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(81) **Designated States** (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

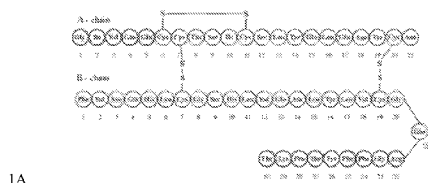
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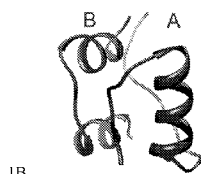
— with international search report (Art. 21(3))

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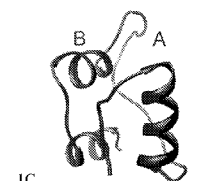
(54) **Title:** CHEMICALLY AND THERMODYNAMICALLY STABLE INSULIN ANALOGUES AND IMPROVED METHODS FOR THEIR PRODUCTION



1A.



1B.



1C.

Figure 1

(57) **Abstract:** The subject matter of this invention is directed towards chemically and thermodynamically stable single-chain insulin (SCI) analogues that are resistant to deamidation and fibrillation. The invention further discloses improved methods for the recombinant expression, purification and refolding of SCI.



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- *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*
- *with sequence listing part of description (Rule 5.2(a))*
- (88) Date of publication of the international search report:**
5 March 2015

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US14/34411

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 38/28 (2014.01)

CPC - A61K 38/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8): A61K 38/28; C07K 5/00; C12P 21/06 (2014.01)

CPC: A61K 38/00; C07K 2319/00, 14/47, 14/62, 14/625; USPC: 530/303; 435/69.1, 41

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MicroPatent (US-G, US-A, EP-A, EP-B, WO, JP-bib, DE-C,B, DE-A, DE-T, DE-U, GB-A, FR-A); Google Scholar; PubMed; ScienceDirect; 'single chain insulin,' 'insulin analog,' 'GEETLW,' oleosin, 'tumor necrosis factor,' 'fusion tag,' affinity tag, thermostability

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y --- A	US 8192957 B2 (WEISS, M) June 25, 2012; column 6, lines 63-67; figure 7; column 7, lines 30-36; column 9, lines 60-65	38-41, 43, 44 ----- 1, 2, 8, 15, 21-27, 29-37, 51-54 ----- 28
Y --- A	US 2007/0129284 A1 (KJELDSEN, TB et al.) June 7, 2007; paragraphs [0003], [0009], [0030]-[0033], [0042], [0059], [0107], [0118]	1, 2, 8, 15, 21-27, 29-37, 51-54 ----- 28
Y --- A	US 2011/0112990 A1 (STOWELL, MHB et al.) May 12, 2011; paragraphs [0030], [0031], [0068], [0076]	24-30, 35-37 ----- 28
Y	BHATLA, S et al. Use Of Oil Bodies And Oleosins In Recombinant Protein Production And Other Biotechnological Applications. Biotechnology Advances. January 11, 2010; Vol. 28; pages 293-300; abstract.	27
Y	US 2004/0014169 A1 (VOGELI, G et al.) January 22, 2004; paragraph [0101]; page 60, SEQ ID NO: 75	30

☐ Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

26 December 2014 (26.12.2014)

Date of mailing of the international search report

12 JAN 2015

Name and mailing address of the ISA/US

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P.O. Box 1450, Alexandria, Virginia 22313-1450
Facsimile No. 571-273-3201

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PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US14/34411

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

-----Please See Supplemental Page-----

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
Groups I+: Claims 1 (in-part), 2, 8, 15, 21-29, 30 (in-part), 31-41, 43, 44, 51-54, SEQ ID NOs: 8, 10, 21, 28

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US14/34411

---Continued from Box No. III: Observations Where Unity of Invention Is Lacking:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Groups I+: Claims 1-54 are directed toward a single chain insulin (SCI) compound of the formula: Stable B chain -C'-Stable A chain Formula (I); having the properties of high affinity for the insulin receptor and low affinity for the IGF-1 receptor with chemical and thermodynamic degradation profiles such that the SCI can be formulated and stored for extended periods of time without refrigeration; as well as related polynucleotides, vectors, cell lines transformed with said vectors and treatment methods.

The single chain insulin (SCI) compound of the formula: Stable B chain -C'-Stable A chain, wherein C' comprises a peptide of about 4 to 9 amino acids according to SEQ ID NO: 28 (synthetic peptide amino acid sequence) will be searched to the extent that the peptide encompasses the first option at each position (e.g. the sequence RRYPGDV). It is believed that Claims 1 (in-part), 2, 8, 15, 21-29, 30 (in-part), 31-41 and 43, 44 and 51-54 encompass this first named invention and thus these claims will be searched without fee to the extent that they encompass SEQ ID NOs: 8 (chemically cleavable amino acid sequence encoded by a fusion tag), 10 (synthetic polypeptide amino acid sequence), 21 (synthetic peptide amino acid sequence), 28 (synthetic peptide amino acid sequence). Applicants must specify the claims that encompass any additionally elected linker and associated full-length peptide sequences. Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An Exemplary Election would be: SEQ ID NO: 16 (synthetic peptide amino acid sequence).

Groups I+ share the technical features including a single chain insulin (SCI) compound of the formula: Stable B chain -C'-Stable A chain Formula (I); having the properties of high affinity for the insulin receptor and low affinity for the IGF-1 receptor with chemical and thermodynamic degradation profiles such that the SCI can be formulated and stored for extended periods of time without refrigeration; wherein Stable B chain and Stable A chain are native or modified human insulin chains; and wherein C' covalently links Stable B chain and Stable A chain, and is a peptide of about 4 to 9 amino acids comprising the following sequence: Y1 Y2 Y3 Y4 Y5 Y6 Y7 (SEQ ID NO: 28); wherein Y1 is R or absent, Y2 is R or absent, Y3 is Y or V, Y4 is P or absent, Y5 is G or D, Y6 is D or G, Y7 is any amino acid; an insulin analogue comprising a single-chain polypeptide having an affinity for insulin receptor greater than or equal to that of natural insulin, as measured in vitro; a polynucleotide comprising a nucleic acid sequence that encodes the single chain insulin compound; a recombinant vector comprising the polynucleotide; a cell line transformed with the vector; a method for treating diabetes in a patient in need thereof comprising administering the single chain insulin compound to said patient; a method for producing single chain insulin comprising introducing a recombinant vector into an expression system, expressing a protein comprising a fusion tag and single chain insulin, cleaving said fusion tag from said single chain insulin, and isolating said single chain insulin; and a method of treating a patient comprising administering a physiologically effective amount of a thermo and chemo stable insulin analogue or a physiologically acceptable salt thereof to the patient, wherein the thermo and chemo stable insulin or a physiologically acceptable salt thereof displays less than 1 percent denaturation with incubation at 40 degrees C for 28 days.

However, these shared technical features are previously disclosed by WO 1995/016708 A1 to Kristensen, et al. (hereinafter 'Kristensen') in view of US 5,962,267 A to Shin, et al. (hereinafter 'Shin'). Kristensen discloses a single chain insulin (SCI) compound (an insulin analogue in which the C-terminal amino acid of the B-chain is connected with the N-terminal amino acid residue of the A-chain by a connecting peptide, in which the C-terminal amino acid residue is different from Lys and Arg (a single chain insulin (SCI) compound); abstract) of the formula: B chain -C' - A chain Formula (I) (analogue in which the C-terminal amino acid of the B-chain is connected with the N-terminal amino acid residue of the A-chain by a connecting peptide (of the formula: B chain -C' - A chain Formula (I)); abstract); having the properties of high affinity for the insulin receptor and low affinity for the IGF-1 receptor (having the properties of high affinity for the insulin receptor and low affinity for the IGF-1 receptor; page 11, line 21 to page 12, line 3); wherein the B chain and A chain are native or modified human insulin chains (wherein the B chain and A chain are native or modified human insulin chains; page 4, lines 4-18); and wherein C' covalently links the B chain and A chain (wherein the connecting peptide is a peptide moiety connecting the C-terminal amino acid of the B-chain with the N-terminal amino acid of the A chain (wherein C' covalently links the B chain and A chain); page 4, lines 19-28), and is a peptide of about 4 to 9 amino acids (and is a peptide of about 1 to 15 amino acids, in a preferred embodiment, 9 amino acids; page 4, lines 22-27); an insulin analogue comprising a single-chain polypeptide (an insulin analogue in which the C-terminal amino acid of the B-chain is connected with the N-terminal amino acid residue of the A-chain by a connecting peptide (an insulin analogue comprising a single-chain polypeptide); abstract) having an affinity for insulin receptor greater than or equal to that of natural insulin, as measured in vitro (having an affinity for insulin receptor greater than or equal to that of natural insulin, as measured in vitro; page 10, line 3 - page 12, line 3); a polynucleotide comprising a nucleic acid sequence that encodes the single chain insulin compound (a synthetic gene encoding the compound (polynucleotide comprising a nucleic acid sequence that encodes for the single chain insulin compound); page 5, lines 23-24); a recombinant vector comprising the polynucleotide (a recombinant vector comprising the synthetic gene (polynucleotide); page 5, lines 17-18); a cell line transformed with the vector (a cell line transformed with the vector; page 5, lines 19-20); a method for treating diabetes in a patient in need thereof comprising administering the single chain insulin compound to said patient (a method for treating diabetes in a patient in need thereof comprising administering the single chain insulin compound to said patient; page 6, line 25 to page 7, line 2); and a method for producing single chain insulin comprising introducing a recombinant vector into an expression system, and isolating said single chain insulin (a method for producing single chain insulin comprising introducing a recombinant vector into an expression system, and isolating said single chain insulin; page 5, lines 13-22).

Kristensen does not disclose an insulin compound of the formula: Stable B chain -C'-Stable A chain; with chemical and thermodynamic degradation profiles such that the SCI can be formulated and stored for extended periods of time without refrigeration; a C' chain comprising the following sequence: Y1 Y2 Y3 Y4 Y5 Y6 Y7 (SEQ ID NO: 28); wherein Y1 is R or absent, Y2 is R or absent, Y3 is Y or V, Y4 is P or absent, Y5 is G or D, Y6 is D or G, Y7 is any amino acid; having an affinity for Insulin-like Growth Factor Receptor no greater than that of natural insulin as measured in vitro; expressing a protein comprising a fusion tag and single chain insulin, cleaving said fusion tag from said single chain insulin; and a method of treating a patient comprising administering a physiologically effective amount of a thermo and chemo stable insulin analogue or a physiologically acceptable salt thereof to the patient, wherein the thermo and chemo stable insulin or a physiologically acceptable salt thereof displays less than 1 percent denaturation with incubation at 40 degrees C for 28 days.

---Continued on Next Supplemental Page---

INTERNATIONAL SEARCH REPORT

International application No.

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-***-Continued from Previous Supplemental Page:

Shin discloses a single-chain human proinsulin analog (a single-chain human proinsulin analog; abstract) of the formula chain B - C' - chain A (of the formula chain B - Z - chain A (of the formula chain B - C' - chain A); abstract); wherein the C' connecting chain comprises the following sequence: Y1 Y2 Y3 Y4 Y5 Y6 Y7 (SEQ ID NO: 28); wherein Y1 is R or absent, Y2 is R or absent, Y3 is Y or V, Y4 is P or absent, Y5 is G or D, Y6 is D or G, Y7 is any amino acid (wherein the C' connecting chain comprises the sequence UU-X-P-J-(X')ⁿ-U-U, wherein U is an arginine or lysine residue; X and X' are independently any amino acid residue; P is a proline residue; J is a glycine, arginine or lysine residue; and n is 0 or an integer of 1 to 5, including the sequence Arg-Arg-Tyr-Pro-Gly-Asp-Val-Lys-Arg; abstract, column 10, lines 29-36); and wherein the connecting sequence forms a beta turn (wherein the connecting sequence forms a beta turn; column 2, lines 40-67), and wherein the residues present at the C-terminus of the connector are present to provide for tryptic cleavage (wherein the residues present at the C-terminus of the connector are present to provide for tryptic cleavage; column 3, lines 1-9).

It would have been obvious to a person of ordinary skill in the art, at the time of the invention, to have modified the insulin analog previously disclosed by Kristensen, with the beta-turn comprising analog connecting sequence disclosed by Shin, wherein the C-terminus of the connector was provided without lysine or arginine, as previously disclosed by Kristensen, for enhancing the stability of the single chain analog to cleavage by trypsin or other proteolytic enzymes with similar specificities, thereby increasing the shelf-life of the peptide. Although Kristensen does not disclose an insulin compound of the formula: Stable B chain -C' -Stable A chain; with chemical and thermodynamic degradation profiles such that the SCI can be formulated and stored for extended periods of time without refrigeration, in particular wherein the thermo- and chemo-stable insulin or a physiologically acceptable salt thereof displays less than 1 percent denaturation with incubation at 40 degrees C for 28 days; expressing a protein comprising a fusion tag and single chain insulin, cleaving said fusion tag from said single chain insulin; and a method of treating a patient comprising administering a physiologically effective amount of a thermo- and chemo-stable insulin analogue or a physiologically acceptable salt thereof to the patient. Kristensen does disclose a method of treating a patient comprising administering a physiologically effective amount of an insulin analogue or a physiologically acceptable salt thereof to the patient. Provided that the analog of Kristensen, modified as previously disclosed by Shin, comprises the identical sequence to the claimed sequence (e.g. human B chain - RRYPGDV connector sequence - human A chain), the peptide disclosed, by its chemical composition and structure, would have possessed the properties claimed, including having an affinity for Insulin-like Growth Factor Receptor no greater than that of natural insulin as measured in vitro, and chemo-stable insulin or a physiologically acceptable salt thereof displays less than 1 percent denaturation with incubation at 40 degrees C for 28 days. Furthermore, the use of affinity or epitope tags to enable and simplify the purification of expressed polypeptides was common and well-known in the art, at the time the invention, and, accordingly, it would have been obvious to a person of ordinary skill in the art, at the time of the invention, to have implemented the expression of a protein comprising a fusion tag and single chain insulin, cleaving said fusion tag from said single chain insulin to provide a chain lacking the additional sequences, for obtaining an analog useful in pharmaceutical formulations.

Since none of the special technical features of the Groups I+ inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by a combination of the Kristensen and Shin references, unity of invention is lacking.