# Supporting Information For:

# Discovery of High Potency, Single-Chain Insulin Analogs with a Shortened B-Chain and Non-Peptide Linker

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**Synthesis of V8 standards:** The segments HLCGAE and CCFRSCD were synthesized on 0.2mM preloaded PAM Resin by automated Boc/Bzl chemistry with Acm (S-acetamidomethyl) protection for cysteines at positions A6 and A11 (native fragment) and A7 and A11 (isomer). The single cysteine in the B-chain segment remained unprotected along with one cysteine (A6 or A7) in the A-chain. 110mg of segment HLNCGAE and 67.8mg DTNP (2,2'-Dithiobis(5-nitropyridine) were combined in 5mL DMSO and stirred overnight at RT. Both the Npys activated B-chain and A-chain segments were purified by preparative HPLC using an Axia Packed Luna C18 column (21.2 x 250 mm) with a gradient of 0-40%B/120mins. Purity was determined by analytical HPLC and MALDI-TOF mass spectrometry. Interchain disulfide bonds were unequivocally formed by combination of the appropriate A and B segments at a concentration of 20mg total peptide in 1.0mL DMSO and stirring for 3-4 hrs. The reaction mixture was then diluted 20 fold, loaded directly onto a C18 preparative column purified as described above and lyophilized. The remaining disulfide bond was formed by iodine oxidation as described previously[1]. The peptide was then purified again by preparative HPLC using the C18 column and the 0-40%B/120min gradient to give a white powder.

**Preparative HPLC (Segments)** 80-100mg of crude peptide material was loaded onto prep column (Axia Packed Luna C8 or C5 column of dimension 21.2 x 250 mm) and eluted at a flow rate of 12 mL per minute with gradient 10%B-40%B over 120 minutes. Solvent A: 0.1% TFA in 10% aqueous acetonitrile, Solvent B: 1% TFA in acetonitrile. Fractions containing the desired peptide were identified by analytical HPLC and MALDI-TOF mass spectrometry, pooled then lyophilized to give a white fluffy powder.

**Analytical HPLC (Segments)**: Analysis was performed on a Beckman System Gold 166 instrument equipped with a Bio-Rad autosampler and using a Zorbax $C_8$ column (4.6 X 50.0mm) programmed for a 1.0ml/min. flow rate and a gradient of 10% - 80%B/ 10 min. System A : 0.1%TFA in water, system B : 0.1%TFA in 90% aqueous acetonitrile.

**Endoprotease Lys C cleavage:** The peptide was dissolved in phosphate buffer solution pH 7.8, and the concentration determined by use of a Nanodrop UV-Vis spectrometer.

Endoproteinase Lys-C enzyme from *Lysobacter enzymogenes* (Sigma, St. Louis) was added to the solution at an enzyme-to-peptide ratio 0.5 units:1.5mg and incubated at 37°C for 6-12 hrs. Digestion was monitored by analytical HPLC and confirmed by MALDI-TOF mass spectrometry. The fully cleaved peptide was frozen at -20°C until ready to use for *in vitro* studies.

**V8 Protease Digestion:** 80ug of protein was dissolved into 0.1M sodium citrate buffered solution pH 7.0. 3.0ug of Endoproteinase Glu-C enzyme from *Staphylococcus aureus* V8 (Sigma, St. Louis) was added, and the digest was placed at 37ºC overnight. Samples were taken from the reaction and run on analytical HPLC and Mass Spec.

**ELISA Phosphorylation Assay:** Transfected IR-A or IGF-1R HEK 293 cells were maintained in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% bovine growth serum containing antibiotics, 10mM HEPES, and 125ug/mL Zeocine. Cells were plated in 80µL serum deprived medium at 4.0 x $10^4$ cells/well on lysine coated 96 well plates (Corning, Corning, NY). Cells were serum deprived for 16 hours by culturing in DMEM (Hyclone, Logan UT) supplemented with 0.25% Bovine Growth Serum, antibiotics and 10mM HEPES. Serial dilutions were done with insulin/IGF-1 standards and Insulin analogs using DMEM containing 0.5% BSA. 20µL was added to the plate containing the transfect cells and incubated for 15 minutes at 37°C, 5% $CO_2$. Cells were then fixed to the plate by adding 100µL of a 10% formylin solution for 20 minutes. Cells were washed with a wash solution (1X PBS containing 0.1% Trition X100) and then blocked using 2% BSA in wash solution at RT for 1 hr. After several washes, 50µL of antibody (4G10 anti-phosphotyrosine-HRP conjugate (Millipore) for IR-A and PY20 anti-phosphotyrosine-HRP conjugate (R&D systems) for IGF-1) diluted 10,000 fold was added to the plates and incubated at RT for 3 hrs. The cell are washed several times before 100µL of 3,3',5,5',TetraMethylBenzidine (TMB)(Invitrogen) substrate was added and incubated for 5-10 minutes. The reaction was stopped by adding 50µL of 1M HCl to each well, the plate is scanned at 540nm by a Titerteck multiscan MCC340 plate reader. Effective 50% concentrations (EC50) were calculated by Origin software (OriginLab, Northampton, MA).

**Insulin Receptor A Binding Assay:** The affinity of each peptide for the insulin receptor was measured in a competition binding assay utilizing scintillation proximity technology. Serial 3 or 5 fold dilutions were made in Tris-Cl buffer (0.05 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.1% w/v bovine serum albumin) and mixed in 96 well plates (Corning Inc., Acton, MA) with 0.05 nM 3-($^{125}$I)-iodotyrosyl) TyrA14 insulin (Perkin-Elmer,Waltham, MA). An aliquot of 1-6 micrograms of plasma membrane fragments (prepared similar to previous literature[2]) from HEK 293 cells over-expressing the human insulin receptors were present in each well and 0.25 mg/well polyethyleneimine-treated wheat germ agglutinin type A scintillation proximity assay beads (Amersham Biosciences, Piscataway, NJ) were added. After five minutes of shaking at 800 rpm the plate was incubated for 12 hrs at room temperature and radioactivity was measured with MicroBeta1450 liquid scintillation counter (Perkin-Elmer, Wellesley, MA). Non-specifically bound (NSB) radioactivity was measured in the wells with a four-fold concentration excess of "cold" native ligand than the highest concentration in test samples. Total bound radioactivity was detected in the wells with no competitor. Percent specific binding was calculated as following: % Specific Binding = (Bound-NSB / Total bound-NSB) x 100. $IC_{50}$ values were determined by using Origin software (OriginLab, Northampton, MA).

***In Vivo* Mouse Studies:** Animal Studies were done by Melior Discovery, Inc. All experiments were conducted in accordance with the National Institutes of Health regulations of animal care covered in "Principles of Laboratory Animal Care," National Institutes of Health publication 85-23 and were approved by the Institutional Animal Care and Use Committee. C57/BL6 male mice (Ace Animals, Boyertown, PA) were 6 weeks of age at time of study. Mice were maintained on Harlan Chow (2016 Teklad Global 16% Protein Rodent Diet) and kept on a 12 hrs light-dark

cycle. On the day of the study, mice were fasted for 4 hrs prior to administration of test compound and maintained fasted until the completion of the study. After the 4 hrs fast, mice were administered test compounds via subcutaneous administration. Blood glucose levels were measured beginning just prior to test compound administration and then periodically up to 8 hrs after administration. Blood glucose levels were measured from a drop of blood (1-3 μL) collected from the tail using Accu-Chek Aviva Glucometers (Roche Diagnostics). Glucometers were calibrated prior to each study. Blood glucose levels are reported as the area under the curve (mg x dL/hr).

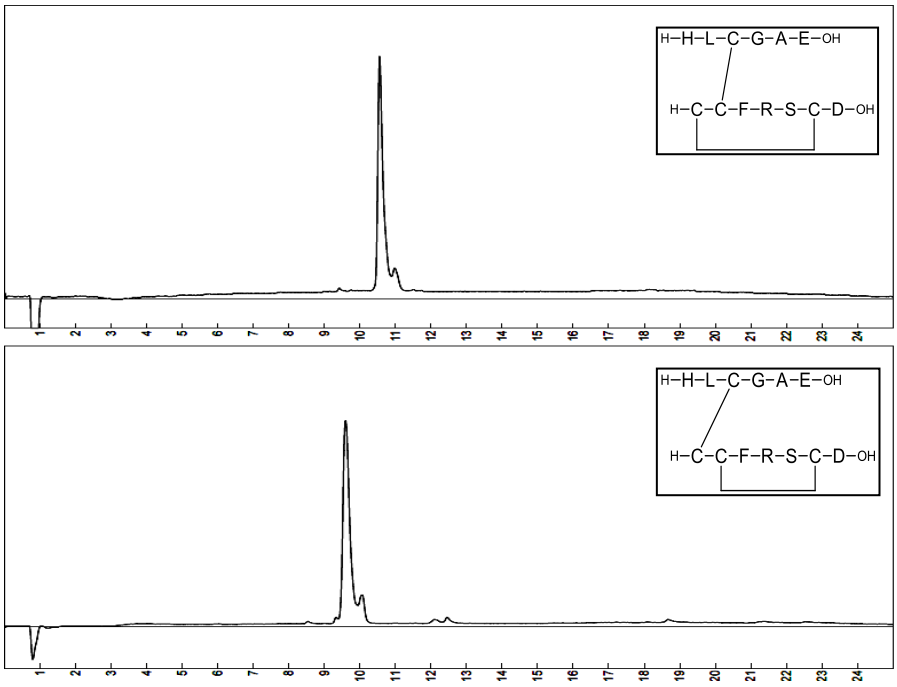


**Figure S1**. HPLC analysis of chemically made disulfide segments. Top: Correct disulfide fragment. Bottom: Isoform (B7-A6, A7-A11) disulfide fragment. HPLC gradient of 20-60%B in 30/mins. B= 90%ACN, 0.1%TFA.

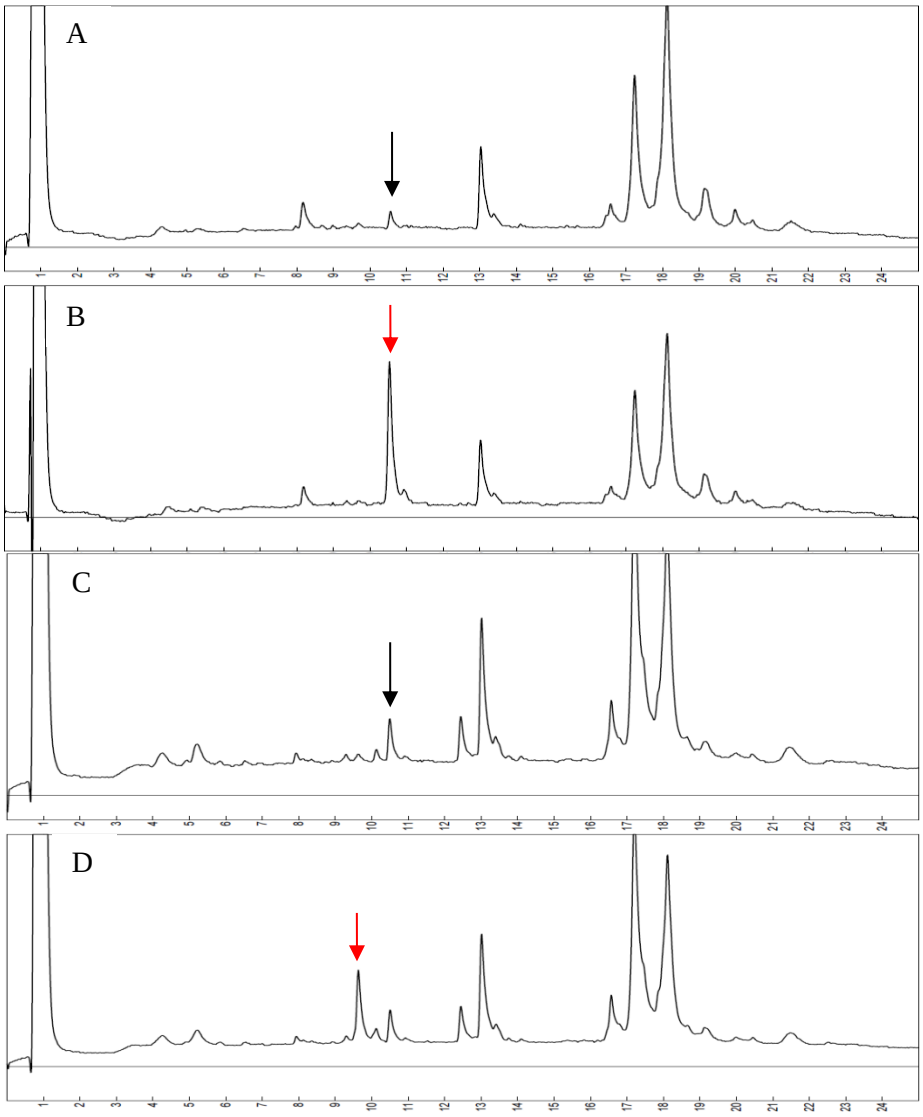


**Figure S2**. HPLC analysis of V8 digested $PEG_{16}$ compared to chemical disulfide segments. **A)** $PEG_{16}$ digested for 16 hours. Black arrow denotes the segment peak. **B)** $PEG_{16}$ digested for 16 hours + correctly formed disulfide fragment (red arrow). **C)** $PEG_{16}$ digested for 32 hours. Black arrow denotes the segment peak. **D)** $PEG_{16}$ digested for 32 hours + isoform disulfide segment (red arrow). HPLC gradient of 20-60% B/30 mins. B= 90% ACN, 0.1% TFA.

**References:**

1. Kamber, B.; Hartmann, A.; Eisler, K.; Riniker, B.; Rink, H.; Sieber, P.; Rittel, W., The Synthesis of Cystine Peptides by Iodine Oxidation of S-Trityl-Cysteine and S-Acetamidomethyl-Cysteine Peptides. *Helv. Chim. Acta.* 1980, *63* (4), 899-915.
2. Nagamatsu, S.; Kornhauser, J. M.; Burant, C. F.; Seino, S.; Mayo, K. E.; Bell, G. I., Glucose transporter expression in brain. cDNA sequence of mouse GLUT3, the brain facilitative glucose transporter isoform, and identification of sites of expression by in situ hybridization. *J. Biol. Chem.* 1992, *267* (1), 467-72.