreted by pancreatic α-cells in response to low blood glucose levels [1]. Glucagon's immediate physiological action at the liver stimulates glycogenolysis and gluconeogenesis while inhibiting glycogen synthesis to rapidly elevate glucose [2]. Insulin-dependent diabetes is a well-recognized risk factor for severe hypoglycemia due to the inherently low therapeutic index of insulin. Excessive action can lead to severe hypoglycemia where blood glucose concentration can fall below 50 mg/dl, and where cognitive capacity is impaired. This represents a particular challenge to insulin-dependent patients as the normal glucagon physiological response is often absent, or sizably diminished [3,4]. This is the central limiting factor to achievement of ideal glycemic control in conventional insulin therapy [5,6] where the avoidance of acute life-threatening hypoglycemia places a patient at increased risk for development of microvascular complications [6].

The standard treatment of a severe insulin-induced hypoglycemic episode is acute subcutaneous administration of glucagon. A common commercial glucagon kit contains a single milligram of lyophilized glucagon powder, along with a one milliliter syringe of dilute hydrochloric acid diluent. The hormone is solubilized in the supplied vial and transferred to the syringe for immediate administration. Dilute hydrochloric acid is employed since glucagon is only sparingly soluble in buffers at physiological pH [7]. Once solubilized, the peptide needs to be immediately used or discarded due to chemical and biophysical instability in dilute acid [8–12]. Previously, we reported two different chemical means of enhancing chemical stability by increasing the aqueous solubility at physiological pH values [13]. Addition of a C-terminal exendin-4 derived sequence [14–16] and substitution of Asn28 with Asp resulted in greatly improved mid-pH solubility as well as enhanced stability relative to native hormone when the latter was formulated in the commercial acidic diluent.

In this report we continue our optimization of the glucagon sequence for the purpose of identifying peptides still more suitable for clinical study. The central challenge is the identification of an aqueous formulation of a specific glucagon analog that achieves sufficient chemical stability while maintaining the native hormone's biopotency and time action when administered repeatedly over an extended period of time. Previously identified analogs proved of insufficient stability in extended study at

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Received December 19, 2013 • Revision received January 6, 2014 • Accepted January 7, 2014 • Available online 22 January 2014

http://dx.doi.org/10.1016/j.molmet.2014.01.006

ambient temperature and prompted us to employ additional modifications to further stabilize the chemical and biophysical properties of the hormone. Of particular importance was the discovery that placement of Aib at position 16 enhanced the biophysical stability of the peptide, rendering it of much superior quality when assessed in six and twelve month aqueous stability studies.

2. MATERIALS AND METHODS

2.1. Peptide synthesis/cleavage/extraction

Glucagon and glucagon analogs with anionic substitutions were synthesized using 0.2 mmol of 4-hydroxymethyl-phenylacetamidomethyl (PAM) resin (Midwest Biotech, Fishers, Indiana) on a modified Applied Biosystems 430A peptide synthesizer (Foster City, CA). The solid phase peptide synthesis protocol utilized *in situ* neutralization for Boc-chemistry as described by Kent [17]. Aib substituted analogs required extended time couplings of four hours prior to and following this residue. Completed peptidyl-resins were treated with HF/*p*-cresol (10:0.5 v/v) at 0 °C for 1 h. The HF was removed in vacuo and the deprotected peptide was precipitated and washed in diethyl ether. The peptide was dissolved in 20% acetonitrile/1% acetic acid and lyophilized.

2.2. *In vivo* pharmacodynamics of glucagon analogs

The *in vivo* experiments were conducted at a certified Contract Research Laboratory named Seventh Wave Laboratories, LLC in Chesterfield, Missouri. This non-clinical laboratory study was exploratory in nature and was conducted in accordance with the principles set forth in the United States Food and Drug Administration (FDA) Good Laboratory Practice (GLP) Regulations, 21 Code of Federal Regulations (CFR) Part 58.

The dogs were individually housed in a setting where the room temperature was maintained in a range of 72 ± 4 °F and relative humidity of 30–70%. There was a 12-h light, 12-h dark cycle employed. Water was provided by the city of St. Louis and meets human drinking standards. The diet was certified Purina Canine Chow.

8–12 kg healthy Canine/Beagle dogs, 8–16 months of age were used to determine the pharmacokinetics and pharmacodynamics properties of the glucagon analogs. The peptides were dissolved in 0.01 N HCl at a concentration of 0.1667 mg/ml and the animals were dosed at 0.03 ml/kg. The animals were administered a 1.5, 5, or 15 ug/kg dose intramuscularly of either glucagon or Asp28. All animals were fasted overnight and bled at the following time points following each dose: 0 h. (pre-dose), 5, 10, 20, 30, 45, 60, 90, 120, 240 min post-dose. Six animals were used for each dose group and approximately 1.0 mL whole blood was placed in K_2EDTA tubes containing a sufficient volume of Trasylol (aprotinin) to yield at least 500 KIU/mL of whole blood. Approximately, 500 uL plasma was collected by centrifuging at 3000*g* for 15 min, at 4 °C. Aliquots of plasma were stored in sealed plastic vials at −70 °C. The remaining whole blood was converted to serum by placing the blood in an empty tube and letting it sit at ambient temperature for 20 min followed by centrifuging at 3000*g* for 15 min, at 4 °C. Aliquots of serum were stored in sealed plastic vials at −70 °C. The glucose responses to glucagon and IUB76 were evaluated in accordance with the collection schedule and procedures listed below. All animals were bled prior to each dose and at nine time points relative to dosing on Days 1 and 5. On Days 2, 3, and 4 all animals were bled at the same time as the first dose was administered on Day 1. The time of dosing and the actual time of each bleed were recorded in the raw data for each animal. Blood samples for glucose measurements were collected at $t=0$ (before dosing) and 5, 10, 20, 30, 45, 60, 90, 120, and 240 min after dosing and measured using an Abbott Freestyle Lite glucometer. Statistical analysis was performed on T_{max}, and all glucodynamic values as observed and after log-transformation using a one tailed Student's *t* test.

2.3. Solubility of glucagon and Asp28 in SDS vs. Tween 20

Glucagon and Asp28 were dissolved at a concentration of 1 mg/ml in 50 mM aqueous triethanolamine (TEA). The stock solution of each formulation was equally divided and the pH was adjusted to 8.5 or 7.0 for either half. The surfactants SDS and Tween-20 were individually added to 1 ml samples of the peptide solution at concentrations of either 0.1% or 0.01% and evaluated in comparison to a control solution with no additive. Solubility of the samples was measured via UV absorbance at 280 nm after 24 h at room temperature.

2.4. Asp28 excipient screen

Asp28 was dissolved in 20 mM tris buffer at pH 8.0 at a concentration of 1.5 mg/ml. Excipients were added to the formulation as follows: buffer alone, 0.1% Tween 80, 0.1% SDS, 0.1% HSA, 5% Sucrose, 5% PPG, 5% PEG, and 0.1% F68. Samples were divided in agitated and non-agitated conditions and incubated at either 4 °C (data not shown), ambient temperature, or 37 °C for the duration of the study. Analysis was performed via RP-HPLC and SEC-HPLC at select time points. The RP-HPLC assay was performed on a Phenomenex Luna C18 analytical column (4.6×250 mm^2) (Torrance, CA) employing a linear gradient from 30% to 53% B (A = 0.025 M NH_4HCO_3, pH 8.0, B = 90% ACN aq) over 15 min. The SEC assay was performed on a GE Healthcare Superdex 75 10/300 GL column (10×300 mm^2) employing a 25 mM ammonium bicarbonate mobile phase over 75 min.

2.5. Long-term stability study of glucagon analogs

Peptide concentrations of one milligram per milliliter were prepared in 20 mM Tris buffer with 0.1% sodium dodecyl sulfate at pH 8.6. These solutions were divided into 250 μl aliquots in cartridges representative of clinical use. The samples were then divided and the aliquots were incubated at 4, 25, 30 and 40 °C temperatures. In addition, the 25 °C samples were further divided into agitated and non-agitated groups for the duration of the study. Tests and assays included visual observation for turbidity, particulates, or discoloration, as well as alkaline RP-HPLC and SEC chromatography. The RP-HPLC assay was performed on a Phenomenex Luna C18 analytical column (4.6×250 mm^2) (Torrance, CA) employing a linear gradient from 30% to 53% B (A = 0.025 M NH_4HCO_3, pH 8.0, B = 90% ACN aq) over 15 min. The SEC assay was performed on a GE Healthcare Superdex 75 10/300 GL column (10×300 mm^2) employing a 25 mM ammonium bicarbonate mobile phase over 75 min.

2.6. Measure of maximum solubility

Saturated solutions of the Asp28 and Asp28,Glu29 analogs were prepared in 10 mM Tris base and the pH of each solution was adjusted to 7.4. Solutions of each peptide were stored at 4 °C and ambient temperature for forty eight hours. After forty eight hours the pH of each solution was measured again. Serial dilutions were made until the point where no visible precipitate could be visualized and a linear correlation of UV absorbance was observed. The peptide concentration was determined using the calculated extinction coefficient at 280 nm for glucagon of 8480/M × cm Abs 0.1% (=1 g/L) 2.435.

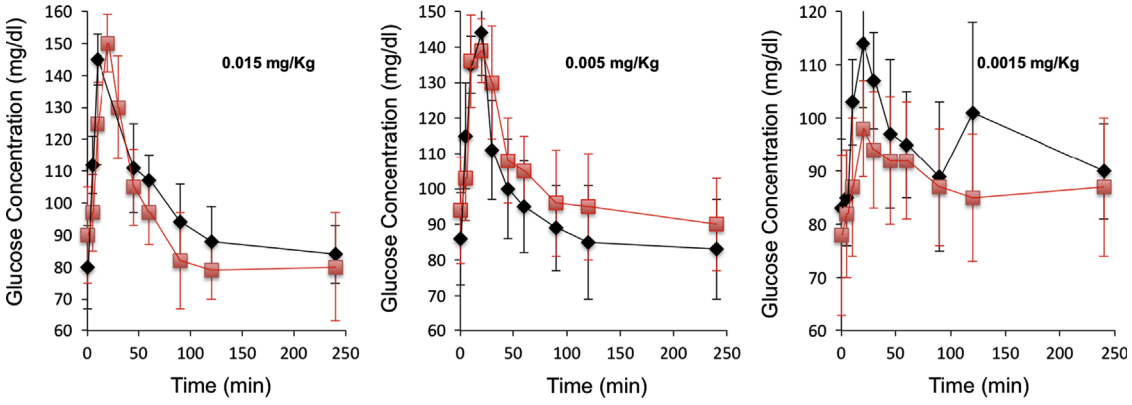

Figure 1: Comparison of pharmacodynamic response of glucagon (black) and Asp28 (red) in fasted beagle dogs ($n=6$) after single IM injection.

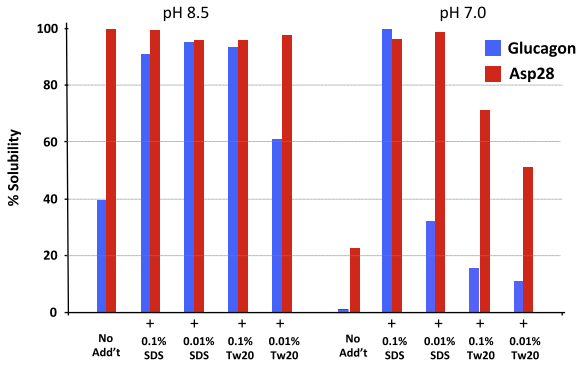

Figure 2: Percent solubility of glucagon peptides in 50 mM triethanolamine with SDS or Tween at 25 °C, 24 h and 1 mg/ml. Native glucagon (blue) and Asp28 (red). The reported observations represent single point experimental results and as such there is no standard deviation.

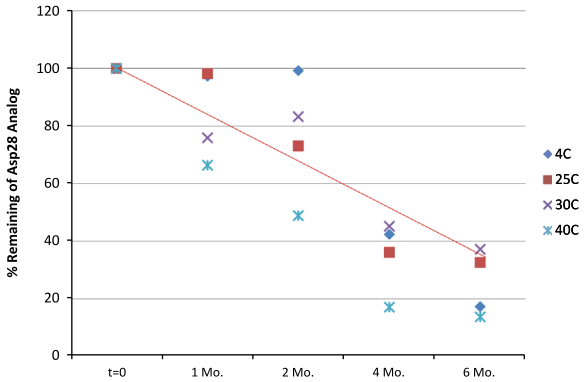

Figure 3: Stability of Asp28 as a percent of the $t=0$ starting concentration. Analysis of 4, 25, 30, and 40 °C samples illustrates disappearance with extended duration at elevated temperature. The reported observations represent single point experimental results and as such there is no standard deviation.

2.7. Environmental stress study of Aib substituted analogs

Each peptide was dissolved in 0.01 N HCl at a concentration of 1.0 mg/ml in low retention Eppendorf vials. An aliquot of the solution was frozen and stored to serve as an initial time-point standard. The vials were placed on a Roto Mix apparatus to agitate the samples at 200 rpm. The samples were shaken for seven weeks with no visible aggregate was observed in any sample. After seven weeks the samples from the initial standards and the agitated peptide solutions were analyzed via RP-HPLC and MALDI-tof MS. The RP-HPLC assay was performed on a Phenomenex Luna C18 analytical column (4.6×250 mm^2) (Torrance, CA) employing a linear gradient from 30 to –53% B (A = 0.025 M NH_4HCO_3, pH 8.0, B = 90% ACN aq) over 15 min.

2.8. Glucagon receptor-mediated cAMP synthesis

Each peptide analog was tested for its ability to stimulate cAMP production through the glucagon receptor. HEK293 cells were co-transfected with the GCGR cDNAs and a luciferase reporter gene-linked to a cAMP response element (CRE). Cells were serum deprived for 16 h by culturing in DMEM (Invitrogen, Carlsbad, CA) supplemented with 0.25% Bovine Growth Serum (HyClone, Logan, UT). Serial dilutions of Glucagon analogs were added to 96-well poly-D-Lysine-coated plates (BD Biosciences, San Jose, CA) containing co-transfected HEK293 cells, and plates were incubated for 5 h at 37 °C, 5% CO_2. Following incubation, an equivalent volume (100 µl) of LucLite luminescence substrate reagent (Perkin-Elmer, Wellesley, MA) was added to each well and the plate was shaken for 3 min at 800 rpm. The plate was incubated for 10 min in the dark and light output was quantified on a MicroBeta-1450 liquid scintillation counter (Perkin-Elmer, Wellesley, MA). Effective 50% concentrations (EC_{50}) were calculated by Origin software (OriginLab, Northampton, MA).

3. RESULTS

3.1. Formulation additives enhance analog solubility

Our interest in the Asp28 analog was driven by its much improved aqueous solubility relative to native glucagon in the pH range of 7–8.5 [13,18] and the present study extends the exploration of Asp28 glucagon characteristics. We had previously reported the preserved *in vitro* potency and selectivity of this analog relative to native hormone. The question of *in vivo* pharmacology is addressed in Figure 1 where equivalent dose titrations of glucagon and the Asp28 analog demonstrated comparable time action and slightly lower potency. The observations are of special significance since any analog must achieve equal or faster onset of glucose elevation given its intended role as an emergency rescue medicine. As shown in Figure 2 there is a fivefold enhancement in solubility at pH 7 of Asp28 relative to native hormone. A further increase in concentration to 1 mg/ml (he minimum

5 10 15 20 25
HSQGTFTSDYSKYLDSRRAQDFVQWLMNT

Peptide	Glucagon Receptor EC50: (nM)	Relative Potency %
Glucagon	0.15	100
His-1	1.94	7.7
Ser-2	0.43	34.9
Gln-3	1.59	9.4
Gly-4	2.23	6.7
Thr-5	1.08	13.9
Phe-6	6.95	2.2
Thr-7	0.88	17.0
Ser-8	2.67	5.6
Asp-9	19.7	0.8
Tyr-10	13.4	1.1
Ser-11	1.14	13.2
Lys-12	2.93	5.1
Tyr-13	0.47	31.9
Leu-14	2.67	5.6

Peptide	Glucagon Receptor EC50: (nM)	Relative Potency %
Glucagon	0.15	100
Asp-15	8.89	1.7
Ser-16	0.17	88.2
Arg-17	0.46	32.6
Arg-18	0.21	71.4
Gln-20	0.22	68.2
Asp-21	0.26	57.7
Phe-22	93.4	0.2
Val-23	13.8	1.1
Gln-24	0.15	100
Trp-25	2.15	7.0
Leu-26	6.44	2.3
Met-27	0.66	22.7
Asn-28	0.21	71.4
Thr-29	0.31	48.4

Figure 4: Sequence and alanine scan of glucagon. Glucagon receptor EC50 values reported in nM concentration. Sequence in red indicates high potency with alanine substitution.

concentration suitable for routine clinical use) was achieved through elevation of the pH to 8.5 or addition of 0.01% sodium dodecyl sulfate (SDS). Once again, the native hormone proved to be of less than half the solubility of Asp28. Tween-20, a non-ionic detergent significantly enhanced the aqueous solubility of both peptides but was less effective than SDS. Additional surface active entities that have been previously reported to enhance aqueous solubility were assessed over an eight week stability study at a peptide concentration of 1.5 mg/ml and pH 8 (Supplementary Figures 1 and 2). This included human serum albumin, sucrose, propylene glycol, polyethylene glycol and a pluronic acid (F68). The relative performance as assessed by reverse-phase HPLC and size-exclusion chromatography was found to be similar with a few subtle differences. A particular focus on the 8 week time ambient and 4 week 37C conditions demonstrated a slightly greater stability for the SDS formulation (Supplementary Figures 1 and 2).

3.2. Asp28 long-term stability

To explore the longer-term chemical stability of the Asp28 analog a six-month stability study at various temperatures was conducted under conditions where solubility at 1 mg/ml was highly likely to be maintained. The experiment was conducted at pH 8.6 in the presence of 0.1% SDS. One month incubation at ambient and refrigerator temperatures did not reveal any apparent loss in the integrity of the peptide. Temperature elevation to 30 °C and 40 °C demonstrated progressively greater deterioration that exceeded 20% reduction of parent peak as measured by RP-HPLC integrated area against an external standard (Figure 3). Analysis at two months differentiated the ambient temperature sample from the 4 °C cohort with continual destruction at 40 °C to a point where only half remained intact. Additional analysis at four and six months demonstrated the continued linear destruction at the elevated temperatures and the surprisingly accelerated disappearance of the refrigerated sample. The chemical basis for the instability was investigated by high performance chromatographic analysis. It was apparent that two earlier eluting components, as well a single later eluting peak significantly contributed to the increased degradation. In total, the integrated area of the chromatographically eluting peptides suggested higher purity than measurement against an external standard.

3.3. Identifying Asp28 chemical degradation

To ascertain the chemical basis for the loss in the integrity of the Asp28 analog as a function of temperature and time a set of hypothetical degradation products were chemically synthesized for chromatographic studies. One of the purported degradation pathways impacting native glucagon chemical stability is glutamine deamidation [19]. To confirm this possibility, the Asp28 peptides individually substituted with glutamic acid at positions 3, 20, and 24 were prepared. The three deamidated analogs of the Asp28 peptide were cleanly resolved from the parent peptide using reverse-phase analysis in an alkaline buffer (Supplementary Figure 3). All three of the glutamic acid containing peptides eluted earlier than the parent, which is consistent with one additional negative charge when analyzed at pH 8. It should be noted that the deamidated analogs resolved poorly from the parent peptides under acidic conditions typically used in conventional reverse-phase analysis. The Glu20 and Glu24 analogs appeared to co-elute, while the Glu3 separated clearly, eluting more closely to the parent Asp28 analog. Presumably, the negative charge when placed in the C-terminal region at residues 20 and 24 has a more common effect than the same chemical change near the N-terminus. An additional entity that trailed the Asp28 parent peak was distinct from the three deamidated peptides (Supplementary Figure 4). The possible rearrangement to isoaspartic acid (beta-peptide) was suspected at position 15 since it is followed by a serine, constituting a dipeptide sequence prone to form the cyclic five-member imide-intermediate [20,21]. The isoAsp15 beta-peptide was synthesized using α-t-butyl carboxylate protection in otherwise comparable fashion to what was employed for the parent peptide. Alkaline RP-HPLC of the synthetic material confirmed it to be identical to the late eluting impurity.

3.4. Identifying Asp28 physical degradation

The presumed impurities, identified by chemical synthesis and chromatographic analysis, were consistent with the observed impurities but did not fully account for the magnitude of loss in the parent peak as assessed against an external standard. Clearly, there was an additional source of degradation that remained unaccounted for by reverse-phase analysis and it was clear that this did not result from the formation of a

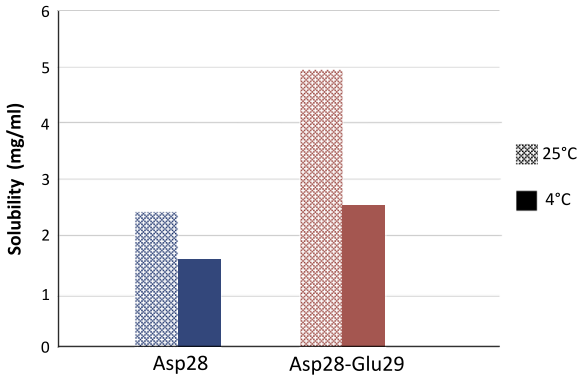

Figure 5: Maximum solubility of Asp28 and Asp28,Glu29 at ambient temperature (shaded bar) and 4 °C (solid bar), in pH 7.4 10 mM Tris, after forty eight hours. The reported observations represent single point experimental results and as such there is no standard deviation.

Peptide	Glucagon Receptor EC50: (nM)	Relative Potency %
Glucagon	0.14	100
Aib16	0.37	37.8
Aib19	13.5	1.0
Aib20	0.32	43.8
Aib21	0.55	25.5
Aib24	0.22	63.6
Aib16,20	0.26	53.8
Aib16,24	0.18	77.8
Aib20,24	0.34	41.2
Aib16,20,24	0.42	33.3

Figure 6: Biological activity (*in vitro*) of glucagon Aib analogs.

Peptide	Sequence	Glucagon receptor EC50 (nM)	Relative potency (%)
Glucagon	HSQGTFTSDYSKYLDSR-RAQDFVQWLMNT	0.062	100
Asp28	HSQGTFTSDYSKYLDSR-RAQDFVQWLM**D**T	0.067	92
Asp28,Glu29	HSQGTFTSDYSKYLDSR-RAQDFVQWLM**DE**	0.070	89
IUB75	HSQGTFTSDYSKYLD**X**R-RAQDFVQWLM**DE**	0.105	59
IUB76	HS**Z**GTFTSDYSKYLD**X**R-RA**A**DFV**A**WL**LDE**	0.105	67

Table 1: Glucagon analog sequence expressed as single amino acid abbreviations and *in vitro* potency. X = Aib and Z = acetyl, diaminobutyric acid.

precipitate. The prospect of this additional loss being a function of higher molecular weight aggregates despite the inclusion of 0.1% SDS was contemplated. It is well known that native glucagon has a propensity to self-aggregate and form high molecular weight fibrils [10,12]. SEC-HPLC of the incubated Asp28 analog indicated an increase in high molecular weight aggregates as a function of time and temperature. The sample incubated at ambient temperature revealed that as much as 10–12% of the parent peptide formed high molecular weight aggregates (Supplementary Figure 5). It became apparent that the single alteration of the isoelectric point which had minimal effect on the native biochemical properties of glucagon and its *in vivo* pharmacological activity was insufficient for improving its long-term stability, as it still remained susceptible to low level deamidation and isoaspartate formation. Additionally, the propensity for degradation through self-association was still evident at slightly alkaline pH even in the presence of 0.1% SDS, although far less so than what has been reported in acidic buffers [12]. Consequently, while the Asp28 modification provides enhanced aqueous solubility and opportunity for acute study an additional level of peptide sequence optimization is needed to maintain integrity for storage over extended periods measured in months, or at body temperature.

3.5. Searching for additional stability

The search for an additional structural element began with a full alanine scan of native glucagon. As shown in Figure 4 the molecule can be divided in two nearly equal lengths. The C-terminal region with few exceptions, most notably residues Asp15, Phe22, Val23, Trp25 and Leu26 can be substituted with alanine with only modest changes in inherent potency. In contrast the N-terminal region is highly intolerant to alanine substitution with only Ser2 and Tyr13 demonstrating modest changes in biopotency. Of the known instability sites documented previously the glutamines at 20 and 24, as well as the asparagine at 28 can be substituted with alanine. However, Gln3 and Asp15 are significantly reduced in potency when substituted with alanine. The prospect of adding anionic character to Asp28 glucagon was envisioned as a means to further enhance the aqueous solubility in the physiological pH range. Given the apparent ability to substitute the native Thr29 with alanine without deleterious effect we explored additional change to Glu29. The additional negative charge resulted in nearly twice the maximal solubility of the Asp28 peptide when assessed at pH 7.4 and no appreciable change in receptor potency (Figure 5 and Table 1). The doubling in apparent peptide solubility with the Asp28,Glu29 mutations relative to Asp28 alone was maintained at 4 °C. This enhanced solubility provides an opportunity to formulate the peptide for administration in a smaller volume or to further lower the pH of the formulation to possibly enhance chemical stability.

3.6. Aib stabilizes physical structure

Recognizing that the biophysical instability of glucagon represents a source of degradation that rivals the total chemical instability we explored whether the peptide conformation could be enhanced through Aib substitutions at sites where alanine had been tolerated. Specifically single Aib substitutions at positions 16, 19, 20, 21, and 24 were studied. Three analogs with double Aib-mutation at two residues represented by 16, 20, and 24 were prepared, along with a single triple mutant. Assessment of *in vitro* potency indicated that four of the five single site substitutions were well tolerated, with the exception being position 19 (Figure 6). This same set of glucagon Aib-analogs was tested for physical degradation in conditions known to promote the formation of glucagon aggregates. The peptides were dissolved at 1 mg/ml in dilute hydrochloric acid and agitated for six weeks at ambient temperature. Mass spectral analysis at study completion indicated the characteristic aspartic acid peptide backbone cleavage in native glucagon between residues 15 and 16 (Figure 7). All analogs containing Aib at position 16 suppressed the cleavage at this particular site (Figure 7 and Supplementary Figure 6a). No other single site Aib-substitution suppressed this specific cleavage to any apparent degree (Supplementary Figure 6b). No enhanced stability was observed for the

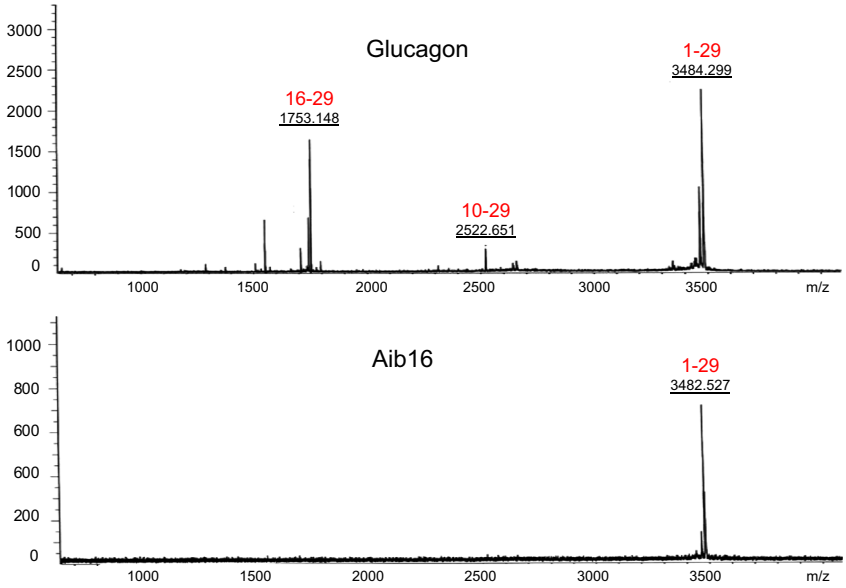

Figure 7: MALDI-tof analysis of glucagon (top) and Aib16 after 7 weeks of agitation in 0.01 N HCl at ambient temperature.

Aib16 analogs with additional Aib substitutions at second and third sites (Supplementary Figure 6a). We observed limited cleavage at the Asp9-Tyr10 amide bond which is typically observed to a slightly greater extent in native glucagon, and cleavage was virtually absent in the single Aib16 mutant. The Aib effectively stabilizes against formation of the five-membered aspartimide intermediate with subsequent bond cleavage prevented through steric hindrance of the dimethyl substitution at its alpha-carbon [22]. Furthermore, the Aib substitution is purported to enhance conformational stability of the peptide which can indirectly minimize structural rearrangement [23]. Clearly, the Aib16 substitution offers sufficient additional stability that it constitutes an additional element for inclusion in pursuit of an optimally stable aqueous formulation.

3.7. Stabilizing the chemical structure and biological activity of Asp28 analogs

Integrating Aib16 with the previously identified Asp28,Glu29 substitutions yielded the peptide analog IUB75. This peptide represents a minimalist approach to native glucagon modification, employing just two changes to enhance anionic character for purposes of aqueous solubility and an additional substitution to stabilize conformation at the highly sensitive mid-region Asp-Ser bond. For comparative purposes a more aggressively modified peptide was synthesized that entailed a more comprehensive set of sites for potential degradation. This second analog started with IUB75 and included substitutions at three deamidation prone glutamine residues (positions 3, 20, and 24) as well as the single oxidation susceptible methionine at position 27. Positions 20 and 24 were substituted with alanine as the alanine scan demonstrated tolerance to individual substitution at these two sites. Position 3 did not tolerate an alanine and through an iterative assessment of suitable glutamine surrogates we identified that acetylated 2,4-diaminobutryic acid (Dab(Ac)) residue which could be introduced without significant change in biopotency. The last substitution involved leucine to replace methionine. Methionine is recognized to possess greater potential to form beta structure and with it promote aggregation [24]. In total, peptide IUB76 contains seven changes to the native sequence and provides a comparative means for assessing the magnitude of instability associated with these putative secondary degradation sites. The two peptides were full *in vitro* agonists and of approximately 40% reduced potency relative to native glucagon (Table 1). This potency is consistent with the comparable reduction observed for the single site Aib16 modification (Figure 6). All of the additional modifications to IUB76 did not result in appreciable changes in potency. The inherent absolute potency of these two analogs remains subnanomolar and is suitable for continued development.

3.8. Enhanced stability of IUB analogs

The stability of these two analogs relative to Asp28 was assessed in a six month study that was extended for an additional six months for IUB76 (Supplementary Figures 7 and 8). When formulated at the same pH and ambient temperature IUB75 was approximately twice as stable as the previously studied Asp28 analog. The total degradation was slightly more than 20% at six months and IUB76 displayed approximately half this degree of degradation, and its study was consequently continued for an additional half-year (Figure 8). The increased stability of IUB75 relative to Asp28 can be attributed mainly to the Aib16 substitution since the other modification (Glu for Thr at the C-terminus which enhanced inherent solubility) was not a factor in this study where the pH and concentration were held constant. The additional modifications present in IUB76 further mitigate instability related to the three glutamine residues and the single methionine residue. Collectively all seven changes reduce the total degradation to approximately 10% at six months and twice that amount at one year. Refrigeration at 4 °C further diminished degradation to less than 10% at one year. Assessment of *in vitro* biopotency at conclusion of the study revealed a comparable loss in the integrity of the starting material at ambient and refrigerated temperatures (data not shown). A central consideration in this stability study was the magnitude of high molecular weight aggregate observed for each analog, with each starting as monomeric material. The results expressed as percent increase through six months of study are dramatically different for the two Aib16 analogs and Asp28. There was a steady and nearly linear progression of high molecular aggregate

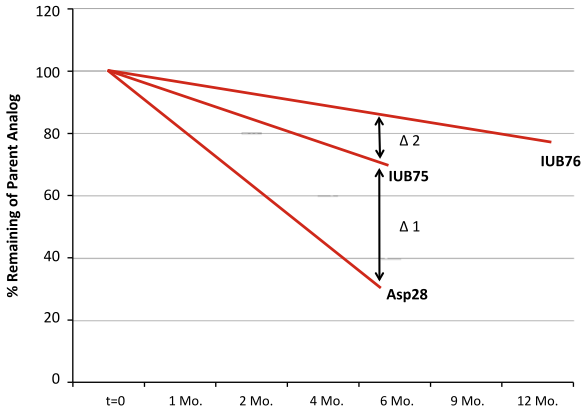

Figure 8: Trend-line depiction of degradation slopes of Asp28, IUB75, and IUB76. Δ1 represents a 40% difference in stability at six months between Asp28 and IUB75. Δ2 represents a 10% difference in stability at six months between IUB75 and IUB76.

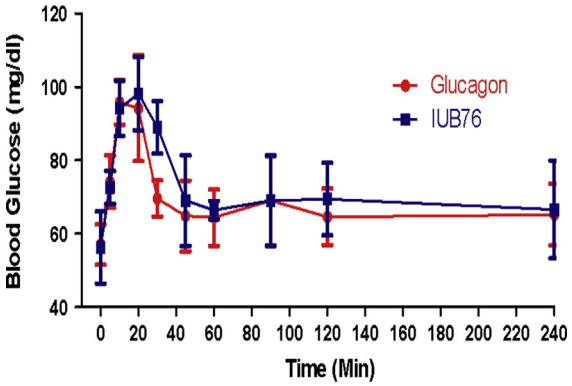

Figure 9: Effect of IM injection (0.014 mg/kg) of glucagon (red) and IUB76 (blue) on the mean blood glucose concentration versus time in five normal beagle dogs.

observed for glucagon-Asp28 which was not apparent for Aib16 analogs, and shown for IUB76 (Supplementary Figure 9). The total aggregate content in the Asp28 formulation at six months accounted for approximately 13% of starting material.

3.9. *In vivo* activity of IUB76 analog

The sharply enhanced relative stability of IUB76 prompted us to conduct a pilot *in vivo* study to assess potency in fasted male beagle dogs relative to native glucagon, when administered as an intramuscular injection (Figure 9). The two peptides were dissolved at identical concentrations but while the native hormone was dissolved in the conventional dilute hydrochloric acid diluent, IUB76 was formulated in slightly alkaline Tris buffer previously employed in the stability studies. Administration of glucagon at a dose equivalent to human dosing on a body weight basis (0.014 mg/kg) led to a rapid increase in glucose concentration that peaked within 20 min after administration. The magnitude and timing to maximal effect as well as return to baseline in IUB76 administration was comparable to native hormone. Subtle differences would require a comparative dose titration and this will be considered at the point when a more optimal formulation and any additional chemical refinements are introduced.

4. DISCUSSION

Glucagon's poor solubility and stability are well known problems and present a considerable challenge to its availability and application at time of therapeutic need. The inherent limitations reside in the fact that the native sequence has evolved to meet its physiological role, as opposed to clinical application. Glucagon is poorly soluble at physiological pH and it displays limited chemical and physical stability in acidic and alkaline diluents. Consequently, our work has focused upon increasing the aqueous solubility of glucagon near physiological pH by increasing its anionic character. The single introduction of Asp28 provided a glucagon analog with enhanced solubility and biological properties that were nearly identical to the native hormone [13]. However, when its stability was examined in the six month study we report here it was observed to be of insufficient quality to justify continued development. In pursuit of other structural refinement we made the seminal observation that the substitution of Ser16 with Aib provided a much enhanced chemical and physical stability. Aib is known to stabilize helical secondary structure and disrupt beta-sheets in peptides [22,25–27]. Structurally Aib is similar to alanine, differing by an additional methyl group at the alpha carbon in place of hydrogen. The application of Aib substitutions has been previously shown to prevent peptide fibril formation [28].

The integration of the Asp28 and Aib16 modifications was pursued in combination with additional amino acid substitution directed at further enhancement of the physical properties of these glucagon agonists. The substitution of the C-terminal threonine with a glutamic acid further lowered the isoelectric point of the peptide such that its solubility near neutral pH was enhanced. This single peptide (IUB75) possessing three amino acid changes relative to native sequence was sizably more stable when studied relative to the singly substituted Asp28 analog. However, there was still slightly more than 20% total degradation at ambient temperature when observed in a six-month stability study. Additional substitutions that changed three glutamines (residues 3, 20 and 24) and a single methionine (residue 27) to inherently more stable aliphatic amino acids yielded an analog (IUB76) that demonstrated approximately half the degradation relative to IUB75. Each of these glucagon agonists was highly potent when assessed by *in vitro* activity and maintained selectivity for the glucagon receptor. When studied in fasted beagle dogs, IUB76 demonstrated comparable potency and pharmacodynamics relative to the native hormone. It is important to note that IUB76 was administered as a pre-formulated peptide at slightly alkaline pH while the native hormone was dissolved in dilute hydrochloric acid. While this is an appropriate means to assess relative performance inherent to the peptides it does not reflect clinical reality where the native hormone requires a timely delay for solubilization of hormone as supplied as a lyophilized powder. Consequently, the bioequivalence that we report understates the true medicinal optimization that resides within the of IUB76 peptide sequence.

Having achieved the discovery of novel glucagon agonists that promise superior clinical performance it is appropriate to address the remaining obstacles to their development. The number of changes in the sequence creates a peptide that is appreciably foreign from an immunological perspective. Consequently, the immunogenicity of such an analog requires detailed characterization relative to the native hormone. Secondly, the formulation that was used in the extended stability studies used low concentrations of SDS. While this is permissible for an infrequently used, life-saving medication it is not optimal for chronic use. Consequently, further refinement of the peptide formulation in concert with possible additional sequence refinement remains to be completed. Finally, it seems prudent to use IUB76 as a benchmark for identification of additional analogs of comparable or enhanced stability that might be achieved through fewer structural changes. In conclusion,

we viewed these results as directional in the search for an optimal glucagon-based medicine. In a relative sense when compared to the investment that has been made in the optimization of insulin as a medicinal agent glucagon is but a mere fraction and yet its impact promises to be equally life saving.

CONFLICT OF INTEREST

Research support was provided by Marcadia Biotech.

APPENDIX A. SUPPLEMENTARY MATERIALS

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.molmet.2014.01.006.

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