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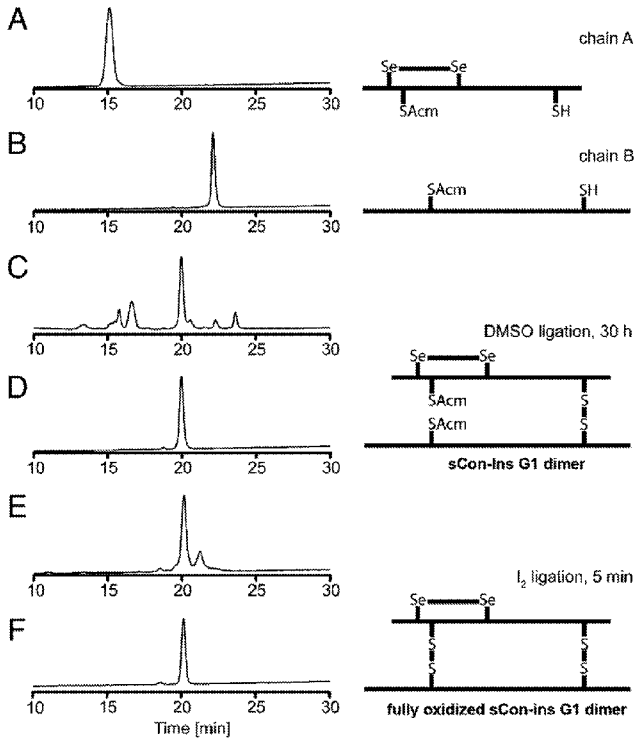

FIG. 1

(57) Abstract: Insulin analogs having shortened B chain peptides are disclosed and discussed, including insulin analog formulations and methods of treatment thereof.

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INSULIN ANALOGS HAVING SHORTENED B CHAIN PEPTIDES AND ASSOCIATED METHODS

BACKGROUND

More than 100 species of venomous cone snails (genus Conus) are highly effective predators of fish. The vast majority of venom components identified and functionally characterized to date are neurotoxins specifically targeted to receptors, ion channels, and transporters in the nervous system of prey, predators, or competitors. Many of these venoms are remarkably potent and diverse. Most bioactive venom components are small disulfide-rich peptides termed conotoxins, which target specific receptors and ion channel subtypes located in the prey's nervous system.

The epithelial cells lining the duct of a cone snail's venom gland are rich in messenger RNAs (mRNAs) encoding conotoxins. These mRNAs are translated initially as inactive precursor peptides that require post-translational processing prior to secretion from the cell as the bioactive mature peptides. Conotoxin precursors exhibit a characteristic primary structure: a hydrophobic signal peptide (prepeptide) sequence, followed by a propeptide region and commonly a cysteine-rich mature peptide region. The signal sequence of a precursor peptide is responsible for targeting it to the cellular secretory pathway, but is removed prior to secretion of the mature peptide. Conotoxins can be classified into gene superfamilies according to this signal peptide sequence. Members of a conotoxin superfamily share a high percentage of sequence identity in their signal peptide sequence but less so in their pro-peptide sequence, and can be highly variable in their mature peptide sequence, (often with the exception of the cysteine framework).

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A is a graphical representation of data according to an example embodiment;

FIG. 1B is a graphical representation of data according to an example embodiment;

FIG. 1C is a graphical representation of data according to an example embodiment;

FIG. 1D is a graphical representation of data according to an example embodiment;

FIG. 1E is a graphical representation of data according to an example embodiment;

FIG. 1F is a graphical representation of data according to an example embodiment;

FIG. 2 is a graphical representation of mass spec data according to an example embodiment;

FIG. 3A is a graphical representation of experimental data according to an example embodiment;

FIG. 3B is a graphical representation of experimental data according to an example embodiment;

FIG. 4A is a graphical representation of an insulin analog sequence according to an example embodiment;

FIG. 4B is a graphical representation of HPLC data according to an example embodiment;

FIG. 5A is a graphical representation of an insulin analog sequence according to an example embodiment;

FIG. 5B is a graphical representation of HPLC data according to an example embodiment;

FIG. 6A is a graphical representation of data according to an example embodiment;

FIG. 6B is a graphical representation of data according to an example embodiment;

FIG. 7A is a graphical representation of peptide sequences according to an example embodiment;

FIG. 7B is a graphical representation of data according to an example embodiment;

FIG. 7C is a graphical representation of data according to an example embodiment; and

FIG. 7D is a graphical representation of data according to an example embodiment.

DESCRIPTION OF EMBODIMENTS

Although the following detailed description contains many specifics for the purpose of illustration, a person of ordinary skill in the art will appreciate that many

variations and alterations to the following details can be made and are considered included herein.

Accordingly, the following embodiments are set forth without any loss of generality to, and without imposing limitations upon, any claims set forth. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs.

In this application, "comprises," "comprising," "containing" and "having" and the like can have the meaning ascribed to them in U.S. Patent law and can mean "includes," "including," and the like, and are generally interpreted to be open ended terms. The terms "consisting of" or "consists of" are closed terms, and include only the components, structures, steps, or the like specifically listed in conjunction with such terms, as well as that which is in accordance with U.S. Patent law. "Consisting essentially of" or "consists essentially of" have the meaning generally ascribed to them by U.S. Patent law. In particular, such terms are generally closed terms, with the exception of allowing inclusion of additional items, materials, components, steps, or elements, that do not materially affect the basic and novel characteristics or function of the item(s) used in connection therewith. For example, trace elements present in a composition, but not affecting the compositions nature or characteristics would be permissible if present under the "consisting essentially of" language, even though not expressly recited in a list of items following such terminology. When using an open ended term in this specification, like "comprising" or "including," it is understood that direct support should be afforded also to "consisting essentially of" language as well as "consisting of" language as if stated explicitly and vice versa.

"The terms "first," "second," "third," "fourth," and the like in the description and in the claims, if any, are used for distinguishing between similar elements and not necessarily for describing a particular sequential or chronological order. It is to be understood that the terms so used are interchangeable under appropriate circumstances such that the embodiments described herein are, for example, capable of operation in sequences other than those illustrated or otherwise described herein. Similarly, if a method is described herein as comprising a series of steps, the order of such steps as presented herein is not necessarily the only order in which such steps

may be performed, and certain of the stated steps may possibly be omitted and/or certain other steps not described herein may possibly be added to the method.

As used herein, the term "substantially" refers to the complete or nearly complete extent or degree of an action, characteristic, property, state, structure, item, or result. For example, an object that is "substantially" enclosed would mean that the object is either completely enclosed or nearly completely enclosed. The exact allowable degree of deviation from absolute completeness may in some cases depend on the specific context. However, generally speaking the nearness of completion will be so as to have the same overall result as if absolute and total completion were obtained. The use of "substantially" is equally applicable when used in a negative connotation to refer to the complete or near complete lack of an action, characteristic, property, state, structure, item, or result. For example, a composition that is "substantially free of" particles would either completely lack particles, or so nearly completely lack particles that the effect would be the same as if it completely lacked particles. In other words, a composition that is "substantially free of" an ingredient or element may still actually contain such item as long as there is no measurable effect thereof.

As used herein, a plurality of items, structural elements, compositional elements, and/or materials may be presented in a common list for convenience. However, these lists should be construed as though each member of the list is individually identified as a separate and unique member. Thus, no individual member of such list should be construed as a de facto equivalent of any other member of the same list solely based on their presentation in a common group without indications to the contrary.

Concentrations, amounts, and other numerical data may be expressed or presented herein in a range format. It is to be understood that such a range format is used merely for convenience and brevity and thus should be interpreted flexibly to include not only the numerical values explicitly recited as the limits of the range, but also to include all the individual numerical values or sub-ranges encompassed within that range as if each numerical value and sub-range is explicitly recited. As an illustration, a numerical range of "about 1 to about 5" should be interpreted to include not only the explicitly recited values of about 1 to about 5, but also include individual values and sub-ranges within the indicated range. Thus, included in this numerical

range are individual values such as 2, 3, and 4 and sub-ranges such as from 1-3, from 2-4, and from 3-5, etc., as well as 1, 1.5, 2, 2.3, 3, 3.8, 4, 4.6, 5, and 5.1 individually.

This same principle applies to ranges reciting only one numerical value as a minimum or a maximum. Furthermore, such an interpretation should apply regardless of the breadth of the range or the characteristics being described.

Reference throughout this specification to "an example" means that a particular feature, structure, or characteristic described in connection with the example is included in at least one embodiment. Thus, appearances of the phrases "in an example" in various places throughout this specification are not necessarily all referring to the same embodiment.

Example Embodiments

An initial overview of technology embodiments is provided below and specific technology embodiments are then described in further detail. This initial summary is intended to aid readers in understanding the technology more quickly, but is not intended to identify key or essential technological features, nor is it intended to limit the scope of the claimed subject matter.

Insulin is amongst the most versatile hormones described to date, which plays central physiological roles in regulating glucose metabolism, reproduction, cognition, and the like. Vertebrate insulin is versatile hormone that is synthesized in pancreatic β cells, and is the key hormone regulator of carbohydrate and fat metabolism. In the brain, insulin functions as a neuromodulator of energy homeostasis and cognition. Insulin is initially synthesized as a precursor comprising three regions, A, B, and C, from which proteolytic cleavage of the C peptide in the Golgi releases the mature insulin heterodimer with an A and B chain connected by disulfide bonds.

Disturbance of insulin regulation is associated with often severe clinical manifestations, as diabetes myelitis, hyperglycemia, as well as other similar conditions. Administration of insulin remains the most effective therapy for the treatment of conditions such as diabetes, which is generally delivered via subcutaneous injection, insulin pump, or the like. Depending on the type of diabetes, the severity of the condition, and an affected individual's lifestyle, various insulin analog formulations are available for use that differ in their onset and length of action. Rapid-acting insulins, for example, have a fast onset of activity, and are thus typically given before meals. Structurally, these insulin analogs differ from normal human

insulin by having amino acid substitutions that are deleterious to insulin multimerization.

Human insulin is a protein complex that includes two chains, an A chain (21 residues) and a B chain (30 residues), which are cross-linked by two disulfide bridges, with a third, intra-chain disulfide bridge occurring within the A chain. Residues at the C-terminus of the B chain promote insulin dimerization through an anti-parallel β-strand interaction. In pancreatic β-cells, insulin is stored as a hexamer comprising three insulin dimers held together by two central zinc ions that coordinate a histidine at position B10 of each monomer. Hexamer-to-monomer conversion is thus crucial to the bioavailability of insulin in many treatment regimes. Rapid-acting insulin analogs have reduced rates of self-association, and are more readily absorbed after subcutaneous injection. For example, in the insulin analog lispro (Eli Lilly), a lysine and proline at position B28 and B29 are reversed, creating steric hindrance and a reduced ability to self-associate.

Notably, however, all rapid-acting insulins analogs created to date retain the classical aromatic triplet PheB24-PheB25-TyrB26 involved in insulin dimer formation—in particular PheB24, which appears to be critical to activity. Attempts to shorten the insulin B chain in order to abolish self-association have resulted in near complete loss of activity. For example, *des*-octapeptide (DOI, B23-30) insulin, a monomeric analog, preserves less than 0.1 % bioactivity.

The present technology provides various insulin analogs that have modified B chains lacking the aromatic triplet, and have, in most cases, shorter B chains compared to previously described insulins. Portions of the presently described insulins have some similarity to insulin peptide found in the venom of fish-hunting cone snails. For example, two fish-hunting cone snails, *Conus geographus* and *Conus tulipa*, have evolved specialized insulins that are expressed as components of their venoms, and that appear to be targeting prey energy metabolism. When injected into fish, these venom insulins elicit hypoglycemic shock, a condition characterized by low blood glucose. It is thus possible that such insulins are used as a weapon by a subset of fish-hunting cone snails that use a net strategy to capture prey. As one specific example, Con-Ins G1 (A chain, SEQ ID NO: 001; B chain SEQ ID NO: 002) is a specialized *C. geographus* insulin, having characteristic conotoxin amino acid modifications of a γ-carboxyglutamate (Gla) at the A04 and B12 positions of the A

and B chains, and a hydroxyproline (Hyp) at the B05 position of the B chain, as well as an amidated cysteine (*) at the terminal end of the A chain.

(SEQ ID NO: 001)

Gly-Val-Val-Gla-His-Cys-Cys-His-Arg-Pro-Cys-Ser-Asn-Ala-Glu-Phe-Lys-Lys-Tyr-Cys*

(SEQ ID NO: 002)

Thr-Phe-Asp-Thr-Hyp-Lys-His-Arg-Cys-Gly-Ser-Gla-Ile-Thr-Asn-Ser-Tyr-Met-Asp-Leu-Cys-Tyr-Arg

Despite the short B chain and the aromatic PheB24-PheB25-TyrB26 triplet found in all human insulins, the *C. geographus* venom insulin Con-Ins G1 is only thirty-fold less active against the human IR-B receptor than hIns (Con-Ins G1 IC_{50} = 17 nM, hIns IC_{50} = 0.56 nM.

In one example embodiment, insulin analogs are disclosed that can include an A chain peptide and a B chain peptide bonded together across at least one pair of Cys residues. In another embodiment, the A chain peptide and the B chain peptide can be bonded together across multiple pairs of Cys residues. Such bonding can be a disulfide bridge, or any other known and useful bridge-type bond. Such bonding can be accomplished across Cys residues, modified Cys residues, or other compatible amino acid or molecule with sufficient similarity to Cys so as to not destroy the functionality of the insulin analog. Non-limiting examples of such bonds can include dicarba, lactam, diselenide, triazole, and the like, including combinations thereof. Furthermore, in some examples, an insulin analog can include an intra-chain disulfide bridge occurring within one of the peptide chains, which in some cases is the A chain.

In another embodiment, the A chain peptide and the B chain peptide can be linked together at one or more terminal ends. Thus, while in some cases the insulin analog is acyclic, in other cases derivatives of the insulin analogs can be cyclic, while retaining the Cys-bridging pattern described. In such cases, the insulin analog has an amide cyclized backbone such that the A and B chains have no free N- or C-terminus (for the embodiment whereby both terminal ends are linked). The linkage at the one or more terminal ends can be directly between amino acids of the A and B chain peptide backbones, or there can be a linker of one or more amino acids or other linker molecules bonded therebetween.

Furthermore, residues or groups of residues known to the skilled artisan to improve stability can be added to the C-terminus and/or N-terminus. Also, residues or groups of residues known to the skilled artisan to improve bioavailability can be added to the C-terminus and/or N-terminus. Furthermore, such residues or groups that can be added to the N-terminus can also replace Gly within the insulin analogs. Fluorescent tags are additionally contemplated for attachment to either the C- or N-terminus.

As one example, the A chain peptide can include the sequence Gly-X_{A2}-X_{A3}-X_{A4}-X_{A5}-Cys_{A6}-Cys_{A7}-X_{A8}-X_{A9}-X_{A10}-Cys_{A11}-X_{A12}-X_{A13}-X_{A14}-X_{A15}-X_{A16}-X_{A17}-X_{A18}-X_{A19}-Cys_{A20}-X_{A21}-X_{A22}-X_{A23}-X_{A24}-X_{A25}-X_{A26}-X_{A27}-X_{A28}-X_{A29}-X_{A30}-X_{A31}-X_{A32}-X_{A33}-X_{A34} (SEQ ID NO: 003), where X_{A2} = Val or Ile; X_{A3} = Val or Ala; X_{A4} = Glu or Cys; X_{A5} = Glu, His or Val; Cys_{A6}, Cys_{A7}, and C_{A11} are independently Cys or selenocysteine; X_{A8} = His, Asp, Gln, Tyr, Lys or Val; X_{A9} = Arg, Asn, His or Lys; X_{A10} = Pro, Tyr, Ala, Ser, Phe, His or Thr; X_{A12} = Ser or Thr; X_{A13} = Asn, Val or Asp; X_{A14} = Ala, Gln, Asp or Glu; X_{A15} = Glu or Thr; X_{A16} = Phe, Leu, or Ala; X_{A17} = Lys, Arg, Ile, Met, Thr or Ser; X_{A18} = Lys, Thr, Asn, Gln or Glu; X_{A19} = Tyr or Phe; Cys_{A20} = Cys, selenocysteine, amidated Cys, or amidated selenocysteine; X_{A21} = Pro, His, Ser, Gly, Ala, or is absent; X_{A22} = Pro, Asn, Thr, Leu, Ser or is absent; X_{A23} = Thr, Leu, Val, Ser or is absent; X_{A24} = Arg, Thr, Met, Gln, Leu or is absent; X_{A25} = Glu, Gly or is absent; from X_{A26} = Ser, Leu or is absent; X_{A27} to X_{A31} are independently Ser or are absent; X_{A32} = Ala, Ser or is absent; X_{A33} = Ala, Val or is absent; and X_{A34} = Ala or is absent.

In another example, the A chain peptide can include the sequence Gly-Val-Val-Glu-His-Cys_{A6}-Cys_{A7}-X_{A8}-Arg-X_{A10} -Cys_{A11}-Ser-Asn-Ala-Glu-Phe-X_{A17}-X_{A18}-Phe-Cys_{A20} (SEQ ID NO: 004), wherein Cys_{A6}, Cys_{A7}, and C_{A11} are independently Cys or selenocysteine; X_{A8} = His, Tyr or Lys; X_{A10} = Pro or Ala; X_{A17} = Lys or Met; X_{A18} = Lys or Gln; X_{A19} = Tyr or Phe; Cys_{A20} = Cys, selenocysteine, amidated Cys, or amidated selenocysteine; X_{A21} = Ser, Gly or is absent; X_{A22} = Asn or is absent; and X_{A23} = Ser or is absent.

In yet another example, the A chain peptide can include the sequence Gly-Ile-X_{A3}-X_{A4}-Glu-$Cys_{A6}Cys_{A7}$-X_{A8}-X_{A9}-X_{A10}-Cys_{A11}-Thr-X_{A13}-X_{A14}-Glu-X_{A16} -X_{A17}-X_{A18}-Tyr-Cys_{A20} (SEQ ID 005), wherein X_{A3} = -Val or A; X_{A4} = -Glu or C; Cys_{A6}, Cys_{A7}, and C_{A11} are independently Cys or selenocysteine; X_{A8} = -His, Asp, Gln, Lys or -Val; X_{A9} = Asn or Lys; X_{A10} = Tyr, Ser, Phe, -His or Thr; X_{A13} = Asn or Asp; X_{A14} = A,

Gln, Asp or -Glu; X_{A16} = Phe or A; X_{A17} = Arg, Met, Thr or Ser; X_{A18} = Lys, Gln or -Glu; Cys_{A20} = Cys, selenocysteine, amidated Cys, or amidated selenocysteine; X_{A21} = Pro, His, Ser, Ala, or is absent; X_{A22} = Pro, Thr, Leu, Ser or is absent; X_{A23} = Thr, Leu, Val, or is absent; X_{A24} = Arg, Thr, Met, Gln, Leu or is absent; X_{A25} = Glu, Gly or is absent; from X_{A26} = Ser, Leu or is absent; X_{A27} to X_{A32} are independently Ser or is absent; X_{A33} = Ala or is absent; and X_{A34} = Ala or is absent.

The B chain peptide, for example, can include the sequence X_{B1}-X_{B2}-X_{B3}-X_{B4}-X_{B5}-X_{B6}-X_{B7}-X_{B8}-Cys_{B9}-X_{B10}-X_{B11}-X_{B12}-X_{B13}-X_{B14}-X_{B15}-X_{B16}-X_{B17}-X_{B18}-X_{B19}-X_{B20}-Cys_{B21}-X_{B22}-X_{B23}-X_{B24}-X_{B25}-X_{B26}-X_{B27}-X_{B28}-X_{B29}-X_{B30}-X_{B31}-X_{B32}-X_{B33}-X_{B34}-X_{B35}-X_{B36}-X_{B37}-X_{B38}-X_{B39} (SEQ ID NO: 006), where X_{B1} = Thr, Asn, Ser or is absent; X_{B2} = Phe, Ser, Asn, Thr, Gln or is absent; X_{B3} = Asp, Gly, Pro, Leu, Phe, or His; X_{B4} = Thr, Pro, Asp, Val or Gly; X_{B5} = Asn, Pro, His, Thr, Arg, Ser or hydroxyproline; X_{B6} = Lys, Glu, Asn, Asp, Arg, Gln or Gly; X_{B7} = His, Tyr, Arg or Ile; X_{B8} = Arg, Thr, Ile, Ser, Leu, Tyr or Lys; Cys_{B9} = Cys or selenocysteine; X_{B10} = Gly, Gln or Asp; X_{B11} = Ser, Leu, Gly or Pro; X_{B12} = His, Glu, Asp, or Asn; X_{B13} = Ile, Leu, Asp, Val or Ala; X_{B14} = Thr, Ala, Pro, Val or Arg; X_{B15} = Asn, Asp, Ala, Val, Thr, Pro or Glu; X_{B16} = Ala, Ser, Gln, His, Tyr, Arg or Gly; X_{B17} = Thr, Tyr, Pro, Leu or Gly; X_{B18} = Tyr, Met, Val, Gln, Ile, Asp, Gly, Asn or Leu; X_{B19} = Leu, Asp, Gln, Gly, Lys, Glu, Arg or Thr; X_{B20} = Val, Leu or Lys; C_{B21} = Cys, amidated Cys, selenocysteine or amidated selenocysteine; X_{B22} = Val, Tyr, Phe, His, Gly, Leu, amidated His, amidated Val or is absent; X_{B23} = Glu, Arg, Ser, Gly or is absent; X_{B24} = Asp, Val or is absent; X_{B25} = Leu, Val or is absent; X_{B26} = Val, Ile or is absent; X_{B27} = Asn, Pro, Glu or is absent; X_{B28} = Tyr, C, His or is absent; X_{B29} = His, Leu, Tyr or is absent; X_{B30} = Glu, Leu, Ile, Arg or is absent; X_{B31} = Ile, Lys or is absent; X_{B32} = Lys, Leu, Gln or is absent; X_{B33} = Cys or is absent; X_{B34} = Glu, Pro, Val or is absent; X_{B35} = Glu, Gly or is absent; X_{B36} = Glu, Gly or is absent; X_{B37} = Glu, Val or is absent; X_{B38} = Ala, Asp or is absent; and X_{B39} = Ala or is absent.

In another example, the B chain peptide can include the sequence X_{B1}-X_{B2}-Asp-Thr-Pro-Lys-His-Arg-Cys_{B9}-Gly-Ser-Glu-X_{B13}-X_{B14}-X_{B15}-X_{B16}-Tyr-X_{B18}-X_{B19}-Leu-Cys_{B21}-X_{B22} (SEQ ID NO: 007), wherein X_{B1} = Thr or Asn; X_{B2} = Phe or Ser; Cys_{B9} = Cys or selenocysteine; X_{B13} = Ile or Leu; X_{B14} = Thr or Ala; X_{B15} = Asn or Asp; X_{B16} = Ser or Gln; X_{B18} = Met or Val; X_{B19} = Asp or Gln; C_{B21} = Cys, amidated Cys, selenocysteine or amidated selenocysteine; X_{B22} = His, Tyr or is absent; and X_{B23} = Arg or is absent.

In yet another example, the B chain peptide can include the sequence X_{B1}-X_{B2}-X_{B3}-X_{B4}-X_{B5}-X_{B6}-X_{B7}-X_{B8}-Cys_{B9}-Gly-Ser-X_{B12}-X_{B13}-X_{B14}-X_{B15}-X_{B16}-X_{B17}-X_{B18}-X_{B19}-X_{B20}-Cys_{B21}-X_{B22}-X_{B23} (SEQ ID 008), wherein X_{B1} = Thr, Asn or is absent; X_{B2} = Phe, Ser or is absent; X_{B3} = Phe or Asp; X_{B4} = Thr or Val; X_{B5} = Pro, Asn or hydroxyproline; X_{B6} = Lys, Asn or Gln; X_{B7} = His or Tyr; X_{B8} = Arg, Ile or Leu; X_{B12} = His, Asp, Glu or gamma carboxyglutamate; Cys_{B9} = Cys or selenocysteine; X_{B13} = Val, Ile or Leu; X_{B14} = Thr, Ala, Pro, or Val; X_{B15} = Glu, Val, Asn or Asp; X_{B16} = Ser, Gln, Tyr or Ala; X_{B17} = Tyr or Leu; X_{B18} = Tyr, Asp, Met or Val; X_{B19} = Leu, Asp, Gln or Lys; X_{B20} = Leu or Val; C_{B21} = Cys, amidated Cys, selenocysteine or amidated selenocysteine; X_{B22} = Gly, Val, Phe, His, amidated His, amidated Val or is absent; and X_{B23} = Glu, Arg, Gly or is absent.

In a further example, the B chain peptide can include the sequence Asn-Ser-Asp-Thr-Pro-Lys-Tyr-Arg-Cys_{B9}-Gly-Ser-X_{B12}-Ile-Pro-Asn-Ser-Tyr-X_{B18}-Asp-Leu-Cys_{B21} (SEQ ID NO: 009), wherein Cys_{B9} = Cys or selenocysteine; X_{B12} = Glu or Asp; X_{B18} = Met or Ile; and C_{B21} = Cys, amidated Cys, selenocysteine or amidated selenocysteine.

In another example, the B chain peptide can include the sequence X_{B1}-Asn-Gly-Pro-Thr-Asn-His-Ile-Cys_{B9}-Gly-Ser-Asp-Val-Val-Val-Tyr-Tyr-Asp-Lys-Leu-Cys_{B21}-X_{B22}-X_{B23} (SEQ ID NO: 010), wherein X_{B1} is absent; Cys_{B9} = Cys or selenocysteine; C_{B21} = Cys, amidated Cys, selenocysteine or amidated selenocysteine, X_{B22} = Val, Pro or is absent; X_{B23} = Gly, amidated Gly or is absent.

In yet another example, the B chain peptide can include the sequence X_{B1}-Ser-Phe-Gly-Ser-X_{B6}-His-X_{B8}-Cys_{B9}-X_{B10}-Pro-X_{B12}-X_{B13}-X_{B14}-X_{B15}-X_{B16}-X_{B17}-X_{B18}-X_{B19}-X_{B20}-Cys_{B21}- X_{B22}-X_{B23}-X_{B24}-X_{B25}-X_{B26}-X_{B27}-X_{B28}-X_{B29}-X_{B30}-Lys-X_{B32}-Cys_{B33}-X_{B34}-X_{B35}-X_{B36} -X_{B37}-X_{B38}-X_{B39} (SEQ ID 011), wherein X_{B1} = Ser or is absent; X_{B6} = Arg, Gly or Gln; X_{B8} = Thr, Lys or Tyr; Cys_{B9} = C or selenocysteine; X_{B10} = Asp or Gly; X_{B12} = Asp; Glu or Asn; X_{B13} = Asp, Leu or Ala; X_{B14} = Thr or Arg; X_{B15} = Ala, Glu or Pro; X_{B16} = His, Tyr or Glu; X_{B17} = Pro, Leu or Gly; X_{B18} = Gln, Leu or Asn; X_{B19} = Gly, Thr or Arg; X_{B20} = Leu or Lys; C_{B21} = Cys, amidated Cys, selenocysteine or amidated selenocysteine; X_{B22} = Gly, Leu or is absent; X_{B23} = Ser, Glu or is absent; X_{B24} = Asp, Val or is absent; X_{B25} = Leu, Val or is absent; X_{B26} = Val, Ile or is absent; X_{B27} = Pro, Glu or is absent; X_{B28} = Tyr, Cys, His or is absent; X_{B29} = Leu, Tyr or is absent; X_{B30} = Leu, Ile, Arg or is absent; X_{B32} = Leu, Gln or is absent; X_{B34} = Pro, Val or is absent; X_{B35} = Gly, amidated Gly or is absent; X_{B36} = Gly or is absent;

X_{B37} = Val or is absent; X_{B38} = Asp or is absent; and X_{B39} = Ala, amidated Ala, or is absent.

In another embodiment, an A chain peptide can include a sequence selected from the following sequences. It is noted that a "_" is a space indicator that is intended to facilitated alignment of residues.

(SEQ ID NO: 012)

Gly-Val-Val-Glu-His-Cys-Cys-His-Arg-Pro-Cys-Ser-Asn-Ala-Glu-Phe-Lys-Lys-Tyr-Cys

(SEQ ID NO: 013)

Gly-Ile-Val-Glu-Val-Cys-Cys-Asp-Asn-Pro-Cys-Thr-Val-Ala-Thr-Leu-Arg-Thr-Phe-Cys-His

(SEQ ID NO: 014)

Gly-Ile-Val-Glu-Val-Cys-Cys-Asp-Asn-Pro-Cys-Thr-Val-Ala-Thr-Leu-Arg-Thr-Phe-Cys-His

(SEQ ID NO: 015)

Gly-Val-Val-Glu-His-Cys-Cys-His-Arg-Pro-Cys-Ser-Asn-Ala-Glu-Phe-Arg-Lys-Tyr-Cys-Gly

(SEQ ID NO: 016)

Gly-Val-Val-Glu-His-Cys-Cys-His-Arg-Pro-Cys-Ser-Asn-Ala-Glu-Phe-Lys-Lys-Tyr-Cys

(SEQ ID NO: 017)

Gly-Ile-Ala-Cys-Glu-Cys-Cys-Gln-His-Tyr-Cys-Thr-Asp-Gln-Glu-Phe-Ile-Asn-Tyr-Cys

(SEQ ID NO: 018)

Gly-Ile-Ala-Cys-Glu-Cys-Cys-Gln-His-Tyr-Cys-Thr-Asp-Gln-Glu-Phe-Ile-Asn-Tyr-Cys

(SEQ ID NO: 019)

Gly-Ile-Val-Glu-Val-Cys-Cys-Asp-Asn-Pro-Cys-Thr-Val-Ala-Thr-Leu-Met-Thr-Phe-Cys-His

(SEQ ID NO: 020)

Gly-Val-Val-Glu-His-Cys-Cys-His-Arg-Pro-Cys-Ser-Asn-Ala-Glu-Phe-Lys-Lys-Phe-Cys

(SEQ ID NO: 021)

Gly-Val-Val-Glu-His-Cys-Cys-Tyr-Arg-Pro-Cys-Ser-Asn-Ala-Glu-Phe-Lys-Lys-Phe-Cys

(SEQ ID NO: 022)

Gly-Val-Val-Glu-His-Cys-Cys-Lys-Arg-Ala-Cys-Ser-Asn-Ala-Glu-Phe-Met-Gln-Phe-Cys

(SEQ ID NO: 023)

Gly-Ile-Val-Glu-Glu-Cys-Cys-Val-Lys-Ser-Cys-Thr-Asn-Gln-Glu-Phe-Met-Gln-Tyr-Cys

(SEQ ID NO: 024)

Gly-Ile-Val-Glu-Glu-Cys-Cys-Val-Lys-Ser-Cys-Thr-Asn-Gln-Glu-Phe-Met-Gln-Tyr-Cys

(SEQ ID NO: 025)

Gly-Ile-Val-Glu-Glu-Cys-Cys-Asp-Lys-Phe-Cys-Thr-Asp-Asp-Glu-Ala-Arg-Lys-Tyr-Cys

(SEQ ID NO: 026)

Gly-Ile-Val-Cys-Glu-Cys-Cys-Lys-Asn-His-Cys-Thr-Asp-Glu-Glu-Phe-Thr-Glu-Tyr-Cys

(SEQ ID NO: 027)

Gly-Ile-Ala-Cys-Glu-Cys-Cys-Gln-Asn-Tyr-Cys-Thr-Asp-Ala-Glu-Phe-Ser-Lys-Tyr-Cys

(SEQ ID NO: 028)

Gly-Ile-Ala-Cys-Glu-Cys-Cys-Gln-Asn-Tyr-Cys-Thr-Asp-Ala-Glu-Phe-Ser-Lys-Tyr-Cys

(SEQ ID NO: 029)

Gly-Ile-Val-Cys-Glu-Cys-Cys-Lys-Asn-His-Cys-Thr-Asp-Glu-Glu-Phe-Thr-Glu-Tyr-Cys

(SEQ ID NO: 030)

Gly-Ile-Val-Glu-Glu-Cys-Cys-His-Lys-Thr-Cys-Thr-Asp-Asp-Glu-Ala-Arg-Lys-Tyr-Cys

(SEQ ID NO: 031)

Gly-Ile-Val-Cys-Glu-Cys-Cys-Lys-Asn-His-Cys-Thr-Asp-Glu-Glu-Phe-Thr-Glu-Tyr-Cys

(SEQ ID NO: 032)

Val-Ile-Val-Gly-Asp-Cys-Cys-Asp-Asn-Tyr-Cys-Thr-Asp-Glu-Arg-Leu-Lys-Gly-Tyr-Cys

(SEQ ID NO: 033)

Gly-Ile-Val-Glu-Asp-Cys-Cys-Tyr-Asn-Asp-Cys-Thr-Asp-Glu-Lys-Leu-Lys-Glu-Tyr-Cys-His

In another embodiment, a B chain peptide can include a sequence selected from the following. It is noted that a "_" is a space indicator that is intended to facilitated alignment of residues.

(SEQ ID NO: 034)

Thr-Phe-Asp-Thr-Pro-Lys-His-Arg-Cys-Gly-Ser-Glu-Ile-Thr-Asn-Ser-Tyr-Met-Asp-Leu-Cys-Tyr-Arg

(SEQ ID NO: 035)

Asn-Ser-Asp-Thr-Pro-Lys-His-Arg-Cys-Gly-Ser-Glu-Leu-Ala-Asp-Gln-Tyr-Val-Gln-Leu-Cys-His*

(SEQ ID NO: 036)

Asn-Ser-Asp-Thr-Pro-Lys-His-Arg-Cys-Gly-Ser-Glu-Leu-Ala-Asp-Gln-Tyr-Val-Gln-Leu-Cys-His

(SEQ ID NO: 037)

Thr-Phe-Asp-Thr-Pro-Lys-His-Arg-Cys-Gly-Ser-Glu-Ile-Thr-Asn-Ser-Tyr-Met-Asp-Leu-Cys-Tyr-Arg

(SEQ ID NO: 038)

Thr-Phe-Asp-Thr-Pro-Lys-His-Arg-Cys-Gly-Ser-Glu-Ile-Thr-Asn-Ser-Tyr-Met-Asp-Leu-Cys-Tyr-Arg

(SEQ ID NO: 039)

_,_,_,-Thr-His-Glu-His-Thr-Cys-Gln-Leu-Asp-Asp-Pro-Ala-His-Pro-Gln-Gly-Lys-Cys-Gly-Ser-Asp-Leu-Val-Asn-Tyr-His-Glu-Glu-Lys-Cys-Glu-Glu-Glu-Glu-Ala

(SEQ ID NO: 040)

_,_,_,-Thr-His-Glu-His-Thr-Cys-Gln-Leu-Asp-Asp-Pro-Ala-His-Pro-Gln-Gly-Lys-Cys-Gly-Ser-Asp-Leu-Val-Asn-Tyr-His-Glu-Glu-Lys-Cys-Glu-Glu-Glu-Glu-Ala

(SEQ ID NO: 041)

Asn-Ser-Asp-Thr-Pro-Lys-His-Arg-Cys-Gly-Ser-Glu-Leu-Ala-Asp-Gln-Tyr-Val-Gln-Leu-Cys-His

(SEQ ID NO: 042)

Asn-Ser-Asp-Thr-Pro-Lys-Tyr-Arg-Cys-Gly-Ser-Glu-Ile-Pro-Asn-Ser-Tyr-Ile-Asp-Leu-Cys

(SEQ ID NO: 043)

Asn-Ser-Asp-Thr-Pro-Lys-Tyr-Arg-Cys-Gly-Ser-Asp-Ile-Pro-Asn-Ser-Tyr-Met-Asp-Leu-Cys

(SEQ ID NO: 044)

Asn-Ser-Asp-Thr-Pro-Lys-Tyr-Arg-Cys-Gly-Ser-Asp-Ile-Pro-Asn-Ser-Tyr-Met-Asp-Leu-Cys

(SEQ ID NO: 045)

_,-Asn-Gly-Pro-Thr-Asn-His-Ile-Cys-Gly-Ser-Asp-Val-Val-Val-Tyr-Tyr-Asp-Lys-Leu-Cys

(SEQ ID NO: 046)

_,-Asn-Gly-Pro-Thr-Asn-His-Ile-Cys-Gly-Ser-Asp-Val-Val-Val-Tyr-Tyr-Asp-Lys-Leu-Cys

(SEQ ID NO: 047)

_,-Ser-Phe-Gly-Ser-Gln-His-Thr-Cys-Asp-Pro-Asn-Ala-Thr-Ala-Gly-Gly-Asn-Arg-Leu-Cys-Gly-Gly-Asp-Val-Ile-Pro-Cys-Leu-Leu-Lys-Leu-Cys-Pro-Gly

(SEQ ID NO: 048)

_,-Ser-Phe-Gly-Ser-Gln-His-Thr-Cys-Asp-Pro-Asn-Ala-Thr-Ala-Gly-Gly-Asn-Arg-Leu-Cys-Gly-Gly-Asp-Val-Ile-Pro-Cys-Leu-Leu-Lys-Leu-Cys-Pro-Gly

(SEQ ID NO: 049)

_,-Ser-Phe-Gly-Ser-Arg-His-Tyr-Cys-Asp-Pro-Asp-Asp-Arg-Pro-His-Pro-Gln-Gly-Lys-Cys-Gly-Ser-Val-Leu-Val-Glu-His-Tyr-Ile-Lys-Gln-Cys-Val-Gly-Gly-Val-Asp-Ala

(SEQ ID NO: 050)

_,-Ser-Phe-Gly-Ser-Arg-His-Tyr-Cys-Asp-Pro-Asp-Asp-Arg-Pro-His-Pro-Gln-Gly-Lys-Cys-Gly-Ser-Asp-Leu-Val-Glu-Tyr-Tyr-Arg-Lys-Gln-Cys-Val-Gly-Gly-Val-Asp-Ala

(SEQ ID NO: 051)

Ser-Phe-Gly-Ser-Gln-His-Thr-Arg-Gly-Ile-Lys-Cys-Gly-Pro-Glu-Leu-Thr-Glu-Tyr-Leu-Leu-Thr-Leu-Cys-Leu

(SEQ ID NO: 052)

_,-Ser-Phe-Gly-Ser-Gln-His-Thr-Cys-Asp-Pro-Asn-Ala-Thr-Ala-Gly-Gly-Asn-Arg-Leu-Cys-Gly-Gly-Asp-Val-Ile-Pro-Cys-Leu-Leu-Lys-Leu-Cys-Pro

(SEQ ID NO: 053)

Ser-Phe-Gly-Ser-Gln-His-Thr-Arg-Gly-Ile-Lys-Cys-Gly-Pro-Glu-Leu-Thr-Glu-Tyr-Leu-Leu-Thr-Leu-Cys-Leu

(SEQ ID NO: 054)

_,-Thr-Pro-Asp-Arg-Asp-His-Ser-Cys-Gly-Gly-Glu-Leu-Val-Asp-Arg-Leu-Val-Lys-Leu-Cys

(SEQ ID NO: 055)

_,-Thr-Leu-Val-Arg-Arg-Arg-Leu-Cys-Gly-Ser-Glu-Leu-Val-Thr-Tyr-Leu-Gly-Glu-Leu-Cys

In some embodiments, the insulin analogs can include various amino acid modifications, some of which are indicated with the sequences herein. For example, in some cases, one or more Glu residues can be replaced with γ-carboxyglutamate (Gla), for example at the A04 or A05 Glu positions of various A chains, or at the B12 Glu position of some B chains (the position may vary, depending on the sequence). In another example, one or more Pro residues can be replaced with hydroxyproline (Hyp), for example at the B05 Pro position of certain B chains. Additionally, C-terminal ends can be amidated, such as an amidated Cys (*) at the terminal end of various A chains, among others.

In another example embodiment, an insulin analog chimera is provided including a native human insulin or modified human insulin A chain peptide and a

synthetic B chain peptide. One non-limiting example of a native human A chain peptide can include the sequence of Gly-Ile-Val-Glu-Gln-Cys-Cys-Thr-Ser-Ile-Cys-Ser-Leu-Tyr-Gln-Leu-Glu-Asn-Tyr-Cys-Asn (SEQ ID NO: 056). The synthetic B chain peptide can include any of the B chain peptides disclosed herein that compliment, and therefore provide insulin functionality with, the native human A chain peptide. In one specific example, however, the synthetic B chain peptide can include the sequence of SEQ ID NO: 008. Other example B chain peptides include SEQ ID NO: 006, SEQ ID NO: 007, SEQ ID NO: 009-011, and SEQ ID NO: 0034-0055.

In another example embodiment, an insulin analog chimera is provided including a human insulin analog A chain peptide and a synthetic B chain peptide. One non-limiting example of a human insulin analog A chain peptide can include the sequence of Gly-X_{A2}-Val-Glu-X_{A5}-Cys_{A6}-Cys_{A7}-X_{A8}-X_{A9}-X_{A10}-Cys_{A11}-Ser-X_{A13}-X_{A14}-X_{A15}-X_{A16}-X_{A17}-X_{A18}-Tyr-Cys_{A20}-X_{A21} (SEQ ID NO: 057), wherein X_{A2} = Ile or Val; X_{A5} = Gln or His; Cys_{A6}, Cys_{A7}, and C_{A11} are independently Cys or selenocysteine; X_{A8} = Thr or His; X_{A9} = Ser or Arg; X_{A10} = Ile or Pro; X_{A13} = Leu or Asn; X_{A14} = Tyr or Ala; X_{A15} = Gln or Glu; X_{A16} = Leu or Phe; X_{A17} = Glu or Lys; X_{A18} = Asn or Lys; Cys_{A20} = Cys, selenocysteine, amidated Cys, or amidated selenocysteine; Cys_{A20} = Cys, selenocysteine, amidated Cys, or amidated selenocysteine; and X_{A21} = Asn or absent. In another embodiment, the human insulin analog A chain peptide can include the sequence Gly-Ile-Val-Glu-His-Cys_{A6}-Cys_{A7}-X_{A8}-X_{A9}-X_{A10}-Cys_{A11}-Ser-Leu-Tyr-Gln-Leu-Glu-Asn-Tyr- Cys_{A20}-Asn (SEQ ID NO: 058), wherein X_{A8} = Asp or His; X_{A9} = Lys, Asn or Arg; X_{A10} = Ser, Phe or Pro; and Cys_{A20} = Cys, selenocysteine, amidated Cys, or amidated selenocysteine.

The synthetic B chain peptide can include any of the B chain peptides disclosed herein that compliment, and therefore provide insulin functionality with, the human insulin analog A chain peptide. In one specific example, however, the synthetic B chain peptide can include the sequence of SEQ ID NO: 008. Other example B chain peptides include SEQ ID NO: 006, SEQ ID NO: 007, SEQ ID NO: 009-011, and SEQ ID NO: 0034-0055.

In another example embodiment, an insulin analog chimera is provided including a cone snail insulin analog A chain peptide and a synthetic B chain peptide derived from a modified human insulin B chain. One non-limiting example of a cone snail insulin analog A chain peptide can include the sequence SEQ ID NO: 001. In

another example, a cone snail insulin analog A chain peptide can include the sequence SEQ ID NO: 012. In other aspects, the cone snail insulin analog A chain peptide can include a sequence selected from SEQ ID NO: 003-005 or SEQ ID NO: 013-033.

The synthetic B chain peptide derived from a modified human insulin B chain can include any modified human B chain sequence that results in an active insulin analog. In one example, the modified human insulin B chain can include the sequence Gly-Val-Val-Glu-Gln-Cys_{A6}-Cys_{A7}-Thr-X_{A9}-X_{A10}-Cys_{A11}-Ser-Leu-X_{A14}-Gln-Leu-Glu-Asn-Tyr-Cys_{A20} (SEQ ID NO: 059), wherein Cys_{A6}, Cys_{A7}, and C_{A11} are independently Cys or selenocysteine; X_{A9} = Ser or Gly; X_{A10} = Ile or Val; X_{A14} = Tyr, Ala or His; and Cys_{A20} = Cys, selenocysteine, amidated Cys, or amidated selenocysteine. In another example, the modified human insulin B chain can include the sequence Gly-Val-Val-Glu-Gln-Cys_{A6}-Cys_{A7}-Thr-Ser-Ile-Cys_{A11}-Ser-Leu-Ala-Gln-Leu-Glu-Asn-Tyr-Cys_{A20} (SEQ ID NO: 060), wherein Cys_{A6}, Cys_{A7}, and C_{A11} are independently Cys or selenocysteine; and Cys_{A20} = Cys, selenocysteine, amidated Cys, or amidated selenocysteine. In yet another example, the modified human insulin B chain can include the sequence Gly-Val-Val-Glu-Gln-Cys_{A6}-Cys_{A7}-Thr-Ser-Ile-Cys_{A11}-Ser-Asn-Ala-Glu-Phe-Glu-Lys-Tyr-Cys_{A20} (SEQ ID NO: 061), wherein Cys_{A6}, Cys_{A7}, and C_{A11} are independently Cys or selenocysteine; and Cys_{A20} = Cys, selenocysteine, amidated Cys, or amidated selenocysteine.

Insulin analog peptides can be made by any known technique or method, and any of such techniques or methods are considered to be within the present scope. Example techniques can include peptide synthesis, recombinant expression, a combination of peptide synthesis and recombinant expression, and the like. Example techniques are described further in the Examples section.

Various insulin analog formulations are provided for the treatment of insulin-related conditions such as diabetes myelitis, hyperglycemia, and the like, as well as for diabetes drug discovery and for other research activities. Such a formulation can generally include any independent combination of A chain peptide sequence and B chain peptide sequence described herein, including equivalents thereof. In some examples, the formulation can be a soluble monomeric insulin analog. In other examples, the formulation can be at least partially in a multimeric configuration. Formulations can generally be for subcutaneous or other parenteral injection, for continuous or intermittent infusion, or any other available administration route.

Such formulations can comprise an insulin analog as described herein, including pharmaceutically acceptable salt of the insulin analog, in a pharmaceutical carrier. In some examples the formulation can independently include various preservatives, stabilizing agents, isotonicity agents, solubilizers, metal ions, and the like, including combinations thereof. Such ingredients are generally well known in the art. Pharmaceutical compositions can generally be prepared according to conventional pharmaceutical techniques. See, for example, Remington: The Science and Practice of Pharmacy, 21st Ed., Lippincott Williams & Wilkins, Philadelphia, 2005.

Any pharmaceutical carrier that can be used in an insulin analog formulation is considered to be within the present scope. Non-limiting examples of carriers can include sodium phosphate, sodium acetate, sodium citrate, TRIS, arginine, such as L-arginine, and the like, including combinations thereof. The pH of the formulations is controlled by the buffering of the pharmaceutical carrier. The concentration of the buffering component of the carrier can be such as to provide adequate buffering of the pH during storage, which is well known to those skilled in the art. It is noted that the term "TRIS" refers to 2-amino-2-hydroxymethyl-1,3,-propanediol, and to any pharmacologically acceptable salt thereof. The free base and the hydrochloride form are two common forms of TRIS. TRIS is also known in the art as trimethylol aminomethane, tromethamine, and tris(hydroxymethyl)aminomethane.

An optional isotonicity agent is a compound that is physiologically tolerated, and that imparts a suitable tonicity to a formulation to prevent or otherwise limit the net flow of water across cell membranes that are in contact with the formulation. Compounds such as glycerin, for example, are commonly used, concentrations of which are well known. Other potential isotonicity agents include salts, such as sodium chloride, dextrose, lactose, and the like.

The term "salt", as used herein, denotes acidic and/or basic salts, formed with inorganic or organic acids and/or bases, preferably basic salts. While pharmaceutically acceptable salts are generally preferred, particularly when employing the insulin analogs as medicaments, other salts find utility, for example, in processing these compounds, or where non-medicament-type uses are contemplated. Salts of these compounds may be prepared by art-recognized techniques.

The insulin analog is preferably administered in a therapeutically effective amount. By a "therapeutically effective amount" or simply "effective amount" of the

insulin analog is meant a sufficient amount of the compound to treat the desired condition at a reasonable benefit/risk ratio applicable to any medical treatment. The actual amount administered, and the rate and time-course of administration, will depend on the nature and severity of the condition being treated. Prescription of treatment, e.g. decisions on dosage, timing, etc., is within the responsibility of general practitioners or specialists, and typically takes account of the nature of the insulin disorder, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners.

Examples

The following examples pertain to specific embodiments and point out specific features, elements, or steps that can be used or otherwise combined in achieving such embodiments.

Con-Ins G1 Synthesized Using a Selenocysteine-Based Strategy.

A strategy incorporating a pair of A chain selenocysteines (Sec) was used to synthesize Con-Ins G1 (A chain SEQ ID NO: 012) (B chain SEQ ID NO: 034). Diselenide bond-containing peptide analogs have similar biological activities to their native peptides and, in some cases, even improved potency or selectivity. Due to the lower redox potential, diselenide bond formation is favored over the disulfide bond formation under acidic conditions. This Cys-to-Sec replacement strategy was combined with orthogonal protection of the remaining two pairs of cysteines to sequentially form intra- and intermolecular disulfide bridges, as shown in FIGs. 1A-F. CysA6 and CysA11 were replaced with Sec residues, which formed an intradiselenide bridge after peptide cleavage and reduction (FIG. 1A). CysA20 and CysB21 of the respective chains (synthesized with a trityl (Trt) protecting group) were used to form the first intermolecular disulfide bridge on DMSO treatment (FIG. 1C). CysA7 and CysB9, bearing acetaminomethyl (Acm) groups, were subjected to iodine oxidation to remove Acm protection and simultaneously form the second disulfide bridge (FIG. 1E). The two-step ligation protocol led to the purified product, synthetic selenocysteine-Con-Ins G1 (sCon-Ins G1) Gly-Val-Val-Glu-His-Sec-Cys-His-Arg-Pro-Sec-Ser-Asn-Ala-Glu-Phe-Lys-Lys-Tyr-Cys (A chain SEQ ID NO: 062) (B chain SEQ ID NO: 034), in a total yield of 10.5%, based on the starting amount of purified

chain A. RP-HPLC confirmed the purity (95%), and electrospray ionization MS sequencing confirmed the correct identity of the product (FIG. 2).

FIGs. 1A-F show synthesis of sCon-Ins G1. (A) Purified chain A with the intramolecular Sec-Sec bridge formed and Cys-7 protected with the Acm group. (B) Purified chain B with Cys-9 protected with the Acm group. (C) sCon-Ins G1 heterodimer formation by treatment with 20% DMSO for 30 h in 0.1 M Tris·HCl containing 1 mM EDTA, pH 7.5. (D) Purified sCon-Ins G1 heterodimer. (E) Oxidation of remaining disulfide bond of the heterodimer of sCon-Ins G1 by treatment with I2 in the mixture of acetonitrile, water, and TFA at 200 μM for 5 min. (F) Purified final sCon-Ins G1.

FIG. 2 shows MS analysis of synthetic sCon-Ins G1. The integrity of synthetic sCon-Ins G1 was determined by electrospray ionization MS at the Salk Institute for Biological Studies, La Jolla, CA. The monoisotopic MH+1 ion was 5238.1004. The inset shows the isotopic distribution.

sCon-Ins G1 Lowers Blood Glucose and Alters Swimming Activity in Fish.

The streptozotocin (STZ)-induced model of hyperglycemia was used to assess whether sCon-Ins G1 could effectively lower blood glucose levels in a model prey: adult zebrafish. Animals were first rendered hyperglycemic through i.p. injection of the β-cell poison STZ (1.5 g/kg), and the effect of subsequent injection of sCon-Ins G1 was examined. Following STZ treatment, blood glucose levels were significantly elevated from 65.9 ±4.75 ($n = 11$) to 273.0 ± 34.08 mg/dL ($n = 5$, $P < 0.01$; FIG. 3A). Administration of sCon-Ins G1 at 65 ng peptide/g body weight significantly lowered blood glucose to 74.0 ± 8.051 mg/dL ($n = 8$, $P < 0.01$). The ability to effectively lower blood glucose was similar to human insulin (92.0 ± 17.35 mg/d, $n = 6$, $P < 0.01$).

To determine whether sCon-Ins G1 elicits an effect when applied to the water column, the spontaneous swimming behavior of fish larvae (7 d after fertilization, $n = 3$) was monitored before (baseline) and following application of 25 nmol/mL peptide. Administration of sCon-Ins G1 reduced overall locomotor activity, observed as a significant decrease in the percentage of time spent swimming ($P = 0.038$) and movement frequency ($P = 0.003$) compared with baseline values for the same animals (FIG. 3B). It has previously been reported that externally applied human insulin can induce insulin shock in fish via direct absorption through the gills into the

bloodstream. The present findings suggest that sCon-Ins G1 also efficiently crosses the endothelium of the gill plexus.

FIGs. 3A-B show that sCon-Ins G1 lowers blood glucose and disrupts swimming behavior in zebrafish. (A) Insulin activity was determined using the streptozotocin (STZ)-induced model of hyperglycemia. Injection of 1.5 g/kg STZ into adult zebrafish caused hyperglycemia as evident by a significant increase of blood glucose (n = 5 fish, P = 0.003) compared with controls (n = 11). Hyperglycemia was successfully reversed on administration of 65 ng/g sCon-Ins G1 (n = 8, P = 0.003) and human insulin (65 ng/g, n = 6, P < 0.003). Data were analyzed in Prism Graphpad software (version 6.0) using unpaired t tests with Welch's correction. Plotted dots represent individual fish. Bold lines and error bars represent means ± SD. (B) Application of sCon-Ins G1 into the water column (25 nmol/mL) significantly reduced overall locomotor activity, observed as a significant decrease in the percentage of time spent swimming (P = 0.038, left y axis) and movement frequency (P = 0.003, right y axis) compared with baseline values for the same animals. Values represent means ± SEM (n = 3).

Solid phase peptide synthesis and purification of selenocysteine-containing Con-Ins G1 (sCon-Ins G1), sCon-Ins without modifications (sCon-Ins G1[^{A4}E;^{B5}P;^{B12}E]) and native-like Con-Ins G1

sCon-Ins G1 (A chain SEQ ID NO: 062) (B chain SEQ ID NO: 034) containing Cys^{A6,11} to Sec ^{A6,11} modifications in the A chain was chemically synthesized, purified and oxidized as described above with the exception that corrected extinction coefficients were used for the quantification of the B chain (2,980 $M^{-1}\cdot cm^{-1}$) and fully oxidized sCon-Ins G1 (4,470 $M^{-1}\cdot cm^{-1}$). Synthesis of sCon-Ins G1[^{A4}E;^{B5}P;^{B12}E] was performed as described for sCon-Ins G1. The stepwise formation of disulfide bonds of sCon-Ins G1[^{A4}E;^{B5}P;^{B12}E] and synthesis of native like Con-Ins G1 are is described in detail below.

sCon-Ins G1[^{A4}E] chain A: cleavage, DTT reduction and purification.

The peptide was cleaved from 125 mg of resin for 1.5 h using 1 mL of enriched Reagent K. Reagent K was prepared using 2 mL TFA (Fisher Scientific, Fair Lawn, NJ), 66 µL H_2O, 12 mg 2,2-dithiobis(5-nitropyridine) (DTNP; Aldrich; Saint Louis, MO), and 150 mg phenol, followed by addition of 25 µL thioanisole. The

cleavage mixture was filtered and precipitated with 10 mL of cold methyl-tert-butyl ether (MTBE; Fisher Scientific, Fair Lawn, NJ). The crude peptide was precipitated by centrifugation at 7,000 × g for 6 min and washed once with 10 mL cold MTBE. To induce the intramolecular diselenide bond formation (Sec^{A6} to Sec^{A10}), the washed peptide pellet was dissolved in 50% acetonitrile (ACN, Fisher Scientific; Fair Lawn, NJ) (vol/vol) in water and 2 mL of 100 mM dithiotreitol (DTT, EMD Chemicals, Gibbstown, NJ) in 1 mL 0.2 M Tris·HCl (Sigma, St Louis, MO) containing 2 mM EDTA (Malinckrodt, St. Louis, MO), pH 7.5, 1 mL of water was added and vortexed gently, and the reaction was allowed to proceed for 2 h. It was then quenched with 8% formic acid (vol/vol), diluted with 0.1% TFA (vol/vol) in water, and purified by reversed-phase (RP) HPLC using a semi-preparative C18 Vydac column (218TP510, 250 × 10 mm, 5-μm particle size; Grace, Columbia, MD) eluted with a linear gradient ranging from 10% to 40% solvent B in 30 min at a flow rate 4 mL/min. The HPLC solvents were 0.1% (vol/vol) TFA in water (solvent A) and 0.1% TFA (vol/vol) in 90% aqueous ACN (vol/vol) (solvent B). The eluent was monitored by measuring absorbance at 220 and 280 nm. Purity of the peptide was assessed by analytical C18 Vydac RP-HPLC (218TP54, 250 × 4.6 mm, 5-μm particle size, Grace, Columbia, MD) using a linear gradient ranging from 10% to 40% of solvent B in 30 min with a flow rate 1 mL/min. The peptide was quantified by UV absorbance at 280 nm using an extinction coefficient (ε) of 1,490 $M^{-1}\cdot cm^{-1}$. Out of 135 mg of the resin 3.8 mg of chain A was obtained. The mass of the peptide was confirmed by electrospray ionization (ESI)-MS (calculated monoisotopic MH^{+1}: 2,473.674, determined monoisotopic: MH^{+1} 2, 472.924) at the Salk Institute for Biological Studies. Molecular masses were calculated using ProteinProspector (version 5.12.1).

sCon-Ins G1[^{B5}P;^{B12}E] chain B: cleavage and purification

The peptide was cleaved from 94 mg resin by a 3-h treatment with 1 mL of Reagent K (TFA/water/ phenol/ thioanisole/1,2-ethanedithiol 82.5/5/5/5/2.5 by volume) and subsequently filtered, precipitated, and washed as described above. The washed peptide pellet was purified as described above with the exception that the gradient ranged from 15% to 45% solvent B. The same gradient was used to assess the purity of the linear peptide as described above, and peptide quantitation was carried out using ε value of 2,980 $M^{-1}\cdot cm^{-1}$. From 94 mg of the cleaved resin, 2.37 mg of chain B was obtained. The mass of the peptide was confirmed by ESI MS

(calculated monoisotopic MH^{+1}: 2,808.24 Da, determined monoisotopic MH^{+1}: 2,808.25 Da).

Copper (Cu)-assisted chain A and chain B ligation to form sCon-Ins G1[^{A4}E;^{B5}P;^{B12}E]

A total of 100 nmol of each chain was combined and dried using a SpeedVac. The peptide mixture was dissolved in 100 µL of 0.1% TFA (vol/vol) and added to a mixture of 800 µL $CuCl_2 \cdot H_2O$ (J.T. Baker; Phillipsburg, NJ) 100 µL 1M Tris·HCl containing 10 nM EDTA, pH 7.5. The final peptide concentration was 100 µM. The reaction was left for 24 h at room temperature and then quenched with 8% formic acid (vol/vol), diluted with 0.1% TFA and purified by RP-HPLC using a preparative C18 Vydac column eluted with a linear gradient ranging from 15% to 45% of solvent B in 30 min at a flow rate 4 mL/min. The purity of sCon-Ins G1 (including an A and B chain connected by one disulfide bond) was assessed by analytical RP-HPLC using the same gradient as for the semi-preparative purification, at a flow rate 1 mL/min. sCon-Ins G1[^{A4}E;^{B5}P;^{B12}E] was quantified at 280 nm using ε value of 4,470 $M^{-1} \cdot cm^{-1}$. The yield of the reaction was 28% (both 90% pure and 73% pure material was accounted). From 900 nmol of the 1:1 mixture of chain A and B, 1.36 mg of the desired product was obtained. The identity of the peptide was confirmed by ESI-MS (calculated monoisotopic MH^{+1}: 5,278.15; determined monoisotopic MH^{+1}: 5278.15).

Iodine (I_2) assisted formation of fully folded sCon-Ins G1[^{A4}E;^{B5}P;^{B12}E]

A solution of I_2 (Acros Organics, Geel, Belgium) was prepared as follows: 10 mg of I_2 was added to 5 mL of ACN. After 20 min of stirring, the I_2 was completely dissolved, and 15 mL of water and 600 µL of TFA were added. A total of 300 µL of the I_2 mixture was added to 149 nmol (90% purity) and 106 nmol (72% purity) of sCon-Ins G1[^{A4}E;^{B5}P;^{B12}E] dissolved in 300 µL of 0.1% TFA each. Reactions were incubated for 5 min, quenched with 10 µL of 1 M L-ascorbic acid (Sigma, St. Louis, MO), diluted with 0.1% TFA in water to a total volume of 4.5 mL and purified as described for partially folded Con-Ins G1[^{A4}E;^{B5}P;^{B12}E]. The purity of the final product (fully folded sCon-Ins G1[^{A4}E;^{B5}P;^{B12}E]) was assessed by analytical C18 RP-HPLC using the same gradient as for the semi-preparative purification, at a flow rate 1 mL/min, and was determined to be 97% (FIG. 4). sCon-Ins G1[^{A4}E;^{B5}P;^{B12}E] was quantified as described for the partially folded product. The yield of the reaction was

22%. The identity of the peptide was confirmed by ESI-MS (calculated monoisotopic MH^{+1}: 5,134.84; determined monoisotopic MH^{+1}: 5,134.07).

FIG. 4 shows sequence and HPLC profile of fully oxidized sCon-Ins G1[^{A4}E;^{B5}P;^{B12}E]. HPLC conditions: C18 Vydac RP-HPLC column, linear gradient ranging from 15% to 45% of solvent B in 30 min with 1 mL / min flow rate monitored at 220 nm. U: selenocysteines. #: amidated C-terminus

Synthesis and Purification of native like Con-Ins G1

Both A and B chains of Con-Ins G1 were synthesized with Fmoc (9-fluorenmethyloxycarbonyl) chemistry on the CEM Liberty 1 automated microwave peptide synthesizer (CEM Corporation, Matthews, NC). For the synthesis of A chain pre-loaded Fmoc-Cys(Trt)-Rink Amide MBHA resin (0.21 mmol/g) (Peptides International, Louisville, KY) and for the synthesis of B chain pre-loaded Fmoc-Arg(Pbf)-Wang resin (0.4 mmol/g) (AnaSpec, EGT., Freemont, CA) were used. Fmoc-N^{α}-protected amino acids with side chain protection were from commercial sources: Bachem Inc. (Torrance, CA), Chem-Impex International (Wood Dale, IL), Genzyme (Cambridge, MA), Novabiochem (San Diego, CA), P3 Biosystem (Louisville, KY) and Reanal (Budapest, Hungary). Fmoc-γ-carboxy-L-glutamic acid γ,γ-di-t-butyl ester (*Fmoc-Gla(OtBu)$_2$-OH)* was synthesized in house. Side- chain protection for the amino acids was as follows: Lys, *tert*-butyloxycarbonyl (Boc); Hyp, Ser, Thr and Tyr, *tert*-butyl ether (tBu); Asn, Cys, and His trityl (Trt); Arg 2,2,4,6,7-pentamethyl-dihydroxybenzofuran-5-sulfonyl (Pbf); Glu, Gla, and Asp *tert*-butyl ester (OtBu); Cys, acetamidomethyl (Acm); Cys, 4-methoxytrityl (Mmt); Cys, S-*tert*-butylthionyl (S-t-Bu). To be able make the correct intra- and intermolecular disulfide bonds, the side chain of ^{A7}Cys and ^{B9}Cys was Acm, the side chain of ^{A20}Cys and ^{B21}Cys was Trt, the side chain of ^{A11}Cys was Mmt, and the side chain of ^{A6}Cys was S-*t*-Bu protected. Both chains were synthesized on a 0.1 mmol scale. Coupling reactions were performed on the resin in the presence of 5-fold molar excess of Fmoc-protected amino acids dissolved in DMF (except His in NMP) with activation by HATU [2-(1H-9-(Azabenzotriazol-1-yl)-1,1,3,3-tetramethyl-aminium hexafluorophosphate]: DIEA [N,N-diisopropylethylamine] : AA [protected amino acids] (0.9 : 2 : 1) at 0 W for 2 minutes then at 35 W with a maximum temperature of 60 °C for 10 minutes. Arg was always double coupled at room temperature for 25 minutes then at 15 W with a

maximum temperature of 50 °C for 12 min. Cys, His, and Gla were coupled at 40 W with a maximum temperature of 50 °C for 6 min. Deprotection of the Fmoc group was performed with 20% piperidine containing 0.1 M HOBt in DMF in two stages (using a fresh reagent each time): with an initial deprotection of 2 minutes at 35 W followed by 5 min deprotection at 35 W with a maximum temperature of 60 °C.

Con-Ins G1 chain A: cleavage, intramolecular disulfide bond formation and purification

The intramolecular disulfide bridge between ^{A6}Cys and ^{A11}Cys was formed on the resin using a non-oxidative method. In the first step, S-*t*-Bu of ^{A6}Cys was removed by reduction to liberate free thiol by treating the resin (760 mg) with 20% mercaptoethanol (ME) (Fluka) and 1% *N*-Methylmorpholine (NMM) in dimethylformamide (DMF) 8 mL overnight at room temperature. The resin was washed with DMF and dried. The resin was then reacted with a 10-fold excess of 2,2'-dithiobis(5-nitropyridine) (DTNB) ~ 1 mmol (Sigma-Aldrich, St Louis, MO) in dichloromethane (DCM) 8 mL for 1 h to form the S-5-nitropyridin-sufenyl (5-Npys) protected ^{A6}Cys. After washing out the excess of the reagents with DCM, the resin was treated with 1% trifluoroacetic acid (TFA) in dichloromethane (DCM) 8 mL in the presence of 2 µL triisopropylsilane (TIS) as a scavenger for 20 minutes to deprotect ^{A11}Cys(Mmt) and to form the disulfide bridge between ^{A6}Cys and ^{A11}Cys at the same time. Cleavage from the resin (720 mg) and simultaneous deprotection of chain A were performed by stirring the resin with 10 mL of a reagent containing (TFA/water/TIS : 95/2.5/2.5) for 2 h. It was followed by precipitation of the peptide using ice-cold anhydrous ethyl ether, then extraction with 0.1% TFA/40% water/60% acetonitrile and lyophilization. The peptide was purified by preparative Waters HPLC (Milford, MA) on Waters PrepPak cartridge (2.5 x 10 cm) packed with Bondapak C_{18} (15-20 µm particle size, 300 Å) in solvent system A: 0.1% TFA/water, B: 0.1% TFA/40% water/60% acetonitrile with a linear gradient ranging from 5% to 65% solvent B in 60 min at a flow rate 20 mL/min. 18.7 mg (7.7 µmol) of chain A was obtained. The mass of the peptide was confirmed by electrospray ionization (ESI)-MS measured on a ThermoScientific LTQ Orbitrap XL (Waltham, MA) instrument (calculated monoisotopic MH^{+1}: 2422.03 Da; determined monoisotopic MH^{+1}value 2422.01 Da).

Con-Ins G1 chain B: cleavage and purification

Cleavage from the resin and simultaneous deprotection of chain B were performed by stirring the resin 500 mg with 10 mL reagent containing (TFA/thioanisol/3,6-Dioxa-1,8-octanedithol (DODT, TCI America, Portland, OR)/water: 87.5/5/2.5/5) for 2 h. It was followed by precipitation of the peptide using ice-cold anhydrous ethyl ether, then extraction with 0.1% TFA/40% water/60% acetonitrile and lyophilization. The peptide was purified by preparative HPLC (as described for purification of chain A, except that the gradient ranged from 20% to 80%B in 60 min) 25.9 mg (9 μmol) of chain B was obtained. The mass of the peptide was confirmed by ESI-MS (calculated monoisotopic MH^{+1}value 2868.24 Da; determined monoisotopic MH^{+1}value 2868.22 Da).

DMSO assisted chain A and chain B ligation to form partially folded Con-Ins G1 (containing one disulfide bond)

Chain A and chain B (7 μmol each) were dissolved together in 0.1% TFA/water solution (7.1 mL) and added to a mixture of 14.5 mL DMSO, 14.25 mL water, 35.6 mL 0.2 M Tris containing 2 mM EDTA, pH 7.5. The oxidation was monitored by analytical HPLC. After 25 h at room temperature, the reaction was quenched with 8% formic acid (1 mL), diluted with 0.1%TFA to a total volume of 225 mL and purified by preparative HPLC with a gradient ranged from 15% to 75%B in 60 min. 4.5 mg (0.85 μmol, 12.1% yield based on the starting amount of 7 μmol) of heterodimer was obtained. The identity of the peptide was confirmed by ESI-MS (calculated monoisotopic MH^{+1}: 5287.25 Da; determined monoisotopic MH^{+1}: 5287.19 Da).

I_2 assisted oxidation to form fully oxidized Con-Ins G1

4.5 mg (0.85 μmol) of Con-Ins G1 (partially folded) was dissolved in 6.2 mL of 2.5% TFA/water solution and 55 μL of I_2 solution (50 mg I_2 in 5 mL MeOH) was added and stirred for 60 min. It was quenched by adding 1M ascorbic acid solution till the yellow color of the solution became clear. The reaction was diluted with 60 mL water and loaded on preparative RP-HPLC column. 1.5 mg (0.29 μmol) of fully oxidized Con-Ins G1 was obtained (yield 35 % based on the starting amount of the partially folded product containing one interchain disulfide bond and 4 % based on the starting amount of purified chain A). The identity of the peptide was confirmed by

ESI-MS on a ThermoScientific LTQ Orbitrap XL (Waltham, MA) mass spectrometer (calculated monoisotopic MH^{+1}: 5143.16 Da; determined monoisotopic MH^{+1}: 5143.16 Da). Purity of the peptide was assessed by RP-HPLC and capillary electrophoresis. Quantitative RP-HPLC was performed using a GE Healthcare AKTApurifier 10 (Pittsburgh, PA) and a Phenomenex (Torrance, CA) Kinetex XB-C18 column (4.6 x 100 mm, 5.0 µm particle size, 100 Å pore size). The solvent system was compri<sed of solvent A = 0.1% TFA in water and solvent B = 60% CH_3CN, 40% A. A gradient was performed from 20%B to 80%B in 30 min at a flow rate of 1.0 mL/min. Detection was at 214 and 280 nm. The purity of the peptide was determined to be 89% (FIG. 5). Capillary electrophoresis (CE) was performed using a Groton Biosystems GPA 100 instrument. (Boxborough, MA) The electrophoresis buffer was 0.1 M sodium phosphate (15% acetonitrile), pH 2.5. Separation was accomplished by application of 20 kV to the capillary (0.75 µm x 100 cm). Detection was at 214 nm. The assessed purity of the peptide was 80%.

FIG. 5 shows sequence and HPLC profile of fully oxidized Con-Ins G1. HPLC conditions: C18 Vydac RP-HPLC column, linear gradient ranging from 15% to 45% of solvent B in 30 min with 1 mL / min flow rate monitored at 220 nm. O: hydroxyproline, γ: γ-carboxyglutamate, #: amidated C-terminus.

Analytical Ultracentrifugation

Analytical ultracentrifugation was conducted at 20°C using a Beckman XLI analytical centrifuge in 12 mm path-length cells. ConInsG1 was diluted from a 10 mg/ml stock in 10 mM HCl into 10 mM Tris, 50 mM NaCl, pH 7.4 to a final concentration of 100 µg/ml. An equal volume of 10 mM NaOH was added to neutralize any pH change. A total sample volume of 100 µl was used. Identical samples were prepared also containing 0.2 mM $ZnCl_2$, 2 mM $CaCl_2$, 1 mM sodium phosphate (pH 7.4) or 0.1 M ammonium sulfate. Radial concentration distributions were measured by absorbance at 220 nm. Sedimentation equilibrium was established at 30,000 and 45,000 rpm, as assessed by sequential absorbance scans 1 h apart. Data at both speeds were jointly fit to a single ideal sedimenting species in SEDPHAT, using values of solution density and solvent partial specific volume estimated from composition using SEDNTERP. With the exception of the disulfides, all post-translational modifications were neglected in the estimation of ConInsG1 partial

specific volume. Reported errors describe the precision of the fit at 0.68 confidence level, estimated from Monte Carlo simulations as implemented in SEDPHAT.

Although the fit is excellent (reduced Chi2 = 0.95), the best-fit mass is slightly but significantly higher than expected. It is likely that this reflects an inaccurate estimate of the protein partial specific volume, which we have determined from amino acid composition, neglecting the post-translational modifications present. Similar analyses of other γ-carboxy glutamate-containing peptides, which have likewise neglected this modification in estimating partial specific volume, have also resulted in systematic overestimations of the expected peptide mass. Nonetheless, the data do not allow us to rule out the possibility that the observed mass discrepancy reflects a small amount of a higher-mass species; approximately 5% dimer by mass would be sufficient to account for the difference. We also tested whether Zn^{2+}, Ca^{2+}, SO_4^{2-} or PO_4^{3-} altered the aggregation state of ConInsG1 (data not shown). In the presence of each of these ions, we observed similar sedimentation equilibrium profiles, equally well described by single sedimenting species and with no significant change in apparent MW. Accordingly, we conclude that ConInsG1 remains predominantly monomeric in the presence of each of these ions, at least up to 100 μg/ml.

Insulin signaling activation assay

To determine the extent of insulin signaling induced by conus insulin molecules, pAkt Ser473 levels were measured in a mouse fibroblast cell line, NIH 3T3, overexpressed with human IR-B. The cell line was cultured in DMEM with 10% FBS, pen/strep and 2ug/mL puromycin. For the assay, 40,000 cells per well were plated in a 96-well plates with culture media containing 1% FBS. 24 hours later, 50uL of insulin solution was pipetted into each well after the removal of the original media. After a 30-min treatment, the insulin solution was aspirated and the HTRF pAkt Ser473 kit (Cisbio, Massachusetts, USA) was used to measure the intracellular level of pAkt Ser473. Briefly, the cells were first treated with cell lysis buffer (50uL per well) for 1 hour under mild shaking. 16uL of cell lysate was then added to 4uL of detecting reagent in a white 384-well plate. After 4-hour incubation, the plate was read in a Synergy Neo plate reader (Biotek, Vermont, USA). The data was processed according to the manufacturer's protocol.

Cone snail insulin vs. mammalian insulin receptor

Insulin signaling activation assay - To determine the extent of insulin signaling induced by conus insulin molecules, pAkt Ser473 levels were measured in a mouse fibroblast cell line, NIH 3T3, overexpressed with human IR-B. The cell line was cultured in DMEM with 10% FBS, pen/strep and 2ug/mL puromycin. For the assay, 40,000 cells per well were plated in a 96-well plates with culture media containing 1% FBS. 24 hours later, 50uL of insulin solution was pipetted into each well after the removal of the original media. After a 30-min treatment, the insulin solution was aspirated and the HTRF pAkt Ser473 kit (Cisbio, Massachusetts, USA) was used to measure the intracellular level of pAkt Ser473. Briefly, the cells were first treated with cell lysis buffer (50uL per well) for 1 hour under mild shaking. 16uL of cell lysate was then added to 4uL of detecting reagent in a white 384-well plate. After 4-hour incubation, the plate was read in a Synergy Neo plate reader (Biotek, Vermont, USA). The data was processed according to the manufacturer's protocol. Results are shown in FIG. 6A. It is clear from the data that the cone snail insulin activates the human insulin signaling pathway.

Effect of Con-Ins-G1 in streptozotocin-treated diabetic mice - To evaluate the in vivo effects of Con-Ins-G1, STZ-treated C57BL/6 diabetic mice (Jackson Laboratory, Maine, USA) are used. Briefly, overnight fasted diabetic mice were treated subcutaneously with a single dose of Con-Ins-G1 (5U/kg). Tail vein blood glucose levels were monitored every 15 minutes for 2 hours. Results are shown in FIG. 6B. Cone snail insulin also reduces blood sugar levels in diabetic mice,

Assessment of Con-Ins G1 in solution

The association state of Con-InsG1 in solution was assessed at 100 μg/ml using sedimentation equilibrium analysis at 30,000 and 45,000 rpm. The data are well described by a Con-InsG1 being a single sedimenting species of apparent MW 5380 ± 55 g/mol (FIG. 7C). Based on a calculated theoretical mass of 5143, it is concluded that Con-InsG1 is overwhelmingly monomeric in solution, with at most 5% possibly being dimeric.

Further investigation of the four post-translational modifications (PTMs) within Con-Ins G1, *viz.*, residues A4 and B10 are γ-carboxylated glutamates (Gla), as

opposed to Glu and His (respectively) in hIns, residue B3 is hydroxyproline (Hyp) as opposed to Pro in hIns, and residue A20 (the C-terminus of the A chain) is amidated (numbering the Con-Ins G1 B-chain residues from -2 onwards to allow easy comparison to hIns). Native Con-Ins G1 is found to be four times more active against the human IR-B than an analog for which the PTMs were omitted (FIG. 7B), consistent with results for downstream activity: Con-Ins G1 induces Akt phosphorylation at an EC_{50} of 8.03 nM (the EC_{50} of human insulin is 0.62 nM, FIG. 7D) while phosphorylation efficiency is eight-fold lower in the absence of the PTMs (IC_{50}: 65.18 nM, FIG. 7D). FIG. 7 Characterization of Con-Ins G1. (A) Sequence comparison with human insulin. γ-: γ--carboxylated-glutamate, O: hydroxyproline, *: C-terminal amidation. (B) Competition binding analysis of Con-Ins G1 against human insulin receptor (isoform B) compared to hIns. (C) Sedimentation equilibrium analysis of ConInsG1 at 30,000 rpm (black points) and 45,000 rpm (red points) with the best fit (lines) to a single species of apparent MW 5380 ± 55 g/mol. (D) AKT phosphorylation analysis of Cons-Ins G1 vs hIns.

While the forgoing examples are illustrative of the principles of invention embodiments in one or more particular applications, it will be apparent to those of ordinary skill in the art that numerous modifications in form, usage and details of implementation can be made without the exercise of inventive faculty, and without departing from the principles and concepts of the disclosure.

CLAIMS

What is claimed is:

1. An insulin analog, comprising:

an A chain peptide including the sequence Gly-X_{A2}-X_{A3}-X_{A4}-X_{A5}-Cys_{A6}-Cys_{A7}-X_{A8}-X_{A9}-X_{A10}-Cys_{A11}-X_{A12}-X_{A13}-X_{A14}-X_{A15}-X_{A16}-X_{A17}-X_{A18}-X_{A19}-Cys_{A20}-X_{A21}-X_{A22}-X_{A23}-X_{A24}-X_{A25}-X_{A26}-X_{A27}-X_{A28}-X_{A29}-X_{A30}-X_{A31}-X_{A32}-X_{A33}-X_{A34} (SEQ ID NO: 003), where X_{A2} = Val or Ile; X_{A3} = Val or Ala; X_{A4} = Glu or Cys; X_{A5} = Glu, His or Val; Cys_{A6}, Cys_{A7}, and C_{A11} are independently Cys or selenocysteine; X_{A8} = His, Asp, Gln, Tyr, Lys or Val; X_{A9} = Arg, Asn, His or Lys; X_{A10} = Pro, Tyr, Ala, Ser, Phe, His or Thr; X_{A12} = Ser or Thr; X_{A13} = Asn, Val or Asp; X_{A14} = Ala, Gln, Asp or Glu; X_{A15} = Glu or Thr; X_{A16} = Phe, Leu, or Ala; X_{A17} = Lys, Arg, Ile, Met, Thr or Ser; X_{A18} = Lys, Thr, Asn, Gln or Glu; X_{A19} = Tyr or Phe; Cys_{A20} = Cys, selenocysteine, amidated Cys, or amidated selenocysteine; X_{A21} = Pro, His, Ser, Gly, Ala, or is absent; X_{A22} = Pro, Asn, Thr, Leu, Ser or is absent; X_{A23} = Thr, Leu, Val, Ser or is absent; X_{A24} = Arg, Thr, Met, Gln, Leu or is absent; X_{A25} = Glu, Gly or is absent; from X_{A26} = Ser, Leu or is absent; X_{A27} to X_{A31} are independently Ser or are absent; X_{A32} = Ala, Ser or is absent; X_{A33} = Ala, Val or is absent; and X_{A34} = Ala or is absent; and

a B chain peptide including the sequence X_{B1}-X_{B2}-X_{B3}-X_{B4}-X_{B5}-X_{B6}-X_{B7}-X_{B8}-Cys_{B9}-X_{B10}-X_{B11}-X_{B12}-X_{B13}-X_{B14}-X_{B15}-X_{B16}-X_{B17}-X_{B18}-X_{B19}-X_{B20}-Cys_{B21}-X_{B22}-X_{B23}-X_{B24}-X_{B25}-X_{B26}-X_{B27}-X_{B28}-X_{B29}-X_{B30}-X_{B31}-X_{B32}-X_{B33}-X_{B34}-X_{B35}-X_{B36}-X_{B37}-X_{B38}-X_{B39} (SEQ ID NO: 006), where X_{B1} = Thr, Asn, Ser or is absent; X_{B2} = Phe, Ser, Asn, Thr, Gln or is absent; X_{B3} = Asp, Gly, Pro, Leu, Phe, or His; X_{B4} = Thr, Pro, Asp, Val or Gly; X_{B5} = Asn, Pro, His, Thr, Arg, Ser or hydroxyproline; X_{B6} = Lys, Glu, Asn, Asp, Arg, Gln or Gly; X_{B7} = His, Tyr, Arg or Ile; X_{B8} = Arg, Thr, Ile, Ser, Leu, Tyr or Lys; Cys_{B9} = Cys or selenocysteine; X_{B10} = Gly, Gln or Asp; X_{B11} = Ser, Leu, Gly or Pro; X_{B12} = His, Glu, Asp, or Asn; X_{B13} = Ile, Leu, Asp, Val or Ala; X_{B14} = Thr, Ala, Pro, Val or Arg; X_{B15} = Asn, Asp, Ala, Val, Thr, Pro or Glu; X_{B16} = Ala, Ser, Gln, His, Tyr, Arg or Gly; X_{B17} = Thr, Tyr, Pro, Leu or Gly; X_{B18} = Tyr, Met, Val, Gln, Ile, Asp, Gly, Asn or Leu; X_{B19} = Leu, Asp, Gln, Gly, Lys, Glu, Arg or Thr; X_{B20} = Val, Leu or Lys; C_{B21} = Cys, amidated Cys, selenocysteine or amidated selenocysteine; X_{B22} = Val, Tyr, Phe, His, Gly, Leu, amidated His, amidated Val or is absent; X_{B23} = Glu, Arg, Ser, Gly or is absent; X_{B24} = Asp, Val or is absent; X_{B25} = Leu, Val or is

absent; X_{B26} = Val, Ile or is absent; X_{B27} = Asn, Pro, Glu or is absent; X_{B28} = Tyr, C, His or is absent; X_{B29} = His, Leu, Tyr or is absent; X_{B30} = Glu, Leu, Ile, Arg or is absent; X_{B31} = Ile, Lys or is absent; X_{B32} = Lys, Leu, Gln or is absent; X_{B33} = Cys or is absent; X_{B34} = Glu, Pro, Val or is absent; X_{B35} = Glu, Gly or is absent; X_{B36} = Glu, Gly or is absent; X_{B37} = Glu, Val or is absent; X_{B38} = Ala, Asp or is absent; and X_{B39} = Ala or is absent;

wherein the A chain peptide and the B chain peptide are bonded together across at least one pair of C residues.

2. The insulin analog of claim 1, wherein C_{B9} of the B chain peptide is bonded to C_{A6} of the A chain peptide.

3. The insulin analog of claim 1, wherein C_{B21} of the B chain peptide is bonded to C_{A20} of the A chain peptide.

4. The insulin analog of claim 1, wherein C_{A7} is bonded to C_{A11}.

5. The insulin analog of claim 1, wherein the B chain peptide includes the sequence X_{B1}-X_{B2}-Asp-Thr-Pro-Lys-His-Arg-Cys_{B9}-Gly-Ser-Glu-X_{B13}-X_{B14}-X_{B15}-X_{B16}-Tyr-X_{B18}-X_{B19}-Leu-Cys_{B21}-X_{B22} (SEQ ID NO: 007), wherein X_{B1} = Thr or Asn; X_{B2} = Phe or Ser; Cys_{B9} = Cys or selenocysteine; X_{B13} = Ile or Leu; X_{B14} = Thr or Ala; X_{B15} = Asn or Asp; X_{B16} = Ser or Gln; X_{B18} = Met or Val; X_{B19} = Asp or Gln; C_{B21} = Cys, amidated Cys, selenocysteine or amidated selenocysteine; X_{B22} = His, Tyr or is absent; and X_{B23} = Arg or is absent.

6. The insulin analog of claim 1, wherein the B chain peptide includes the sequence X_{B1}-X_{B2}-X_{B3}-X_{B4}-X_{B5}-X_{B6}-X_{B7}-X_{B8}-Cys_{B9}-Gly-Ser-X_{B12}-X_{B13}-X_{B14}-X_{B15}-X_{B16}-X_{B17}-X_{B18}-X_{B19}-X_{B20}-Cys_{B21}-X_{B22}-X_{B23} (SEQ ID 008), wherein X_{B1} = Thr, Asn or is absent; X_{B2} = Phe, Ser or is absent; X_{B3} = Phe or Asp; X_{B4} = Thr or Val; X_{B5} = Pro, Asn or hydroxyproline; X_{B6} = Lys, Asn or Gln; X_{B7} = His or Tyr; X_{B8} = Arg, Ile or Leu; X_{B12} = His, Asp, Glu or gamma carboxyglutamate; Cys_{B9} = Cys or selenocysteine; X_{B13} = Val, Ile or Leu; X_{B14} = Thr, Ala, Pro, or Val; X_{B15} = Glu, Val, Asn or Asp; X_{B16} = Ser, Gln, Tyr or Ala; X_{B17} = Tyr or Leu; X_{B18} = Tyr, Asp, Met or Val; X_{B19} = Leu, Asp, Gln or Lys; X_{B20} = Leu or Val; C_{B21} = Cys, amidated Cys, selenocysteine or amidated

selenocysteine; X_{B22} = Gly, Val, Phe, His, amidated His, amidated Val or is absent; and X_{B23} = Glu, Arg, Gly or is absent.

7. The insulin analog of claim 1, wherein the B chain peptide includes the sequence Asn-Ser-Asp-Thr-Pro-Lys-Tyr-Arg-Cys_{B9}-Gly-Ser-X_{B12}-Ile-Pro-Asn-Ser-Tyr-X_{B18}-Asp-Leu-Cys_{B21} (SEQ ID NO: 009), wherein Cys_{B9} = Cys or selenocysteine; X_{B12} = Glu or Asp; X_{B18} = Met or Ile; and C_{B21} = Cys, amidated Cys, selenocysteine or amidated selenocysteine.

8. The insulin analog of claim 1, wherein the B chain peptide includes the sequence X_{B1}-Asn-Gly-Pro-Thr-Asn-His-Ile-Cys_{B9}-Gly-Ser-Asp-Val-Val-Val-Tyr-Tyr-Asp-Lys-Leu-Cys_{B21}-X_{B22}-X_{B23} (SEQ ID NO: 010), wherein X_{B1} is absent; Cys_{B9} = Cys or selenocysteine; C_{B21} = Cys, amidated Cys, selenocysteine or amidated selenocysteine, X_{B22} = Val, Pro or is absent; X_{B23} = Gly, amidated Gly or is absent.

9. The insulin analog of claim 1, wherein the B chain peptide includes the sequence X_{B1}-Ser-Phe-Gly-Ser-X_{B6}-His-X_{B8}-Cys_{B9}-X_{B10}-Pro-X_{B12}-X_{B13}-X_{B14}-X_{B15}-X_{B16}-X_{B17}-X_{B18}-X_{B19}-X_{B20}-Cys_{B21}- X_{B22}-X_{B23}-X_{B24}-X_{B25}-X_{B26}-X_{B27}-X_{B28}-X_{B29}-X_{B30}-Lys-X_{B32}-Cys_{B33}-X_{B34}-X_{B35}-X_{B36} -X_{B37}-X_{B38}-X_{B39} (SEQ ID 011), wherein X_{B1} = Ser or is absent; X_{B6} = Arg, Gly or Gln; X_{B8} = Thr, Lys or Tyr; Cys_{B9} = C or selenocysteine; X_{B10} = Asp or Gly; X_{B12} = Asp; Glu or Asn; X_{B13} = Asp, Leu or Ala; X_{B14} = Thr or Arg; X_{B15} = Ala, Glu or Pro; X_{B16} = His, Tyr or Glu; X_{B17} = Pro, Leu or Gly; X_{B18} = Gln, Leu or Asn; X_{B19} = Gly, Thr or Arg; X_{B20} = Leu or Lys; C_{B21} = Cys, amidated Cys, selenocysteine or amidated selenocysteine; X_{B22} = Gly, Leu or is absent; X_{B23} = Ser, Glu or is absent; X_{B24} = Asp, Val or is absent; X_{B25} = Leu, Val or is absent; X_{B26} = Val, Ile or is absent; X_{B27} = Pro, Glu or is absent; X_{B28} = Tyr, Cys, His or is absent; X_{B29} = Leu, Tyr or is absent; X_{B30} = Leu, Ile, Arg or is absent; X_{B32} = Leu, Gln or is absent; X_{B34} = Pro, Val or is absent; X_{B35} = Gly, amidated Gly or is absent; X_{B36} = Gly or is absent; X_{B37} = Val or is absent; X_{B38} = Asp or is absent; and X_{B39} = Ala, amidated Ala, or is absent.

10. The insulin analog of claim 1, wherein the A chain peptide includes the sequence Gly-Val-Val-Glu-His-Cys_{A6}-Cys_{A7}-X_{A8}-Arg-X_{A10} -Cys_{A11}-Ser-Asn-Ala-Glu-Phe-X_{A17}-X_{A18}-Phe-Cys_{A20} (SEQ ID NO: 004), wherein Cys_{A6}, Cys_{A7}, and C_{A11} are

independently Cys or selenocysteine; X_{A8} = His, Tyr or Lys; X_{A10} = Pro or Ala; X_{A17} = Lys or Met; X_{A18} = Lys or Gln; X_{A19} = Tyr or Phe; Cys_{A20} = Cys, selenocysteine, amidated Cys, or amidated selenocysteine; X_{A21} = Ser, Gly or is absent; X_{A22} = Asn or is absent; and X_{A23} = Ser or is absent.

11. The insulin analog of claim 1, wherein the A chain peptide includes the sequence Gly-Ile-X_{A3}-X_{A4}-Glu-$Cys_{A6}$$Cys_{A7}$-$X_{A8}$-$X_{A9}$-$X_{A10}$-$Cys_{A11}$-Thr-$X_{A13}$-$X_{A14}$-Glu-$X_{A16}$ - X_{A17}-X_{A18}-Tyr-Cys_{A20} (SEQ ID 005), wherein X_{A3} = Val or Ala; X_{A4} = Glu or Cys; Cys_{A6}, Cys_{A7}, and C_{A11} are independently Cys or selenocysteine; X_{A8} = His, Asp, Gln, Lys or Val; X_{A9} = Asn or Lys; X_{A10} = Tyr, Ser, Phe, His or Thr; X_{A13} = Asn or Asp; X_{A14} = Ala, Gln, Asp or Glu; X_{A16} = Phe or Ala; X_{A17} = Arg, Met, Thr or Ser; X_{A18} = Lys, Gln or Glu; Cys_{A20} = Cys, selenocysteine, amidated Cys, or amidated selenocysteine; X_{A21} = Pro, His, Ser, Ala, or is absent; X_{A22} = Pro, Thr, Leu, Ser or is absent; X_{A23} = Thr, Leu, Val, or is absent; X_{A24} = Arg, Thr, Met, Gln, Leu or is absent; X_{A25} = Glu, Gly or is absent; from X_{A26} = Ser, Leu or is absent; X_{A27} to X_{A32} are independently Ser or is absent; X_{A33} = Ala or is absent; and X_{A34} = Ala or is absent.

12. The insulin analog of claim 1, wherein the A chain peptide comprises a sequence selected from the group consisting of SEQ ID NO: 012 to SEQ ID NO: 033.

13. The insulin analog of claim 1, wherein the B chain peptide comprises a sequence selected from the group consisting of SEQ ID NO: 034 to SEQ ID NO: 055.

14. The insulin analog of claim 1, wherein the A chain peptide and the B chain peptide are linked together at a terminal end.

15. The insulin analog of claim 1, wherein the A chain peptide and the B chain peptide are linked together at both terminal ends.

16. An insulin analog chimera, comprising:

a human insulin A chain peptide including the sequence Gly-Ile-Val-Glu-Gln-Cys-Cys-Thr-Ser-Ile-Cys-Ser-Leu-Tyr-Gln-Leu-Glu-Asn-Tyr-Cys-Asn (SEQ ID NO: 056);

a synthetic insulin B chain peptide selected from either of a sequence of a B chain peptide of claim 1 or a B chain peptide including the sequence X_{B1}-X_{B2}-X_{B3}-X_{B4}-X_{B5}-X_{B6}-X_{B7}-X_{B8}-Cys_{B9}-Gly-Ser-X_{B12}-X_{B13}-X_{B14}-X_{B15}-X_{B16}-X_{B17}-X_{B18}-X_{B19}-X_{B20}-Cys_{B21}-X_{B22}-X_{B23} (SEQ ID 008), wherein X_{B1} = Thr, Asn or is absent; X_{B2} = Phe, Ser or is absent; X_{B3} = Phe or Asp; X_{B4} = Thr or Val; X_{B5} = Pro, Asn or hydroxyproline; X_{B6} = Lys, Asn or Gln; X_{B7} = His or Tyr; X_{B8} = Arg, Ile or Leu; X_{B12} = His, Asp, Glu or gamma carboxyglutamate; Cys_{B9} = Cys or selenocysteine; X_{B13} = Val, Ile or Leu; X_{B14} = Thr, Ala, Pro, or Val; X_{B15} = Glu, Val, Asn or Asp; X_{B16} = Ser, Gln, Tyr or Ala; X_{B17} = Tyr or Leu; X_{B18} = Tyr, Asp, Met or Val; X_{B19} = Leu, Asp, Gln or Lys; X_{B20} = Leu or Val; C_{B21} = Cys, amidated Cys, selenocysteine or amidated selenocysteine; X_{B22} = Gly, Val, Phe, His, amidated His, amidated Val or is absent; and X_{B23} = Glu, Arg, Gly or is absent;

wherein the A chain and the B chain are bonded together across at least one pair of C residues.

17. The chimera of claim 16, wherein the B chain peptide comprises a sequence selected from the group consisting of SEQ ID NO: 034 to SEQ ID NO: 055.

18. The chimera of claim 16, wherein the B chain peptide comprises a sequence selected from the group consisting of SEQ ID NO: 034 to SEQ ID NO: 041.

19. The chimera of claim 16, wherein the B chain peptide has a sequence SEQ ID NO: 034.

20. An insulin analog chimera, comprising:

a human insulin analog A chain peptide including the sequence Gly-X_{A2}-Val-Glu-X_{A5}-Cys_{A6}-Cys_{A7}-X_{A8}-X_{A9}-X_{A10}-Cys_{A11}-Ser-X_{A13}-X_{A14}-X_{A15}-X_{A16}-X_{A17}-X_{A18}-Tyr-Cys_{A20}-X_{A21} (SEQ ID NO: 057), wherein X_{A2} = Ile or Val; X_{A5} = Gln or His; Cys_{A6}, Cys_{A7}, and C_{A11} are independently Cys or selenocysteine; X_{A8} = Thr or His; X_{A9} = Ser or Arg; X_{A10} = Ile or Pro; X_{A13} = Leu or Asn; X_{A14} = Tyr or Ala; X_{A15} = Gln or Glu; X_{A16} = Leu or Phe; X_{A17} = Glu or Lys; X_{A18} = Asn or Lys; Cys_{A20} = Cys, selenocysteine, amidated Cys, or amidated selenocysteine; Cys_{A20} = Cys, selenocysteine, amidated Cys, or amidated selenocysteine; and X_{A21} = Asn or absent; and

a synthetic insulin B chain peptide selected from either of a sequence of a B chain peptide of claim 1 or a B chain peptide including the sequence X_{B1}-X_{B2}-X_{B3}-X_{B4}-X_{B5}-X_{B6}-X_{B7}-X_{B8}-Cys_{B9}-Gly-Ser-X_{B12}-X_{B13}-X_{B14}-X_{B15}-X_{B16}-X_{B17}-X_{B18}-X_{B19}-X_{B20}-Cys_{B21}-X_{B22}-X_{B23} (SEQ ID 008), wherein X_{B1} = Thr, Asn or is absent; X_{B2} = Phe, Ser or is absent; X_{B3} = Phe or Asp; X_{B4} = Thr or Val; X_{B5} = Pro, Asn or hydroxyproline; X_{B6} = Lys, Asn or Gln; X_{B7} = His or Tyr; X_{B8} = Arg, Ile or Leu; X_{B12} = His, Asp, Glu or gamma carboxyglutamate; Cys_{B9} = Cys or selenocysteine; X_{B13} = Val, Ile or Leu; X_{B14} = Thr, Ala, Pro, or Val; X_{B15} = Glu, Val, Asn or Asp; X_{B16} = Ser, Gln, Tyr or Ala; X_{B17} = Tyr or Leu; X_{B18} = Tyr, Asp, Met or Val; X_{B19} = Leu, Asp, Gln or Lys; X_{B20} = Leu or Val; C_{B21} = Cys, amidated Cys, selenocysteine or amidated selenocysteine; X_{B22} = Gly, Val, Phe, His, amidated His, amidated Val or is absent; and X_{B23} = Glu, Arg, Gly or is absent;

wherein the A chain and the B chain are bonded together across at least one pair of C residues.

21. The chimera of claim 20, wherein the B chain peptide comprises a sequence selected from the group consisting of SEQ ID NO: 034 to SEQ ID NO: 055.

22. The chimera of claim 20, wherein the B chain peptide comprises a sequence selected from the group consisting of SEQ ID NO: 034 to SEQ ID NO: 041.

23. The chimera of claim 20, wherein the B chain peptide has a sequence SEQ ID NO: 034.

24. An insulin analog, comprising:

an A chain peptide including the sequence Gly-Ile-Val-Glu-His-Cys_{A6}-Cys_{A7}-X_{A8}-X_{A9}-X_{A10}-Cys_{A11}-Ser-Leu-Tyr-Gln-Leu-Glu-Asn-Tyr- Cys_{A20}-Asn (SEQ ID NO: 058), wherein X_{A8} = Asp or His; X_{A9} = Lys, Asn or Arg; X_{A10} = Ser, Phe or Pro; and Cys_{A20} = Cys, selenocysteine, amidated Cys, or amidated selenocysteine; and

a synthetic insulin B chain peptide including the sequence X_{B1}-X_{B2}-X_{B3}-X_{B4}-X_{B5}-X_{B6}-X_{B7}-X_{B8}-Cys_{B9}-Gly-Ser-X_{B12}-X_{B13}-X_{B14}-X_{B15}-X_{B16}-X_{B17}-X_{B18}-X_{B19}-X_{B20}-Cys_{B21}-X_{B22}-X_{B23} (SEQ ID 008), wherein X_{B1} = Thr, Asn or is absent; X_{B2} = Phe, Ser or is absent; X_{B3} = Phe or Asp; X_{B4} = Thr or Val; X_{B5} = Pro, Asn or hydroxyproline; X_{B6} = Lys, Asn or Gln; X_{B7} = His or Tyr; X_{B8} = Arg, Ile or Leu; X_{B12} = His, Asp, Glu

or gamma carboxyglutamate; Cys_{B9} = Cys or selenocysteine; X_{B13} = Val, Ile or Leu; X_{B14} = Thr, Ala, Pro, or Val; X_{B15} = Glu, Val, Asn or Asp; X_{B16} = Ser, Gln, Tyr or Ala; X_{B17} = Tyr or Leu; X_{B18} = Tyr, Asp, Met or Val; X_{B19} = Leu, Asp, Gln or Lys; X_{B20} = Leu or Val; C_{B21} = Cys, amidated Cys, selenocysteine or amidated selenocysteine; X_{B22} = Gly, Val, Phe, His, amidated His, amidated Val or is absent; and X_{B23} = Glu, Arg, Gly or is absent;

wherein the A chain and the B chain are bonded together across at least one pair of C residues.

25. An insulin analog formulation, comprising:

an insulin analog as in claim 20, including a pharmaceutically acceptable salt thereof;

a pharmaceutical carrier; and

a preservative.

26. The formulation of claim 25, further comprising an isotonicity agent.

27. The formulation of claim 25, wherein the pharmaceutical carrier includes a member selected from the group consisting of sodium phosphate, sodium acetate, sodium citrate, TRIS, arginine, and combinations thereof.

28. The formulation of claim 25, wherein the preservative is a phenolic preservative.

29. The formulation of claim 25 formulated for parenteral delivery.

30. A method for treating an insulin-related condition, comprising administering the formulation of claim 25 to a subject in need thereof.

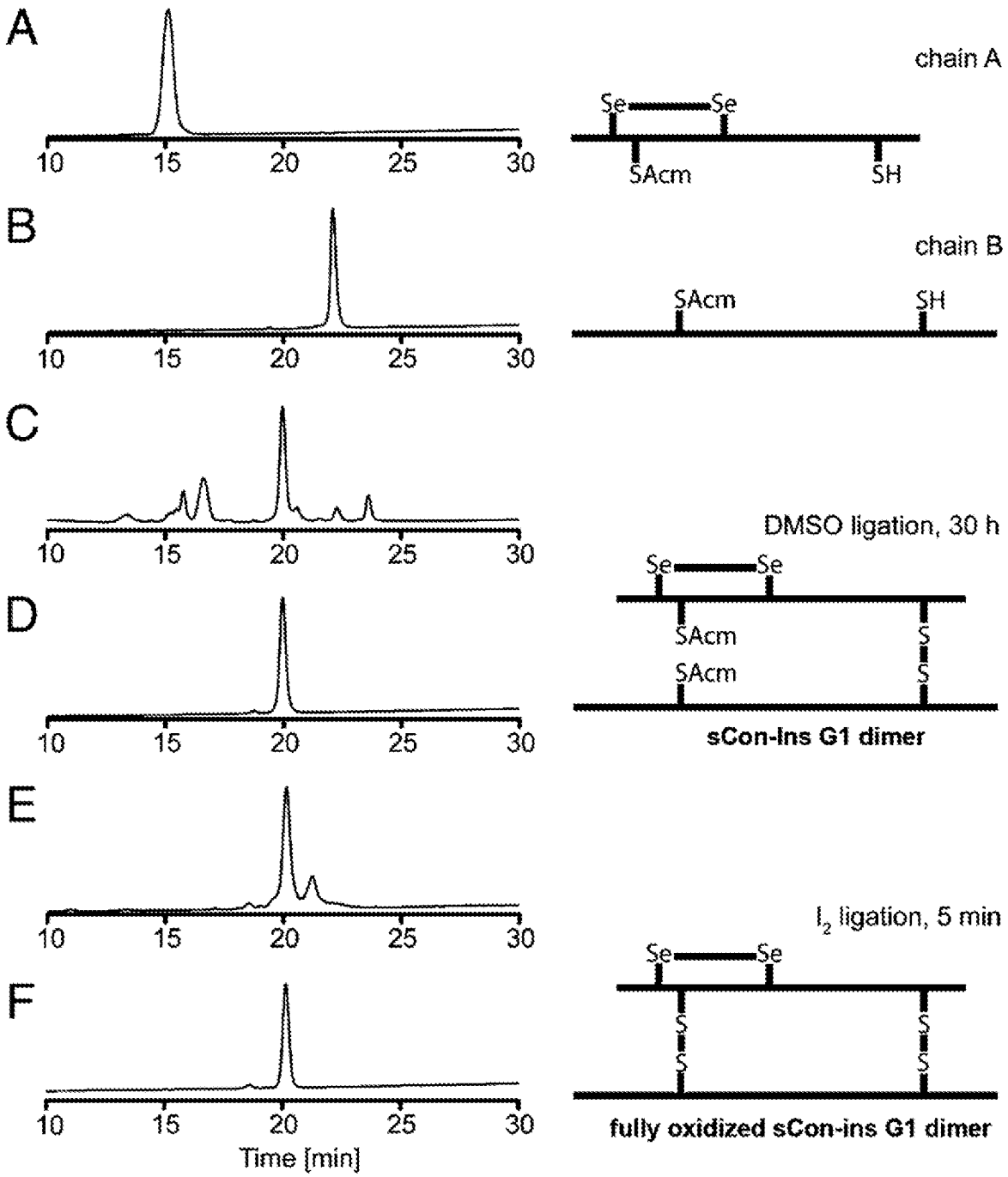
A
chain A
Se
Se
SAcm
SH
B
chain B
SAcm
SH
C
DMSO ligation, 30 h
Se
Se
SAcm
SAcm
S
S
D
sCon-Ins G1 dimer
E
I2 ligation, 5 min
Se
Se
S
S
S
S
F
fully oxidized sCon-ins G1 dimer
10
15
20
25
30
Time [min]

FIG. 1

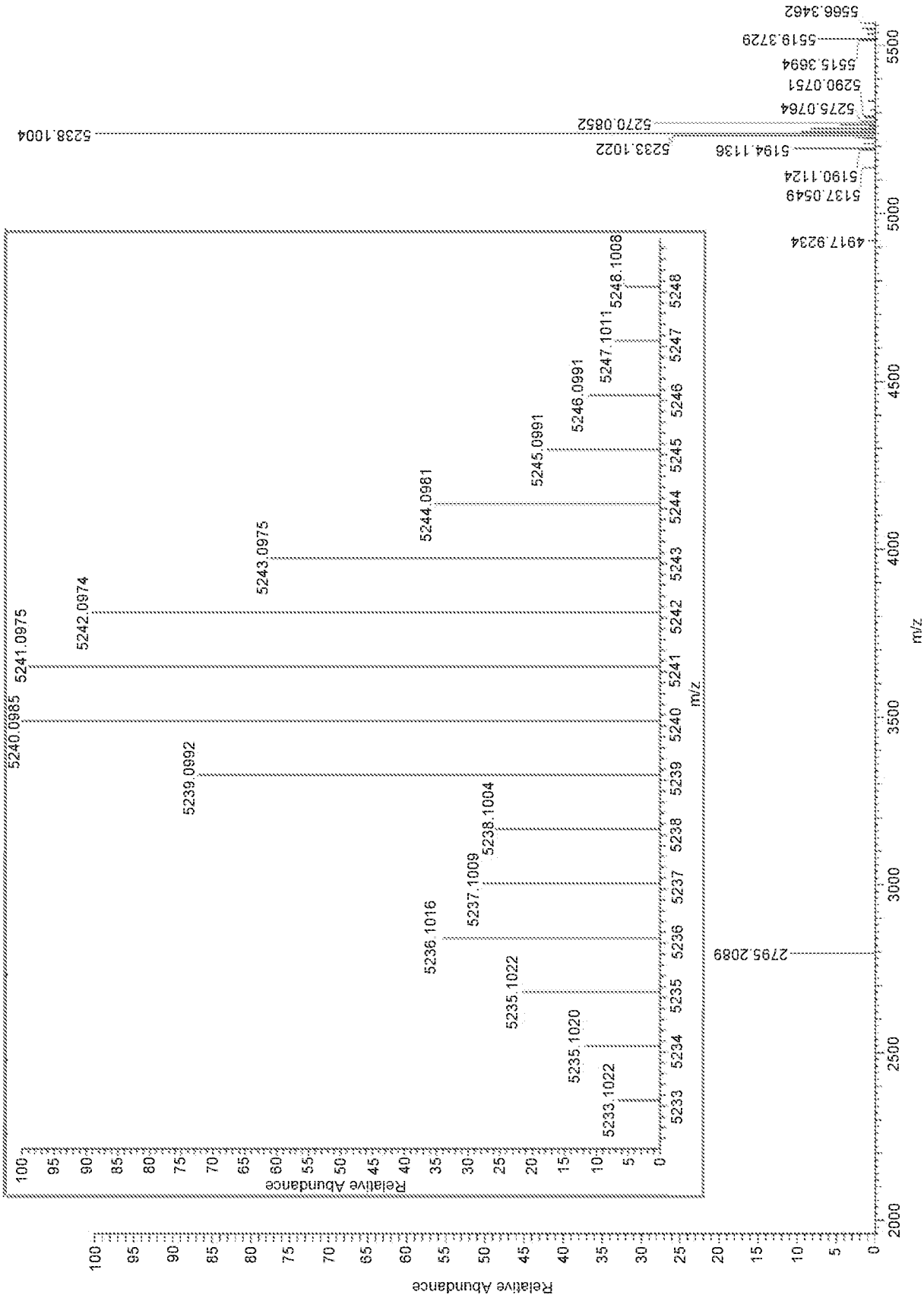
5240.0985
5241.0975
5242.0974
5243.0975
5244.0981
5245.0991
5246.0991
5247.1011
5248.1008
5239.0992
5238.1004
5237.1009
5236.1016
5235.1022
5235.1020
5233.1022
m/z
Relative Abundance
5538.1004
5270.0852
5233.1022
5194.1136
5190.1124
5137.0549
4917.9234
2795.2089
5275.0764
5290.0751
5515.3694
5519.3729
5566.3462
m/z
Relative Abundance

FIG. 2

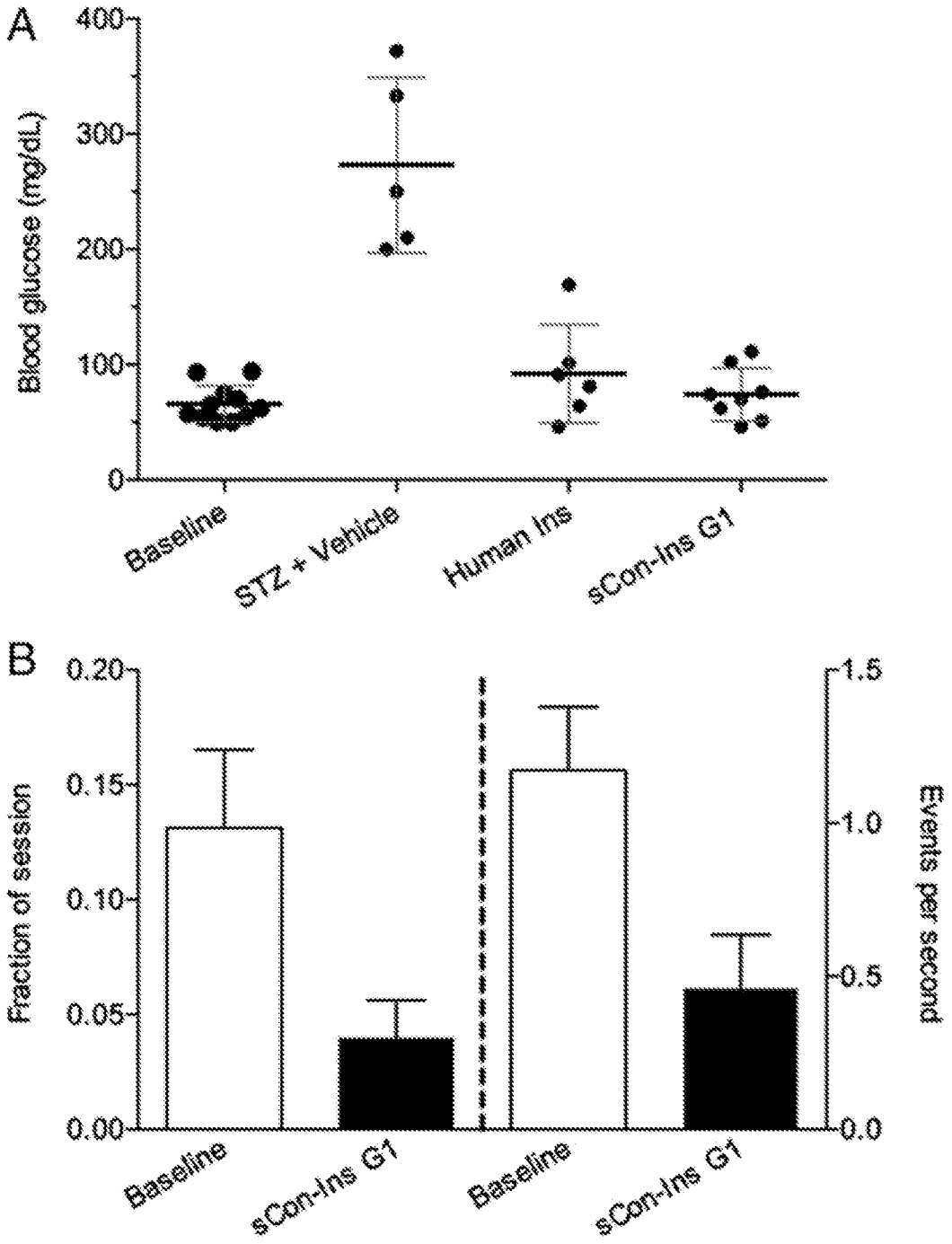
A
Blood glucose (mg/dL)
400
300
200
100
0
Baseline
STZ + Vehicle
Human Ins
sCon-Ins G1
B
Fraction of session
0.20
0.15
0.10
0.05
0.00
Events per second
1.5
1.0
0.5
0.0
Baseline
sCon-Ins G1
Baseline
sCon-Ins G1

FIG. 3

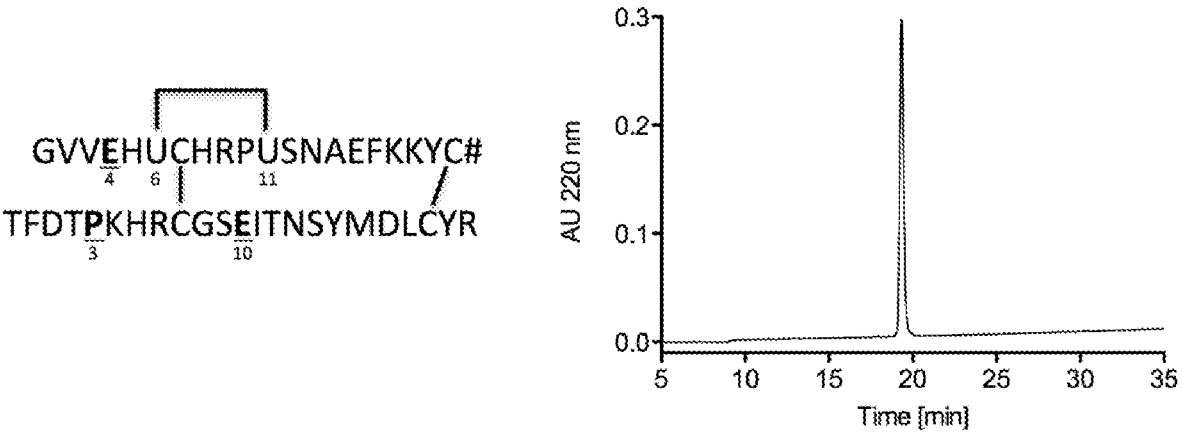
GVVEHUCHRPUSNAEFKKYC#
4 6 11
TFDTPKHRCGSEITNSYMDLCYR
3 10
AU 220 nm
0.3
0.2
0.1
0.0
5 10 15 20 25 30 35
Time [min]

FIG. 4

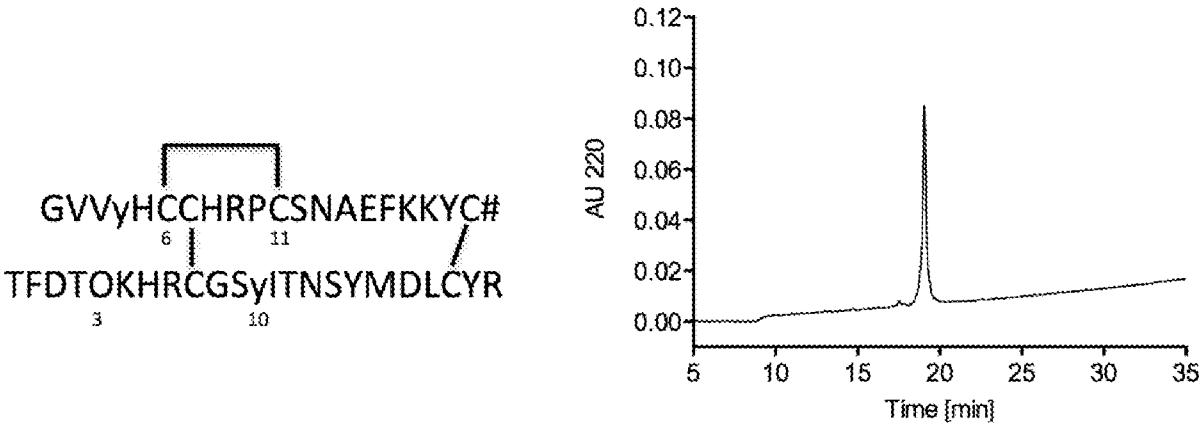
GVVyHCCHRPCSNAEFKKYC#
6 11
TFDTOKHRCGSyITNSYMDLCYR
3 10
AU 220
0.12
0.10
0.08
0.06
0.04
0.02
0.00
5 10 15 20 25 30 35
Time [min]

FIG. 5

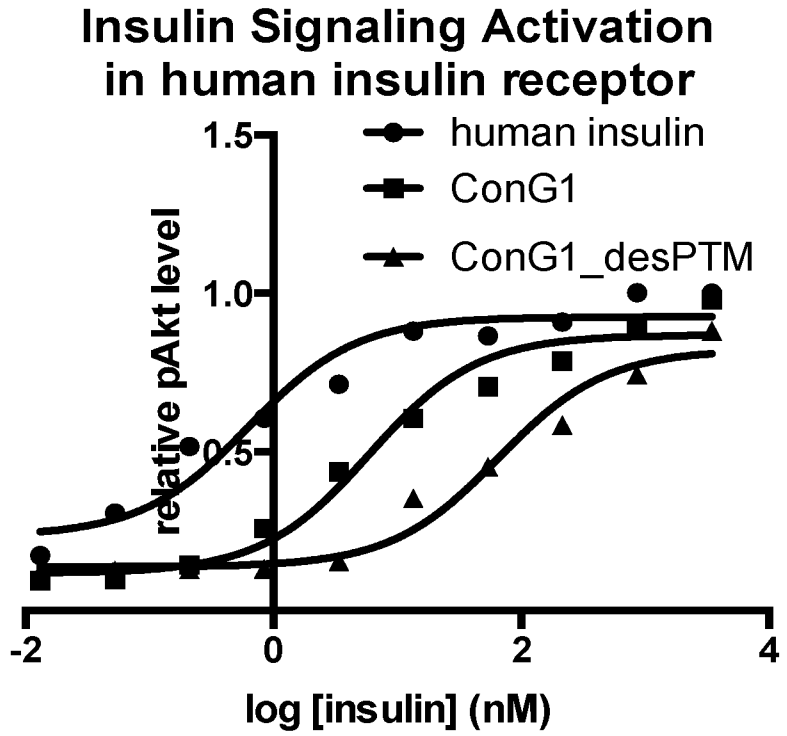
Insulin Signaling Activation
in human insulin receptor
human insulin
ConG1
ConG1_desPTM
relative pAkt level
1.5
1.0
0.5
-2
0
2
4
log [insulin] (nM)

FIG. 6A

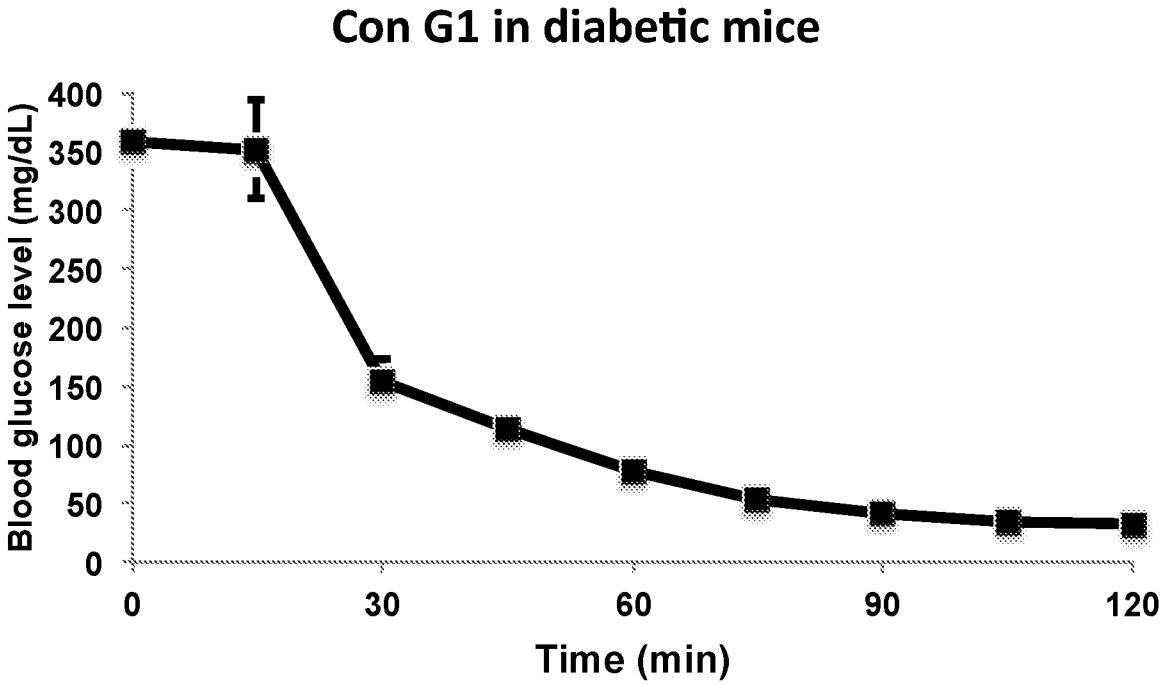
Con G1 in diabetic mice
Blood glucose level (mg/dL)
400
350
300
250
200
150
100
50
0
0
30
60
90
120
Time (min)

FIG. 6B

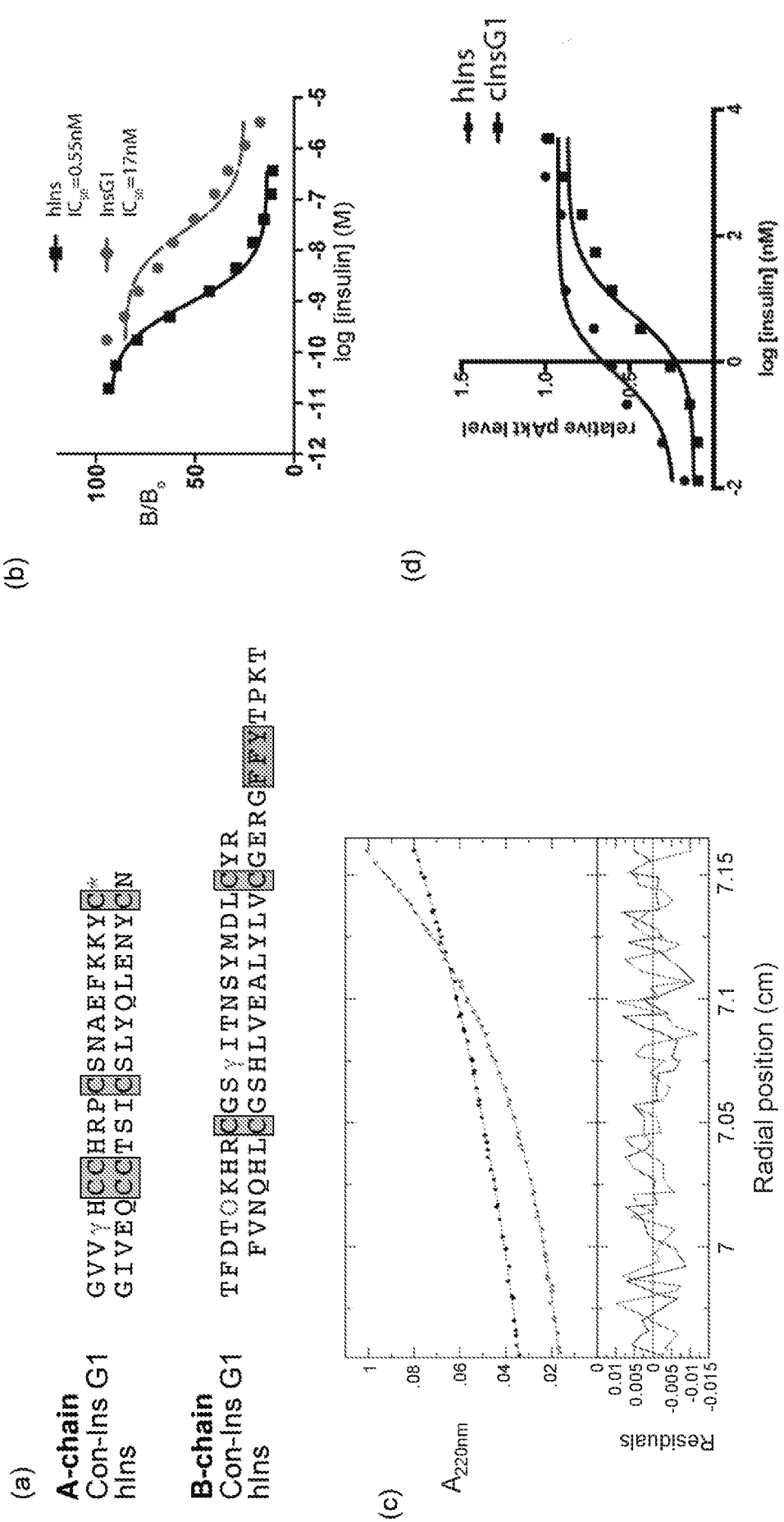
(a)
A-chain
Con-Ins G1
hIns
B-chain
Con-Ins G1
hIns
(b)
hIns
IC50=0.55nM
InsG1
IC50=17nM
B/B0
log [insulin] (M)
(c)
A220nm
Residuals
Radial position (cm)
(d)
hIns
cInsG1
relative pAkt level
log [insulin] (nM)

FIG. 7