Innovation, Sciences et Développement économique Canada
Office de la Propriété Intellectuelle du Canada

Innovation, Science and Economic Development Canada
Canadian Intellectual Property Office

CA 3122636 A1 2020/06/18

(21) 3 122 636

(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION

(13) A1

(86) Date de dépôt PCT/PCT Filing Date: 2019/12/10
(87) Date publication PCT/PCT Publication Date: 2020/06/18
(85) Entrée phase nationale/National Entry: 2021/06/09
(86) N° demande PCT/PCT Application No.: EP 2019/084427
(87) N° publication PCT/PCT Publication No.: 2020/120477
(30) Priorités/Priorities: 2018/12/11 (EP18306659.6); 2018/12/11 (EP18306658.8); 2018/12/11 (EP18306657.0)

(51) Cl.Int./Int.Cl. *C07K 14/62* (2006.01)

(71) Demandeur/Applicant:
SANOFI, FR

(72) Inventeurs/Inventors:
BOEHME, THOMAS, DE;
GUESSREGEN, STEFAN, DE;
KORN, MARCUS HERMANN, DE;
WILL, MARTIN, DE

(74) Agent: SMART & BIGGAR LLP

(54) Titre : ANALOGUES D'INSULINE AYANT UNE AFFINITE DE LIAISON DE RECEPTEUR D'INSULINE REDUITE
(54) Title: INSULIN ANALOGS HAVING REDUCED INSULIN RECEPTOR BINDING AFFINITY

(57) Abrégé/Abstract:
Provided herein are insulin analogs comprising at least one mutation relative to the parent insulin, wherein the insulin analogs comprise a mutation at position B16 which is substituted with a hydrophobic amino acid and/or a mutation at position B25 which is substituted with a hydrophobic amino acid.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
18 June 2020 (18.06.2020)

WIPO | PCT

(10) International Publication Number
WO 2020/120477 A1

(51) International Patent Classification:
C07K 14/62 (2006.01)

(21) International Application Number:
PCT/EP2019/084427

(22) International Filing Date:
10 December 2019 (10.12.2019)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

18306657.0	11 December 2018 (11.12.2018)	EP
18306658.8	11 December 2018 (11.12.2018)	EP
18306659.6	11 December 2018 (11.12.2018)	EP

(71) Applicant: **SANOFI** [FR/FR]; 54, rue La Boétie, 75008 Paris (FR).

(72) Inventors: **BOEHME, Thomas**; c/o Sanofi-Aventis Deutschland GmbH, 65926 Frankfurt am Main (DE). **GUESSREGEN, Stefan**; c/o Sanofi-Aventis Deutschland GmbH, 65926 Frankfurt am Main (DE). **KORN, Marcus Hermann**; c/o Sanofi-Aventis Deutschland GmbH, 65926 Frankfurt am Main (DE). **WILL, Martin**; c/o Sanofi-Aventis Deutschland GmbH, 65926 Frankfurt am Main (DE).

(74) Agent: **ALTMANN STÖSSEL DICK PATENTANWÄLTE PARTG MBB**; Dr. Alexander Dick, Dudenstraße 46, 68167 Mannheim (DE).

(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, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, 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.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:
— *of inventorship (Rule 4.17(iv))*

Published:
— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*

WO 2020/120477 A1

(54) Title: INSULIN ANALOGS HAVING REDUCED INSULIN RECEPTOR BINDING AFFINITY

(57) Abstract: Provided herein are insulin analogs comprising at least one mutation relative to the parent insulin, wherein the insulin analogs comprise a mutation at position B16 which is substituted with a hydrophobic amino acid and/or a mutation at position B25 which is substituted with a hydrophobic amino acid.

Insulin analogs having reduced insulin receptor binding affinity

Description

Provided herein are insulin analogs comprising at least one mutation relative to the parent insulin, wherein the insulin analogs comprise a mutation at position B16 which is substituted with a hydrophobic amino acid and/or a mutation at position B25 which is substituted with a hydrophobic amino acid.

BACKGROUND

Worldwide, more than 400 million people suffer from type 1 or type 2 diabetes mellitus. Type 1 diabetes is treated with insulin substitution. In contrast to type 1 diabetes, there is basically no deficiency of insulin in type 2 diabetes, but in a large number of cases, especially in the advanced stage, type 2 diabetes patients are treated with insulin.

In a healthy person, the release of insulin by the pancreas is strictly coupled to the concentration of the blood glucose. Elevated blood glucose levels, such as occur after meals, and are rapidly compensated by a corresponding increase in insulin secretion. In the fasting state, the plasma insulin level falls to a basal value which is adequate to guarantee a continuous supply of insulin-sensitive organs and tissue with glucose and to keep hepatic glucose production low in the night. Often, the replacement of the endogenous insulin secretion by exogenous, mostly subcutaneous administration of insulin does not achieve the quality of the physiological regulation of the blood glucose described above. Deviations of the blood glucose upward or downward can occur, which in their severest forms can be life-threatening. It is to be derived from this that an improved therapy of diabetes is primarily to be aimed at keeping the blood glucose as closely as possible in the physiological range.

Human insulin is a polypeptide of 51 amino acids, which are divided into 2 amino acid chains: the A chain having 21 amino acids and the B chain having 30 amino acids. The chains are connected to one another by means of 2 disulfide bridges. A third disulfide bridge exists between the cysteines at position 6 and 11 of the A chain. Some products in current use for the treatment of diabetes mellitus are insulin analogs, i.e. insulin variants whose sequence differs from that of human insulin by one or more amino acid substitutions in the A chain and/or in the B chain.

Like many other peptide hormones, human insulin has a short half-life *in vivo*. Thus, it is administered frequently which is associated with discomfort for the patient. Therefore, insulin analogs are desired which have an increased half-life *in vivo* and, thus, a prolonged duration of action.

There are currently different approaches for extending the half-life of insulins.

One approach is based on the development of a soluble formulation at low pH, but of reduced solubility relative to native insulin at physiologic pH. The isoelectric point of the insulin analog is increased through the addition of two arginines to the C-terminus of the B-chain. The addition of two arginines in combination with a glycine substitution at A21 (insulin glargine) provides an insulin with extended duration of action. The insulin analog precipitates in the presence of zinc upon injection in subcutaneous sites and slowly solubilizes, resulting a sustained presence of insulin glargine.

In another approach, a long chain fatty acid group is conjugated to the epsilon amino group of LysB29 of insulin. The presence of this group allows the attachment of the insulin to serum albumin by noncovalent, reversible binding. As a consequence, this insulin analog has a significantly prolonged time–action profile relative to human insulin (see e.g. Mayer et al., Inc. Biopolymers (Pept Sci) 88: 687–713, 2007; or WO 2009/115469).

WO 2016/006963 discloses insulin analogs having a reduced insulin receptor-mediated clearance rate, compared to human insulin.

WO 2018/056764 discloses insulin analogs having a reduced insulin receptor-mediated clearance rate, compared to human insulin.

WO 2008/034881 discloses protease stabilized insulin analogs.

In order to increase the duration of action of a drug, half-life plays a major role. Half-life ($t_{1/2}$) is proportional to the volume of distribution divided by clearance. In the case of human insulin, clearance is mainly driven by binding to the insulin receptor, internalization and subsequent degradation.

Accordingly, there is a need for insulin analogs which have a reduced insulin receptor-binding activity, and thus a reduced receptor-mediated clearance rate, but which have a signal transduction activity which allow for sufficiently lowering the blood glucose level *in vivo*.

SUMMARY

Provided herein are long-acting insulin analogs having a very low binding affinity (hence a lower clearance rate) whilst still maintaining high signal transduction.

Surprisingly, it was shown in the context of the studies underlying the present invention that a substitution at position B16 and/or B25 of human insulin with a hydrophobic amino acid (such as leucine, isoleucine, valine, alanine and tryptophan) resulted in a decrease of insulin receptor binding activity (as compared to the insulin receptor binding activity of the parent insulin, see Examples). The strongest effects on insulin receptor binding activity were observed for substitutions with branched-chain amino acids (leucine, isoleucine and valine). Interestingly, insulin analogs with such substitutions at these positions (such as at position B25) showed up to 6-fold enhancement in signal transduction than expected based on their insulin receptor isoform B (IR-B) binding affinities (see Examples). Further, some tested insulin analogs showed improved proteolytic stability against α-chymotrypsin, cathepsin D and insulin degrading enzyme (see Examples).

Accordingly, provided herein are insulin analogs comprising at least one mutation relative to the parent insulin, wherein the insulin analogs comprise a mutation at position B16 which is substituted with a hydrophobic amino acid, and/or a mutation at position B25 which is substituted with a hydrophobic amino acid. In some embodiments, an insulin analog is provided comprising at least one mutation relative to the parent insulin, wherein the insulin analog comprises a mutation at position B16 which is substituted with a branched-chain amino acid and/or a mutation at position B25 which is substituted with a branched-chain amino acid.

The expression “insulin analog” as used herein refers to a peptide which has a molecular structure which formally can be derived from the structure of a naturally occurring insulin (herein also referred to as “parent insulin”, e.g. human insulin) by deleting and/or substituting at least one amino acid residue occurring in the naturally occurring insulin and/or adding at least one amino acid residue. The added and/or exchanged amino acid residue can either be codable amino acid residues or other naturally occurring residues or purely synthetic amino acid residues. The analog as referred to herein is capable of lowering blood glucose levels in *vivo*, such as in a human subject.

In some embodiments, the insulin analog provided herein comprises two peptide chains, an A-chain and a B-chain. Typically, the two chains are connected by disulfide bridges between cysteine residues. For example, in some embodiments, insulin analogs provided herein comprise three disulfide bridges: one disulfide bridge between the cysteines at position A6 and A11, one disulfide bridge between the cysteine at position A7 of the A-chain and the cysteine at position

B7 of the B-chain, and one between the cysteine at position A20 of the A-chain and the cysteine at position B19 of the B-chain. Accordingly, insulin analogs provided herein may comprise cysteine residues at positions A6, A7, A11, A20, B7 and B19.

In some embodiments provided herein, the insulin analog is a single-chain insulin. A single-chain insulin is a single polypeptide chains in which the insulin B-chain is linked contiguously with the insulin A-chain via an uncleaved connecting peptide.

Mutations of insulin, i.e. mutations of a parent insulin, are indicated herein by referring to the chain, i.e. either the A-chain or the B-chain of the analog, the position of the mutated amino acid residue in the A- or B-chain (such as A14, B16 and B25), and the three letter code for the amino acid substituting the native amino acid in the parent insulin. The term “desB30” refers to an analog lacking the B30 amino acid from the parent insulin (i.e. the amino acid at position B30 is absent). For example, Glu(A14)Ile(B16)desB30 human insulin, is an analog of human insulin in which the amino acid residue at position 14 of the A-chain (A14) of human insulin is substituted with glutamic acid, the amino acid residue at position 16 of the B-chain (B16) is substituted with isoleucine, and the amino acid at position 30 of the B chain is deleted (i.e. is absent).

Insulin analogs provided herein comprise at least one mutation (substitution, deletion, or addition of an amino acid) relative to parent insulin. The term “at least one”, as used herein means one, or more than one, such as “at least two”, “at least three”, “at least four”, ”at least five”, etc. In some embodiments, the insulin analogs provided herein comprise at least one mutation in the B-chain and at least one mutation in the A-chain. In a further embodiment, the insulin analogs provided herein comprise at least two mutations in the B-chain and at least one mutation in the A-chain. For example, the insulin analog may comprise a substitution at position B16, a deletion at position B30 and a substitution at position A14. Alternatively, the insulin analog may comprise a substitution at position B25, a deletion at position B30 and a substitution at position A14. Further, the insulin analog may comprise a substitution at position B16, a substitution at position B25, a deletion at position B30 and a substitution at position A14.

The insulin analogs provided herein may comprise mutations in addition to the mutations above. In some embodiments, the number of mutations does not exceed a certain number. In some embodiments, the insulin analogs comprise less than twelve mutations (i.e. deletions, substitution, additions) relative to the parent insulin. In another embodiment, the analog comprises less than ten mutations relative to the parent insulin. In another embodiment, the analog comprises less than eight mutations relative to the parent insulin. In another embodiment, the analog comprises less than seven mutations relative to the parent insulin. In another embodiment, the analog comprises less than six mutations relative to the parent insulin. In another embodiment, the analog comprises less than five mutations relative to the parent insulin. In another embodiment,

the analog comprises less than four mutations relative to the parent insulin. In another embodiment, the analog comprises less than three mutations relative to the parent insulin.

The expression "parent insulin" as used herein refers to naturally occurring insulin, i.e. to an unmutated wild-type insulin. In some embodiments, the parent insulin is animal insulin, such as mammalian insulin. For example, the parent insulin may be human insulin, porcine insulin, or bovine insulin.

In some embodiments, the parent insulin is human insulin. The sequence of human insulin is well known in the art and shown in Table 1 in the Example section. Human insulin comprises an A chain having an amino acid sequence as shown in SEQ ID NO: 1 (GIVEQCCTSICSLYQLENYCN) and a B chain having an amino acid sequence as shown in SEQ ID NO: 2 (FVNQHLCGSHLVEALYLVCGERGFFYTPKT).

In another embodiment, the parent insulin is bovine insulin. The sequence of bovine insulin is well known in the art. Bovine insulin comprises an A chain having an amino acid sequence as shown in SEQ ID NO: 81 (GIVEQCCASVCSLYQLENYCN) and a B chain having an amino acid sequence as shown in SEQ ID NO: 82 (FVNQHLCGSHLVEALYLVC-GERGFFYTPKA).

In another embodiment, the parent insulin is porcine insulin. The sequence of porcine insulin is well known in the art. Porcine insulin comprises an A chain having an amino acid sequence as shown in SEQ ID NO: 83 (GIVEQCCTSICSLYQLENYCN) and a B chain having an amino acid sequence as shown in SEQ ID NO: 84 (FVNQHLCGSHLVEALYLVC GERGFFYTPKA).

Human, bovine, and porcine insulin comprises three disulfide bridges: one disulfide bridge between the cysteines at position A6 and A11, one disulfide bridge between the cysteine at position A7 of the A-chain and the cysteine at position B7 of the B-chain, and one between the cysteine at position A20 of the A-chain and the cysteine at position B19 of the B-chain.

The insulin analogs provided herein have an insulin receptor binding affinity which is reduced as compared to the insulin receptor binding affinity of the corresponding parent insulin, e.g. of human insulin.

The insulin receptor can be any mammalian insulin receptor, such as a bovine, porcine or human insulin receptor. In some embodiments, the insulin receptor is a human insulin receptor, e.g. human insulin receptor isoform A or human insulin receptor isoform B (which was used in the Examples section).

Advantageously, the human insulin analogs provided herein have a significantly reduced binding affinity to the human insulin receptor as compared to the binding affinity of human insulin to the human insulin receptor (see Examples). Thus, the insulin analogs have a very low clearance rate, i.e. a very low insulin-receptor-mediated clearance rate.

In some embodiments, the insulin analogs have, i.e. exhibit, less than 20 % of the binding affinity to the corresponding insulin receptor compared to its parent insulin. In another embodiment, the insulin analogs provided herein have less than 10 % of the binding affinity to the corresponding insulin receptor compared to its parent insulin. In another embodiment, the insulin analogs provided herein have less than 5 % of the binding affinity to the corresponding insulin receptor compared to its parent insulin, such as less than 3 % of the binding affinity compared to its parent insulin. For example, the insulin analogs provided herein may have between 0.1% to 10 %, such as between 0.3 % to 5 % of the of the binding affinity to the corresponding insulin receptor compared to its parent insulin. Also, the insulin analogs provided herein may have between 0.5% to 3 %, such as between 0.5 % to 2 % of the of the binding affinity to the corresponding insulin receptor compared to its parent insulin.

Methods for determining the binding affinity of an insulin analog to an insulin receptor are well known in the art. For example, the insulin receptor binding affinity can be determined by a scintillation proximity assay which is based on the assessment of competitive binding between [125I]-labelled parent insulin, such as [125I]-labelled human insulin, and the (unlabeled) insulin analog to the insulin receptor. The insulin receptor can be present in a membrane of a cell, e.g. of CHO (Chinese Hamster Ovary) cell, which overexpresses a recombinant insulin receptor. In an embodiment, the insulin receptor binding affinity is determined as described in the Examples section.

Binding of a naturally occurring insulin or an insulin analog to the insulin receptor activates the insulin signaling pathway. The insulin receptor has tyrosine kinase activity. Binding of insulin to its receptor induces a conformational change that stimulates the autophosphorylation of the receptor on tyrosine residues. The autophosphorylation of the insulin receptor stimulates the receptor's tyrosine kinase activity toward intracellular substrates involved in the transduction of the signal. The autophosphorylation of the insulin receptor by an insulin analog is therefore considered as a measure for signal transduction caused by said analog.

The insulin analogs in Table 1 of the Examples section were subjected to autophosphorylation assays. Interestingly, insulin analogs with aliphatic substitutions at positions B16 and B25 caused higher than expected insulin receptor autophosphorylation based on their insulin receptor binding affinities. Thus, the insulin analogs provided herein have a low binding activity, and consequently a lower receptor-mediated clearance rate, but are nevertheless capable of causing a relatively high signal transduction. Therefore, the insulin analogs provided herein could be

used as long-acting insulins. In some embodiments, the insulin analog provided herein are capable of inducing 1 to 10 %, such as 2 to 8 %, insulin receptor autophosphorylation relative to the parent insulin (such as human insulin). Further, in some embodiments, the insulin analogs provided herein are capable of inducing 3 to 7 %, such as 5 to 7% insulin receptor autophosphorylation relative to the parent insulin (such as human insulin). The insulin receptor autophosphorylation relative to a parent insulin can be determined as described in the Examples section.

Insulin analogs provided herein were subjected to protease stability assays. As shown in Table 3, insulin analogs provided herein had higher stability towards at least some of the tested proteases as compared to human insulin. Improved proteolytic stability was observed against α-chymotrypsin, cathepsin D and insulin degrading enzyme (IDE). Accordingly, insulin analogs provided herein are, typically, proteolytically stable insulin analogs. Thus, they are slower degraded by proteases relative to the parent insulin. In some embodiments, the insulin analog provided herein are stabilized against degradation by α-chymotrypsin, cathepsin D and insulin degrading enzyme (IDE) compared to parent insulin.

As set forth above, the insulin analog comprises at least one mutation as compared to the parent insulin.

In some embodiments insulin analogs provided herein comprise a mutation at position B16 which is substituted with a hydrophobic amino acid. Thus, the amino acid at position B16 (tyrosine in human, bovine and porcine insulin) is replaced with a hydrophobic amino acid.

In another embodiment, insulin analogs provided herein comprise a mutation at position B25 which is substituted with a hydrophobic amino acid. Thus, the amino acid at position B25 (phenylalanine in human, bovine and porcine insulin) is replaced with a hydrophobic amino acid.

In another embodiment, insulin analogs provided herein comprise a mutation at position B16 which is substituted with a hydrophobic amino acid and a mutation at position B25 which is substituted with a hydrophobic amino acid.

The hydrophobic amino acid may be any hydrophobic amino acid. For example, the hydrophobic amino acid may be an aliphatic amino acid such as a branched-chain amino acid.

In some embodiments of the insulin analogs provided herein, the hydrophobic amino acid used for the substitution at position B16 and/or B25 is isoleucine, valine, leucine, alanine, tryptophan, methionine, proline, glycine, phenylalanine or tyrosine (or with a derivative of the aforementioned amino acids).

Several parent insulins such as human, bovine and porcine insulin comprise tyrosine at position B16 and phenylalanine at position B25. Thus, the amino acid at position B16 of the parent insulin may be substituted with isoleucine, valine, leucine, alanine, tryptophan, methionine, proline, glycine or phenylalanine (or with a derivative of the aforementioned amino acids). Further, the amino acid at position B25 of the parent insulin may be substituted with isoleucine, valine, leucine, alanine, tryptophan, methionine, proline, glycine, or tyrosine (or with a derivative of the aforementioned amino acids).

Derivatives of the aforementioned amino acids are known in the art.

Derivatives of leucine include, but are not limited to, homo-leucine and tert-leucine. Thus, the amino acid at position B16 and/or B25 may be substituted with homo-leucine or tert-leucine.

A derivative of valine is, e.g., 3-ethyl norvaline. Thus, the amino acid at position B16 and/or B25 may be substituted with 3-ethyl norvaline.

Derivatives of glycine include, but are not limited to, cyclohexyl-glycine cyclopropylglycine, and trifluorethylglycine.

Derivatives of alanine include, but are not limited to, beta-t-butylalanine, cyclobutyl-alanine, cyclopropyl-alanine and homo-cyclohexylalanine.

In some embodiments, the hydrophobic amino acid used for the substitution at position B16 and/or B25 is isoleucine, valine, leucine, alanine, or tryptophan.

In some embodiments, the aliphatic amino acid is not alanine. Accordingly, the hydrophobic amino acid used for the substitution at position B16 and/or B25 may be isoleucine, valine, leucine, or tryptophan.

In some embodiments, the hydrophobic amino acid used for the substitution at position B16 and/or B25 is isoleucine, valine, or leucine.

In some embodiments, the amino acids referred to herein are L-amino acids (such as L-isoleucine, L-valine, or L-leucine). Accordingly, the amino acids (or the derivatives thereof) used for e.g. the substitution at position B16, B25 and/or A14 are typically L-amino acids.

In some embodiments, the hydrophobic amino acid is an aliphatic amino acid. Accordingly, the insulin analogs provided herein comprise a mutation at position B16 which is substituted with an

aliphatic amino acid and a mutation at position B25 which is substituted with an aliphatic amino acid (and optionally further mutations including but not limited to Des(B30) and Glu(A14)).

Aliphatic amino acids are non-polar and hydrophobic amino acids comprising an aliphatic side chain functional group. Hydrophobicity increases with the number of carbon atoms on the hydrocarbon chain increases. A measure for the hydrophobicity of an aliphatic is the hydropathy index according to the Kyte and Doolittle scale which e.g. can be determined as disclosed by Kyte J. *et al.* Journal of Molecular Biology. 1982 157 (1): 105–32. In some embodiments, the aliphatic amino acid is an aliphatic amino acid having a hydropathy index (according to the Kyte and Doolittle scale) of larger than 2.0, such as larger than 3.0 or larger than 3.5.

Aliphatic amino acids include, but are not limited to, isoleucine, valine, leucine, alanine and glycine. For example, the aliphatic amino acid may be an amino acid selected from isoleucine, valine, leucine, and glycine, such as an amino acid selected from isoleucine, valine and leucine.

Isoleucine, valine and leucine are branched-chain amino acids (abbreviated BCAA). Thus, the aliphatic amino acid may be a branched-chain amino acid. In some embodiments the insulin analogs provided herein comprise a mutation at position B16 which is substituted with a branched-chain amino acid and a mutation at position B25 which is substituted with a branched-chain amino acid (and optionally further mutations including but not limited to Des(B30) and Glu(A14)).

BCAAs are amino acids such as isoleucine, valine, and leucine are amino acids having aliphatic side chains that are non-linear, i.e. branched-chain amino acids are amino acid having an aliphatic side-chain with a branch (a central carbon atom bound to three or more carbon atoms).

The branched-chain amino acid may be a proteinogenic BCAA, i.e. an amino acid that is incorporated biosynthetically into proteins during translation, or a non-proteinogenic BCAA, i.e. an amino acid that is not naturally encoded or found in the genetic code of any organism. For example, proteinogenic BCAAs are leucine, isoleucine, and valine. Thus, the hydrophobic/aliphatic amino acid branched-chain amino acid may be leucine, isoleucine or valine (or a derivative of the leucine, isoleucine or valine, such as a derivative of leucine or valine as set forth above).

In some embodiments, the branched-chain amino acid is isoleucine.

In some embodiments, the branched-chain amino acid is valine.

In some embodiments, the branched-chain amino acid is leucine.

In addition to the mutation at position B16 and/or the mutation at position B25 as described above, insulin analogs provided herein may comprise further mutations relative to the parent insulin.

For example, the insulin analog may further comprise a mutation at position A14. Such mutations are known to increase protease stability (see e.g. WO 2008/034881). In some embodiments, the amino acid at position A14 is substituted with glutamic acid (Glu). In some embodiments, the amino acid at position A14 is substituted with aspartic acid (Asp). In some embodiments, the amino acid at position A14 is substituted with histidine (His).

Further, the insulin analogs provided herein may comprise a mutation at position B30. In some embodiment, the mutation at position B30 is the deletion of threonine at position B30 of the parent insulin (also referred to as Des(B30)-mutation).

Further, the insulin analog of the present invention may further comprise a mutation at position B3 which is substituted with a glutamic acid (Glu), and/or a mutation at position A21 which is substituted with glycine (Gly).

In an embodiment, the B chain of the insulin analog of the present invention comprises or consists of the amino acid sequence shown in SEQ ID NO: 22 (FVNQHLCGSHLVEALYLVCGERGFLYTPK).

In another embodiment, the B chain of the insulin analog of the present invention comprises or consists of the amino acid sequence shown in SEQ ID NO: 24 (FVNQHLCGSHLVEALYLVCGERGFVYTPK).

In another embodiment, the B chain of the insulin analog of the present invention comprises or consists of the amino acid sequence shown in SEQ ID NO: 44 (FVNQHLCGSHLVEALYLVCGERGFIYTPK).

In another embodiment, the B chain of the insulin analog of the present invention comprises or consists of the amino acid sequence shown in SEQ ID NO: 48 (FVNQHLCGSHLVEALYLVCGERGFVYTPK).

In another embodiment, the B chain of the insulin analog of the present invention comprises or consists of the amino acid sequence shown in SEQ ID NO: 50 (FVEQHLCGSHLVEALYLVCGERGFVYTPK).

In another embodiment, the B chain of the insulin analog of the present invention comprises or consists of the amino acid sequence shown in SEQ ID NO: 58 (FVNQHLCGSHLVEAL-ILVCGERGFIYTPK).

In another embodiment, the B chain of the insulin analog of the present invention comprises or consists of the amino acid sequence shown in SEQ ID NO: 60 (FVEQHLCGSHLVEAL-ILVCGERGFIYTPK).

In another embodiment, the B chain of the insulin analog of the present invention comprises or consists of the amino acid sequence shown in SEQ ID NO: 64 (FVNQHLCGSHLVEAL-ILVCGERGFVYTPK).

In another embodiment, the B chain of the insulin analog of the present invention comprises or consists of the amino acid sequence shown in SEQ ID NO: 66 (FVEQHLCGSHLVEAL-ILVCGERGFVYTPK).

In another embodiment, the B chain of the insulin analog of the present invention comprises or consists of the amino acid sequence shown in SEQ ID NO: 70 (FVNQHLCGSHLVEALVLVCGERGFIYTPK).

In another embodiment, the B chain of the insulin analog of the present invention comprises or consists of the amino acid sequence shown in SEQ ID NO: 78 (FVEQHLCGSHLVEALVLVCGERGFVYTPK).

In another embodiment, the B chain of the insulin analog of the present invention comprises or consists of the amino acid sequence shown in SEQ ID NO: 80 (FVEQHLCGSHLVEALVLVCGERGFVYTPK).

The B chains summarized above comprise the Des(B30) mutation. Accordingly, the amino acid which is present at position B30 of the parent insulin (threonine in human insulin, and alanine in porcine and bovine insulin) is deleted, i.e. not present. However, it is also envisaged that the B chains of the analogs of the present invention do not comprise this mutation, i.e. comprise a threonine at position 30. Accordingly, the B chain of the insulin analog of the present invention may comprise or consist of an amino acid sequence selected from the group consisting of:

- FVNQHLCGSHLVEALYLVCGERGFLYTPKT (SEQ ID NO: 85)
- FVNQHLCGSHLVEALYLVCGERGFVYTPKT (SEQ ID NO: 86)
- FVNQHLCGSHLVEALYLVCGERGFIYTPKT (SEQ ID NO: 87)
- FVNQHLCGSHLVEALYLVCGERGFVYTPKT (SEQ ID NO: 88)

- FVEQHLCGSHLVEALYLVCGERGFVYTPKT (SEQ ID NO: 89)
- FVNQHLCGSHLVEALILVCGERGFIYTPKT (SEQ ID NO: 90)
- FVEQHLCGSHLVEALILVCGERGFIYTPKT (SEQ ID NO: 91)
- FVNQHLCGSHLVEALILVCGERGFVYTPKT (SEQ ID NO: 92)
- FVEQHLCGSHLVEALILVCGERGFVYTPKT (SEQ ID NO: 93)
- FVNQHLCGSHLVEALVLVCGERGFIYTPKT (SEQ ID NO: 94)
- FVNQHLCGSHLVEALVLVCGERGFVYTPKT (SEQ ID NO: 95)
- FVEQHLCGSHLVEALVLVCGERGFVYTPKT (SEQ ID NO: 96)
- FVEQHLCGSHLVEALVLVCGERGFVYTPKT (SEQ ID NO: 97)

In an embodiment, the A chain of the insulin analog of the present invention comprises or consists of the amino acid sequence shown in SEQ ID NO: 1 (GIVEQCCTSICSLYQLENYCN).

In another embodiment, the A chain of the insulin analog of the present invention comprises or consists of the amino acid sequence shown in SEQ ID NO: 43 (GIVEQCCTSICSLEQLENYCN).

In another embodiment, the A chain of the insulin analog of the present invention comprises or consists of the amino acid sequence shown in SEQ ID NO: 45 (GIVEQCCTSICSLEQLENYCG).

Typically, the insulin analog of the present invention comprises an A-chain and a B-chain as set forth above.

For example, the insulin analog of the present invention is selected from the group consisting of:
Leu(B16)-insulin (e.g. human insulin, i.e. Leu(B16)-human insulin),
Val(B16)-insulin (e.g. human insulin, i.e. Val(B16)-human insulin),
Ile(B16)-insulin (e.g. human insulin),
Leu(B16)Des(B30)-insulin (e.g. human insulin),
Val(B16)Des(B30)-insulin (e.g. human insulin),
Ile(B16)Des(B30)-insulin (e.g. human insulin),
Leu(B25)-insulin (e.g. human insulin),
Val(B25)-insulin (e.g. human insulin),
Ile(B25)-insulin (e.g. human insulin),
Leu(B25)Des(B30)-insulin (e.g. human insulin),
Val(B25)Des(B30)-insulin (e.g. human insulin),
Ile(B25)Des(B30)-insulin (e.g. human insulin),
Glu(A14)Leu(B16)Des(B30)-insulin (e.g. human insulin),
Glu(A14)Ile(B16)Des(B30)-insulin (e.g. human insulin),
Glu(A14)Val(B16)Des(B30)-insulin (e.g. human insulin),
Glu(A14)Leu(B16)-insulin (e.g. human insulin),

Glu(A14)Ile(B16)-insulin (e.g. human insulin),
Glu(A14)Val(B16)-insulin (e.g. human insulin),
Glu(A14)Leu(B25)Des(B30)-insulin (e.g. human insulin),
Glu(A14)Ile(B25)Des(B30)-insulin (e.g. human insulin),
Glu(A14)Val(B25)Des(B30)-insulin (e.g. human insulin),
Glu(A14)Leu(B25)-insulin (e.g. human insulin),
Glu(A14)Ile(B25)-insulin (e.g. human insulin),
Glu(A14)Val(B25)-insulin (e.g. human insulin),
Glu(A14)Gly(A21)Glu(B3)Val(B25)Des(B30)-insulin (e.g. human insulin),
Glu(A14)Ile(B16)Ile(B25)Des(B30)-insulin (e.g. human insulin),
Glu(A14)Glu(B3)Ile(B16)Ile(B25)Des(B30)-insulin (e.g. human insulin),
Glu(A14)Ile(B16)Val(B25)Des(B30)-insulin (e.g. human insulin),
Glu(A14)Gly(A21)Glu(B3)Ile(B16)Val(B25)Des(B30)-insulin (e.g. human insulin),
Glu(A14)Val(B16)Ile(B25)Des(B30)-insulin (e.g. human insulin),
Glu(A14)Val(B16)Val(B25)Des(B30)-insulin (e.g. human insulin),
Glu(A14)Glu(B3)Val(B16)Val(B25)Des(B30)-insulin (e.g. human insulin),
Glu(A14)Gly(A21)Glu(B3)Val(B16)Val(B25)Des(B30)-insulin (e.g. human insulin),
Glu(A14)Gly(A21)Glu(B3)Val(B25)-insulin (e.g. human insulin),
Glu(A14)Ile(B16)Ile(B25)-insulin (e.g. human insulin),
Glu(A14)Glu(B3)Ile(B16)Ile(B25)-insulin (e.g. human insulin),
Glu(A14)Ile(B16)Val(B25)-insulin (e.g. human insulin),
Glu(A14)Gly(A21)Glu(B3)Ile(B16)Val(B25)-insulin (e.g. human insulin),
Glu(A14)Val(B16)Ile(B25)-insulin (e.g. human insulin),
Glu(A14)Val(B16)Val(B25)-insulin (e.g. human insulin),
Glu(A14)Glu(B3)Val(B16)Val(B25)-insulin (e.g. human insulin), and
Glu(A14)Gly(A21)Glu(B3)Val(B16)Val(B25)-insulin (e.g. human insulin).

In another embodiment, the insulin analogs provided herein are selected from the group consisting of:

Asp(A14)Leu(B16)Des(B30)-insulin (e.g. human insulin, i.e. Asp(A14)Leu(B16)Des(B30)-human insulin),
Asp(A14)Ile(B16)Des(B30)-insulin (e.g. human insulin),
Asp(A14)Val(B16)Des(B30)-insulin (e.g. human insulin),
Asp(A14)Leu(B16)-insulin (e.g. human insulin),
Asp(A14)Ile(B16)-insulin (e.g. human insulin),
Asp(A14)Val(B16)-insulin (e.g. human insulin),
Asp(A14)Leu(B25)Des(B30)-insulin (e.g. human insulin),
Asp(A14)Ile(B25)Des(B30)-insulin (e.g. human insulin),
Asp(A14)Val(B25)Des(B30)-insulin (e.g. human insulin),

Asp(A14)Leu(B25)-insulin (e.g. human insulin),
Asp(A14)Ile(B25)-insulin (e.g. human insulin),
Asp(A14)Val(B25)-insulin (e.g. human insulin),
Asp(A14)Gly(A21)Glu(B3)Val(B25)Des(B30)-insulin (e.g. human insulin),
Asp(A14)Ile(B16)Ile(B25)Des(B30)-insulin (e.g. human insulin),
Asp(A14)Glu(B3)Ile(B16)Ile(B25)Des(B30)-insulin (e.g. human insulin),
Asp(A14)Ile(B16)Val(B25)Des(B30)-insulin (e.g. human insulin),
Asp(A14)Gly(A21)Glu(B3)Ile(B16)Val(B25)Des(B30)-insulin (e.g. human insulin),
Asp(A14)Val(B16)Ile(B25)Des(B30)-insulin (e.g. human insulin),
Asp(A14)Val(B16)Val(B25)Des(B30)-insulin (e.g. human insulin),
Asp(A14)Glu(B3)Val(B16)Val(B25)Des(B30)-insulin (e.g. human insulin),
Asp(A14)Gly(A21)Glu(B3)Val(B16)Val(B25)Des(B30)-insulin (e.g. human insulin),
Asp(A14)Gly(A21)Glu(B3)Val(B25)-insulin (e.g. human insulin),
Asp(A14)Ile(B16)Ile(B25)-insulin (e.g. human insulin),
Asp(A14)Glu(B3)Ile(B16)Ile(B25)-insulin (e.g. human insulin),
Asp(A14)Ile(B16)Val(B25)-insulin (e.g. human insulin),
Asp(A14)Gly(A21)Glu(B3)Ile(B16)Val(B25)-insulin (e.g. human insulin),
Asp(A14)Val(B16)Ile(B25)-insulin (e.g. human insulin),
Asp(A14)Val(B16)Val(B25)-insulin (e.g. human insulin),
Asp(A14)Glu(B3)Val(B16)Val(B25)-insulin (e.g. human insulin), and
Asp(A14)Gly(A21)Glu(B3)Val(B16)Val(B25)-insulin (e.g. human insulin).

In another embodiment, the insulin analogs provided herein are selected from the group consisting of:
His(A14)Leu(B16)Des(B30)-insulin (e.g. human insulin),
His(A14)Ile(B16)Des(B30)-insulin (e.g. human insulin),
His(A14)Val(B16)Des(B30)-insulin (e.g. human insulin),
His(A14)Leu(B16)-insulin (e.g. human insulin),
His(A14)Ile(B16)-insulin (e.g. human insulin),
His(A14)Val(B16)-insulin (e.g. human insulin),
His(A14)Leu(B25)Des(B30)-insulin (e.g. human insulin),
His(A14)Ile(B25)Des(B30)-insulin (e.g. human insulin),
His(A14)Val(B25)Des(B30)-insulin (e.g. human insulin),
His(A14)Leu(B25)-insulin (e.g. human insulin),
His(A14)Ile(B25)-insulin (e.g. human insulin),
His(A14)Val(B25)-insulin (e.g. human insulin),
His(A14)Gly(A21)Glu(B3)Val(B25)Des(B30)-insulin (e.g. human insulin),
His(A14)Ile(B16)Ile(B25)Des(B30)-insulin (e.g. human insulin),
His(A14)Glu(B3)Ile(B16)Ile(B25)Des(B30)-insulin (e.g. human insulin),

His(A14)Ile(B16)Val(B25)Des(B30)-insulin (e.g. human insulin),
His(A14)Gly(A21)Glu(B3)Ile(B16)Val(B25)Des(B30)-insulin (e.g. human insulin),
His(A14)Val(B16)Ile(B25)Des(B30)-insulin (e.g. human insulin),
His(A14)Val(B16)Val(B25)Des(B30)-insulin (e.g. human insulin),
His(A14)Glu(B3)Val(B16)Val(B25)Des(B30)-insulin (e.g. human insulin),
His(A14)Gly(A21)Glu(B3)Val(B16)Val(B25)Des(B30)-insulin (e.g. human insulin),
His(A14)Gly(A21)Glu(B3)Val(B25)-insulin (e.g. human insulin),
His(A14)Ile(B16)Ile(B25)-insulin (e.g. human insulin),
His(A14)Glu(B3)Ile(B16)Ile(B25)-insulin (e.g. human insulin),
His(A14)Ile(B16)Val(B25)-insulin (e.g. human insulin),
His(A14)Gly(A21)Glu(B3)Ile(B16)Val(B25)-insulin (e.g. human insulin),
His(A14)Val(B16)Ile(B25)-insulin (e.g. human insulin),
His(A14)Val(B16)Val(B25)-insulin (e.g. human insulin),
His(A14)Glu(B3)Val(B16)Val(B25)-insulin (e.g. human insulin), and
His(A14)Gly(A21)Glu(B3)Val(B16)Val(B25)-insulin (e.g. human insulin).

In another embodiment, the insulin analog is Leu(B25)Des(B30)-Insulin (such as Leu(B25)Des(B30)-human insulin). The sequence of this analog is, e.g., shown in Table 1 of the Examples section (see Analog 11). For example, Leu(B25)Des(B30)-Insulin comprises an A chain having an amino acid sequence as shown in SEQ ID NO: 21 (GIVEQCCTSICSLYQLENYCN) and a B chain having an amino acid sequence as shown in SEQ ID NO: 22 (FVNQHLCGSHLVEALYLVCGERGFLYTPK).

In another embodiment, the insulin analog is Val(B25)Des(B30)-Insulin (such as Val(B25)Des(B30)-human insulin). The sequence of this analog is, e.g., shown in Table 1 of the Examples section (see Analog 12). For example, Val(B25)Des(B30)-Insulin comprises an A chain having an amino acid sequence as shown in SEQ ID NO: 23 (GIVEQCCTSICSLYQLENYCN) and a B chain having an amino acid sequence as shown in SEQ ID NO: 24 (FVNQHLCGSHLVEALYLVCGERGFVYTPK).

In another embodiment, the insulin analog is Glu(A14)Ile(B25)Des(B30)-Insulin (such as Glu(A14)Ile(B25)Des(B30)-human insulin). The sequence of this analog is, e.g., shown in Table 1 of the Examples section (see Analog 22). For example, Glu(A14)Ile(B25)Des(B30)-Insulin comprises an A chain having an amino acid sequence as shown in SEQ ID NO: 43 (GIVEQCCTSICSLEQLENYCN) and a B chain having an amino acid sequence as shown in SEQ ID NO: 44 (FVNQHLCGSHLVEALYLVCGERGFIYTPK).

In another embodiment, the insulin analog is Glu(A14)Val(B25)Des(B30)-Insulin (such as Glu(A14)Val(B25)Des(B30)-human insulin). The sequence of this analog is, e.g., shown in Ta-

ble 1 of the Examples section (see Analog 24). For example, Glu(A14)Val(B25)Des(B30)-Insulin comprises an A chain having an amino acid sequence as shown in SEQ ID NO: 47 (GIVEQCCTSICSLEQLENYCN) and a B chain having an amino acid sequence as shown in SEQ ID NO: 48 (FVNQHLCGSHLVEALYLVCGERGFVYTPK).

In another embodiment, the insulin analog is Glu(A14)Gly(A21)Glu(B3)Val(B25)Des(B30)-Insulin (such as Glu(A14)Gly(A21)Glu(B3) Val(B25)Des(B30)-human insulin). The sequence of this analog is, e.g., shown in Table 1 of the Examples section (see Analog 25). For example, Glu(A14)Gly(A21)Glu(B3) Val(B25)Des(B30)-Insulin comprises an A chain having an amino acid sequence as shown in SEQ ID NO: 49 (GIVEQCCTSICSLEQLENYCG) and a B chain having an amino acid sequence as shown in SEQ ID NO: 50 (FVEQHLCGSHLVEALYLVCGERGFVYTPK).

In another embodiment, the insulin analog is Glu(A14)Ile(B16)Ile(B25)Des(B30)-Insulin(such as Glu(A14)Ile(B16)Ile(B25)Des(B30)-human insulin). The sequence of this analog is, e.g., shown in Table 1 of the Examples section (see Analog 29). For example, Glu(A14)Ile(B16)Ile(B25)Des(B30)-Insulin comprises an A chain having an amino acid sequence as shown in SEQ ID NO: 57 (GIVEQCCTSICSLEQLENYCN) and a B chain having an amino acid sequence as shown in SEQ ID NO: 58 (FVNQHLCGSHLVEALILVCGERGFIYTPK).

In another embodiment, the insulin analog is Glu(A14)Glu(B3)Ile(B16)Ile(B25)Des(B30)-Insulin(such as Glu(A14)Glu(B3)Ile(B16) Ile(B25)Des(B30)-human insulin). The sequence of this analog is, e.g., shown in Table 1 of the Examples section (see Analog 30). For example, Glu(A14)Glu(B3) Ile(B16)Ile(B25)Des(B30)-Insulin comprises an A chain having an amino acid sequence as shown in SEQ ID NO: 56 (GIVEQCCTSICSLEQLENYCN) and a B chain having an amino acid sequence as shown in SEQ ID NO: 60 (FVEQHLCGSHLVEALILVCGERGFIYTPK).

In another embodiment, the insulin analog is Glu(A14)Ile(B16)Val(B25)Des(B30)-Insulin (such as Glu(A14)Ile(B16)Val(B25)Des(B30)-human insulin) The sequence of this analog is, e.g., shown in Table 1 of the Examples section (see Analog 32). For example, Glu(A14)Ile(B16)Val(B25)Des(B30)-Insulin comprises an A chain having an amino acid sequence as shown in SEQ ID NO: 63 (GIVEQCCTSICSLEQLENYCN) and a B chain having an amino acid sequence as shown in SEQ ID NO: 64 (FVNQHLCGSHLVEALILVCGERGFVYTPK).

In another embodiment, the insulin analog is Glu(A14)Gly(A21)Glu(B3)Ile(B16)Val(B25)Des(B30)-Insulin (such as Glu(A14)Gly(A21) Glu(B3)Ile(B16)Val(B25)Des(B30)-human insulin). The sequence of this analog is, e.g., shown in Table 1 of the Examples section (see Analog 33). For example,

Glu(A14)Gly(A21)Glu(B3)Ile(B16)Val(B25)Des(B30)-Insulin comprises an A chain having an amino acid sequence as shown in SEQ ID NO: 65 (GIVEQCCTSICSLEQLENYCG) and a B chain having an amino acid sequence as shown in SEQ ID NO: 66 (FVEQHLCGSHLVEAL-ILVCGERGFVYTPK).

In another embodiment, the insulin analog is Glu(A14)Val(B16)Ile(B25)Des(B30)-Insulin (such as Glu(A14)Val(B16)Ile(B25)Des(B30)-human insulin). The sequence of this analog is, e.g., shown in Table 1 of the Examples section (see Analog 35). For example, Glu(A14)Val(B16)Ile(B25)Des(B30)-Insulin comprises an A chain having an amino acid sequence as shown in SEQ ID NO: 69 (GIVEQCCTSICSLEQLENYCN) and a B chain having an amino acid sequence as shown in SEQ ID NO: 70 (FVNQHLCGSHLVEALVLVCGERGFIYTPK).

In another embodiment, the insulin analog is Glu(A14)Val(B16)Val(B25)Des(B30)-Insulin (such as Glu(A14)Val(B16)Val(B25) Des(B30)-human insulin). The sequence of this analog is, e.g., shown in Table 1 of the Examples section (see Analog 38). For example, Glu(A14)Val(B16)Val(B25)Des(B30)-Insulin comprises an A chain having an amino acid sequence as shown in SEQ ID NO: 75 (GIVEQCCTSICSLEQLENYCN) and a B chain having an amino acid sequence as shown in SEQ ID NO: 76 (FVNQHLCGSHLVEALVLVCGERGFVYTPK).

In another embodiment, the insulin analog is Glu(A14)Glu(B3)Val(B16)Val(B25)Des(B30)-Insulin (such as Glu(A14)Glu(B3)Val(B16) Val(B25)Des(B30)-human insulin). The sequence of this analog is, e.g., shown in Table 1 of the Examples section (see Analog 39). For example, Glu(A14)Glu(B3)Val(B16) Val(B25)Des(B30)-Insulin comprises an A chain having an amino acid sequence as shown in SEQ ID NO: 77 (GIVEQCCTSICSLEQLENYCN) and a B chain having an amino acid sequence as shown in SEQ ID NO: 78 (FVEQHLCGSHLVEALVLVCGERGFVYTPK).

In another embodiment, the insulin analog is Glu(A14)Gly(A21)Glu(B3)Val(B16)Val(B25)Des(B30)-Insulin (such as Glu(A14)Gly(A21) Glu(B3)Val(B16)Val(B25)Des(B30)-human insulin). The sequence of this analog is, e.g., shown in Table 1 of the Examples section (see Analog 40). For example, Glu(A14)Gly(A21)Glu(B3)Val(B16)Val(B25)Des(B30)-Insulin comprises an A chain having an amino acid sequence as shown in SEQ ID NO: 79 (GIVEQCCTSICSLEQLENYCG) and a B chain having an amino acid sequence as shown in SEQ ID NO: 80 (FVEQHLCGSHLVEALVLVCGERGFVYTPK).

The insulin analog can be prepared by any method deemed appropriate. For example, the insulin analog can be prepared by recombinant methods or by solid-phase synthesis.

The definitions and explanations given herein above apply *mutatis mutandis* to the following.

Provided herein are insulin B chains, i.e. insulin B chain peptides, as defined herein above in connection with the B chain of the insulin analog provided herein. Accordingly, provided herein are insulin B chains which comprise at least one mutation relative to the insulin B chain of the parent insulin, wherein the B chains comprise a mutation at position B16 which is substituted with a hydrophobic amino acid, and/or a mutation at position B25 which is substituted with a hydrophobic amino acid. The insulin B chain may comprise further mutations as described herein above such as the des(B30) deletion.

Also provided herein are proinsulins comprising an insulin A chain and/or an insulin B chain of the insulin analogs provided herein. The B chain may be any B chain as defined herein above for the insulin analogs provided herein. For example, provided herein are proinsulins comprising an insulin A chain and an insulin B chain, wherein said B chain comprises at least one mutation relative to B chain of a parent insulin, wherein the mutation is in position B16 which is substituted with a hydrophobic amino acid and/or wherein the mutation is in position B25 which is substituted with a hydrophobic amino acid. The insulin B chain may comprise further mutations as described herein above for the B chain.

The A chain comprised by the proinsulin provided herein may be any A chain as defined herein above for the insulin analogs provided herein. In some embodiments, the A chain of said proinsulin comprises a mutation at position A14 which is substituted with an amino acid selected from glutamic acid (Glu), aspartic acid (Asp) and histidine (His).

In addition to the insulin A chain and/or the insulin B chain, the proinsulins provided herein may comprise further elements such as leader sequences or a C-peptide. In some embodiments, the proinsulin may further comprise a C-peptide which is located between the insulin B chain and the insulin A chain. The C-peptide may have a length of 4-10 amino acids, such as a length of 4 to 9 amino acids. The orientation may be as follows (from N-terminus to C-terminus): B chain, C-peptide, A chain.

Provided herein are polynucleotides encoding the insulin analogs, insulin B chains, and the proinsulins provided herein. Said polynucleotide may be operably linked to a promoter which allows for the expression of said polynucleotide. In some embodiments, the promoter is heterologous with respect to said polynucleotide. In some embodiments, the promoter is a constitutive promoter. In another embodiment, the promoter is an inducible promoter.

Further, provided herein are vectors comprising the polynucleotide encoding the insulin analogs provided herein. In some embodiments, said vector is an expression vector.

Provided herein are host cells comprising nucleic acids encoding the insulin analogs, insulin B chains, and proinsulins, the polynucleotides, and/or the vectors provided herein. In some embodiments, the host cell is a bacterial cell such as a cell of belonging to the genus *Escherichia*, e.g. an *E. coli* cell. In another embodiment, the host cell is a yeast cell, such as a *Pichia pastoris* cell or *Klyveromyces lactis* cell.

Provided herein are pharmaceutical compositions comprising a pharmaceutically effective amount of an insulin analog provided herein and a pharmaceutically acceptable excipient.

Provided herein are methods for treating a disease comprising administering a pharmaceutically effective amount of one or more insulin analogs provided herein or the pharmaceutical composition thereof to a subject.

In some embodiments, the disease is diabetes mellitus such as diabetes type II mellitus.

Provided herein are insulin analogs or the pharmaceutical composition thereof for use in medicine.

Provided herein are insulin analogs or the pharmaceutical composition thereof for use in the treatment of diabetes mellitus, such as of diabetes type II mellitus.

Finally, provided herein are uses of the insulin analogs provided herein or the pharmaceutical compositions thereof for the preparation of a medicament or drug for the treatment of diabetes mellitus, such as of diabetes type II mellitus.

The insulin analogs, insulin B chains, proinsulins, and uses are further illustrated by the following embodiments and combinations of embodiments as indicated by the respective dependencies and back-references. The definitions and explanations given herein above apply *mutatis mutandis* to the following embodiments.

1. An insulin analog comprising at least one mutation relative to the parent insulin, wherein the insulin analog comprises a mutation at position B16 which is substituted with a hydrophobic amino acid and/or a mutation at position B25 which is substituted with a hydrophobic amino acid.

2. The insulin analog of embodiment 1, wherein the parent insulin is human insulin, porcine insulin, or bovine insulin.

3. The insulin analog of embodiments 1 and 2, wherein the hydrophobic amino acid at position B16 and/or position B25 is an aliphatic amino acid.

4. The insulin analog of any one of embodiments 1 to 3, wherein said aliphatic amino acid at position B16 and/or position B25 is a branched-chain amino acid, such as a branched-chain amino acid selected from the group consisting of valine (Val), isoleucine (Ile), and leucine (Leu).

5. The insulin analog of any one of embodiments 1 to 3, wherein said insulin analog further comprises a mutation at position A14 which is substituted with an amino acid selected from the group consisting of glutamic acid (Glu), aspartic acid (Asp) and histidine (His).

6. The insulin analog of any one of embodiments 1 to 5, wherein said insulin analog further comprises a mutation at position B30, e.g. wherein the mutation at position B30 is the deletion of the amino acid at position B30 of the parent insulin (Des(B30)-mutation).

7. The insulin analog of any one of embodiments 1 to 6, wherein said insulin analog further comprises a mutation at position B3 which is substituted with a glutamic acid (Glu).

8. The insulin analog of any one of embodiments 1 to 7, wherein said insulin further comprises a mutation at position A21 which is substituted with glycine (Gly).

9. The insulin analog of any one of embodiments 1 to 8, wherein the B chain of the insulin analog comprises or consists of an amino acid sequence selected from the group consisting of

FVNQHLCGSHLVEALYLVCGERGFLYTPK	(SEQ ID NO: 22)
FVNQHLCGSHLVEALYLVCGERGFIYTPK	(SEQ ID NO: 44)
FVNQHLCGSHLVEALYLVCGERGFVYTPK	(SEQ ID NO: 48)
FVEQHLCGSHLVEALYLVCGERGFVYTPK	(SEQ ID NO: 50)
FVNQHLCGSHLVEALILVCGERGFIYTPK	(SEQ ID NO: 58)
FVEQHLCGSHLVEALILVCGERGFIYTPK	(SEQ ID NO: 60)
FVNQHLCGSHLVEALILVCGERGFVYTPK	(SEQ ID NO: 64)
FVEQHLCGSHLVEALILVCGERGFVYTPK	(SEQ ID NO: 66)
FVNQHLCGSHLVEALVLVCGERGFIYTPK	(SEQ ID NO: 70)
FVNQHLCGSHLVEALVLVCGERGFVYTPK	(SEQ ID NO: 76)
FVEQHLCGSHLVEALVLVCGERGFVYTPK	(SEQ ID NO: 78)
FVEQHLCGSHLVEALVLVCGERGFVYTPK	(SEQ ID NO: 80)
FVNQHLCGSHLVEALYLVCGERGFLYTPKT	(SEQ ID NO: 85)
FVNQHLCGSHLVEALYLVCGERGFVYTPKT	(SEQ ID NO: 86)

FVNQHLCGSHLVEALYLVCGERGFIYTPKT (SEQ ID NO: 87)
FVNQHLCGSHLVEALYLVCGERGFVYTPKT (SEQ ID NO: 88)
FVEQHLCGSHLVEALYLVCGERGFVYTPKT (SEQ ID NO: 89)
FVNQHLCGSHLVEALILVCGERGFIYTPKT (SEQ ID NO: 90)
FVEQHLCGSHLVEALILVCGERGFIYTPKT (SEQ ID NO: 91)
FVNQHLCGSHLVEALILVCGERGFVYTPKT (SEQ ID NO: 92)
FVEQHLCGSHLVEALILVCGERGFVYTPKT (SEQ ID NO: 93)
FVNQHLCGSHLVEALVLVCGERGFIYTPKT (SEQ ID NO: 94)
FVNQHLCGSHLVEALVLVCGERGFVYTPKT (SEQ ID NO: 95)
FVEQHLCGSHLVEALVLVCGERGFVYTPKT (SEQ ID NO: 96), and
FVEQHLCGSHLVEALVLVCGERGFVYTPKT (SEQ ID NO: 97).

10. The insulin analog of any one of embodiments 1 to 9, comprising

(a) an A chain having an amino acid sequence as shown in SEQ ID NO: 43 (GIVEQCCTSICSLEQLENYCN) and a B chain having an amino acid sequence as shown in SEQ ID NO: 44 (FVNQHLCGSHLVEALYLVCGERGFIYTPK),

(b) an A chain having an amino acid sequence as shown in SEQ ID NO: 47 (GIVEQCCTSICSLEQLENYCN) and a B chain having an amino acid sequence as shown in SEQ ID NO: 48 (FVNQHLCGSHLVEALYLVCGERGFVYTPK), or

(c) an A chain having an amino acid sequence as shown in SEQ ID NO: 77 (GIVEQCCTSICSLEQLENYCN) and a B chain having an amino acid sequence as shown in SEQ ID NO: 78 (FVEQHLCGSHLVEALVLVCGERGFVYTPK).

11. An insulin analog selected from the group consisting of

Leu(B16)-human insulin,
Val(B16)-human insulin,
Ile(B16)-human insulin,
Leu(B16)Des(B30)-human insulin,
Val(B16)Des(B30)-human insulin,
Ile(B16)Des(B30)-human insulin,
Leu(B25)-human insulin,
Val(B25)-human insulin,
Ile(B25)-human insulin,
Leu(B25)Des(B30)-human insulin,
Val(B25)Des(B30)-human insulin,
Ile(B25)Des(B30)-human insulin,
Glu(A14)Leu(B16)Des(B30)-human insulin,
Glu(A14)Ile(B16)Des(B30)-human insulin,
Glu(A14)Val(B16)Des(B30)-human insulin,

Glu(A14)Leu(B16)-human insulin,
Glu(A14)Ile(B16)-human insulin,
Glu(A14)Val(B16)-human insulin,
Glu(A14)Leu(B25)Des(B30)-human insulin,
Glu(A14)Ile(B25)Des(B30)-human insulin,
Glu(A14)Val(B25)Des(B30)-human insulin,
Glu(A14)Leu(B25)-human insulin,
Glu(A14)Ile(B25)-human insulin,
Glu(A14)Val(B25)-human insulin,
Glu(A14)Gly(A21)Glu(B3)Val(B25)Des(B30)-human insulin,
Glu(A14)Ile(B16)Ile(B25)Des(B30)-human insulin,
Glu(A14)Glu(B3)Ile(B16)Ile(B25)Des(B30)-human insulin,
Glu(A14)Ile(B16)Val(B25)Des(B30)-human insulin,
Glu(A14)Gly(A21)Glu(B3)Ile(B16)Val(B25)Des(B30)-human insulin,
Glu(A14)Val(B16)Ile(B25)Des(B30)-human insulin,
Glu(A14)Val(B16)Val(B25)Des(B30)-human insulin,
Glu(A14)Glu(B3)Val(B16)Val(B25)Des(B30)-human insulin,
Glu(A14)Gly(A21)Glu(B3)Val(B16)Val(B25)Des(B30)-human insulin,
Glu(A14)Gly(A21)Glu(B3)Val(B25)-human insulin,
Glu(A14)Ile(B16)Ile(B25)-human insulin,
Glu(A14)Glu(B3)Ile(B16)Ile(B25)-human insulin,
Glu(A14)Ile(B16)Val(B25)-human insulin,
Glu(A14)Gly(A21)Glu(B3)Ile(B16)Val(B25)-human insulin,
Glu(A14)Val(B16)Ile(B25)-human insulin,
Glu(A14)Val(B16)Val(B25)-human insulin,
Glu(A14)Glu(B3)Val(B16)Val(B25)-human insulin, and
Glu(A14)Gly(A21)Glu(B3)Val(B16)Val(B25)-human insulin.

12. An insulin B chain comprising at least one mutation relative to the B chain of the parent insulin, wherein the B chain comprises a mutation at position B16 which is substituted with a hydrophobic amino acid, and/or a mutation at position B25 which is substituted with a hydrophobic amino acid.

13. The insulin B chain according to embodiment 12, wherein the parent insulin is human insulin, porcine insulin, or bovine insulin.

14. The insulin B chain according to embodiments 12 and 13, wherein the hydrophobic amino acid at position B16 and/or position B25 is an aliphatic amino acid.

15. The insulin B chain according to any one of embodiments 12 to 14, wherein said aliphatic amino acid in a branched-chain amino acid, such as a branched-chain amino acid selected from the group consisting of valine (Val), isoleucine (Ile), and leucine (Leu).

16. The insulin B chain according to any one of embodiments 12 to 15, wherein said insulin B chain further comprises a mutation at position B3 which is substituted with a glutamic acid (Glu).

17. The insulin B chain according to any one of embodiments 12 to 16, wherein said insulin B chain further comprises a mutation at position B30, wherein the mutation at position B30 is the deletion of the amino acid at position B30 of the parent insulin (Des(B30)-mutation).

18. A proinsulin comprising an insulin A chain and an insulin B chain, wherein the insulin B chain comprises at least one mutation relative to B chain of a parent insulin, wherein the B chain comprises a mutation at position B16 which is substituted with a hydrophobic amino acid and/or a mutation at position B25 which is substituted with a hydrophobic amino acid.

19. The proinsulin of embodiment 18, wherein the insulin A chain of said proinsulin comprises a mutation at position A14 which is substituted with an amino acid selected from glutamic acid (Glu), aspartic acid (Asp) and histidine (His).

20. The proinsulin according to embodiments 18 and 19, wherein the parent insulin is human insulin, porcine insulin, or bovine insulin.

21. The proinsulin according to any one of embodiments 18 to 20, wherein the hydrophobic amino acid at position B16 and/or position B25 is an aliphatic amino acid.

22. The proinsulin according to any one of embodiments 18 to 21, wherein said aliphatic amino acid in a branched-chain amino acid, such as a branched-chain amino acid selected from the group consisting of valine (Val), isoleucine (Ile), and leucine (Leu).

23. The proinsulin according to any one of embodiments 18 to 22, wherein said proinsulin further comprises a mutation at position B3 which is substituted with a glutamic acid (Glu).

24. The proinsulin according to any one of embodiments 18 to 23, wherein said proinsulin further comprises a mutation at position B30, wherein the mutation at position B30 is the deletion of the amino acid at position B30 of the parent insulin (Des(B30)-mutation).

25. A polynucleotide encoding the insulin analog of any one of embodiments 1 to 11, the insulin B chain of any one of embodiments 12 to 17, and/or the proinsulin of any one of embodiment 18 to 24.

26. An expression vector comprising the polynucleotide of embodiment 25.

27. A host cell comprising insulin analog of any one of embodiments 1 to 11, the insulin B chain of any one of embodiments 12 to 17, the proinsulin of any one of embodiments 18 to 24, the polynucleotide of embodiment 25, and/or the expression vector of embodiment 26.

28. A method for treating a patient having diabetes mellitus comprising administering to the patient one or more insulin analog as defined in any one of embodiments 1 to 11.

29. The insulin analog as defined in any one of embodiments 1 to 11 for use in treating diabetes mellitus.

All references cited in this specification are herewith incorporated by reference with respect to their entire disclosure content and the disclosure content specifically mentioned in this specification.

Example 1: Production of human insulin and insulin analogs

Human insulin as well as insulin analogs were produced recombinantly. Polynucleotides encoding pre-pro-insulin were ordered from Geneart®. The designed polynucleotides were optimized for expression in yeast. They were inserted into an expression vector by classical restriction cloning enabling functional expression and secretion in *Klyveromyces lactis* K. As secretion leader, the gene was C-terminally fused to a DNA sequence encoding the alpha mating factor signal of *Saccharomyces cerevisiae*. The recombinant gene expression was controlled by a lactose inducible *K. lactis* promoter.

Human insulin as well as insulin analogs were manufactured as a pre-pro-insulin. A genetically fused N-terminal pre-sequence was used to improve expression and secretion yields and to stabilize the peptide in the culture broth. A broad variety of sequences can be used for this purpose and were tested for efficiency. The proinsulin itself consists of a B-chain fused to a C-peptide followed by the C-terminal A-chain. As C-peptide a variety of amino acid combinations are described. It was shown that short peptides of 1-10 amino acids work well as C-sequences.

For later processing of the insulin the recognition sites for specific proteases, which flank the C-peptide to enable its excision, are important.

K. lactis cells were made competent by chemical means. Subsequently, the cells were transformed with the expression plasmid coding for the respective pre-pro-insulin. After insertion of the plasmid, cells were plated on selective agar plates containing geneticin. Grown colonies were isolated and tested for recombinant gene expression. Cells were grown to sufficiently high cell densities in yeast peptone dextrose medium supplemented with geneticin. After an initial growth phase, a salt-buffered yeast extract medium with geneticin supplemented with lactose was added to the cultures to induce expression of the recombinant gene. Cultures were grown several days and supernatants were harvested by centrifugation.

Purification of the functional insulin or insulin analogs was started by a filtration procedure. Initial chromatographic capturing procedure was made with an ion-exchange resin. Cleavage of pre-pro-insulin to insulin was performed with a highly specific protease. Depletion of host cell protein, pre-sequence and product related products were made by a cascade of two additional chromatographic steps. Next to a hydrophobic interaction chromatography another ion-exchange procedure was applied to achieve this goal. Final polishing was made by reverse phase chromatography. Filtration, precipitation and freeze drying were used to finish the production process of the insulin molecule.

After coupling reactions with an activated carboxylic acid derivative, the solution with conjugated insulin molecules was filtered. Final purification was made by reverse phase chromatography. Filtration, precipitation and freeze drying were used to finish the synthesis of the target molecule.

Various insulins analogs with mutations e.g. at positions B16, B25 and/or A14 were generated. Table 1 provides an overview of the generated insulins.

Table 1: Generated analogs of human Insulin

Analog	Backbone	A14	B16	B25	Amino acid sequence Chain A	SEQ ID NO (A chain)	Amino acid sequence Chain B	SEQ ID NO (B-Chain)
WT	Human Insulin (wild-type)	Tyr	Tyr	Phe	GIVEQCCT-SICSLYQLE-NYCN	1	FVNQHLCGSHLVEA-LYLVCGERGFFYTPKT	2
2	Glu(A14)Des(B30)-Insulin	Glu	Tyr	Phe	GIVEQCCT-SICSLEQLE-NYCN	3	FVNQHLCGSHLVEA-LYLVCGERGFFYTPK	4
3	Leu(B16)Des(B30)-Insulin	Tyr	Leu	Phe	GIVEQCCT-SICSLYQLE-NYCN	5	FVNQHLCGSHLVEALLL-VCGERGFFYTPK	6
4	Gly(A21)Trp(B16)Des(B30)-Insulin	Tyr	Trp	Phe	GIVEQCCT-SICSLYQLE-NYCG	7	FVNQHLCGSHL-VEALWLVCGERGFFYTPK	8
5	His(B16)Des(B30)-Insulin	Tyr	His	Phe	GIVEQCCT-SICSLYQLE-NYCN	9	FVNQHLCGSHLVEALHL-VCGERGFFYTPK	10
6	Val(B16)Des(B30)-Insulin	Tyr	Val	Phe	GIVEQCCT-SICSLYQLE-NYCN	11	FVNQHLCGSHLVEAL-VLVCGERGFFYTPK	12
7	Ala(B25)-Insulin	Tyr	Tyr	Ala	GIVEQCCT-SICSLYQLE-NYCN	13	FVNQHLCGSHLVEA-LYLVCGERGFAYTPKT	14
8	Ala(B25)Des(B30)-Insulin	Tyr	Tyr	Ala	GIVEQCCT-	15	FVNQHLCGSHLVEA-	16

					SICSLYQLE-NYCN		LYLVCGERGFAYTPK	
9	Glu(B25)Des(B30)-Insulin	Tyr	Tyr	Glu	GIVEQCCT-SICSLYQLE-NYCN	17	FVNQHLCGSHLVEA-LYLVCGERGFEYTPK	18
10	His(B25)Des(B30)-Insulin	Tyr	Tyr	His	GIVEQCCT-SICSLYQLE-NYCN	19	FVNQHLCGSHLVEA-LYLVCGERGFHYTPK	20
11	Leu(B25)Des(B30)-Insulin	Tyr	Tyr	Leu	GIVEQCCT-SICSLYQLE-NYCN	21	FVNQHLCGSHLVEA-LYLVCGERGFLYTPK	22
12	Val(B25)Des(B30)-Insulin	Tyr	Tyr	Val	GIVEQCCT-SICSLYQLE-NYCN	23	FVNQHLCGSHLVEA-LYLVCGERGFVYTPK	24
13	His(B16)His(B25)Des(B30)-Insulin	Tyr	His	His	GIVEQCCT-SICSLYQLE-NYCN	25	FVNQHLCGSHLVEALHL-VCGERGFHYTPK	26
14	Gly(A21)Trp(B16)His(B25) Des(B30)-Insulin	Tyr	Trp	His	GIVEQCCT-SICSLYQLE-NYCG	27	FVNQHLCGSHL-VEALWLVCGERGFHYTPK	28
15	Gly(A21)Trp(B16)Trp(B25) Des(B30)-Insulin	Tyr	Trp	Trp	GIVEQCCT-SICSLYQLE-NYCG	29	FVNQHLCGSHL-VEALWLVCGERG-FWYTPK	30
16	Glu(A14)His(B16)Des(B30) -Insulin	Glu	His	Phe	GIVEQCCT-SICSLEQLE-	31	FVNQHLCGSHLVEALHL-VCGERGFFYTPK	32

					NYCN			
17	Glu(A14)Gly(A21)Trp(B16)Des(B30)-Insulin	Glu	Trp	Phe	GIVEQCCT-SICSLEQLE-NYCG	33	FVNQHLCGSHL-VEALWLVCGERGFFYTPK	34
18	Glu(A14)Ile(B16)Des(B30)-Insulin	Glu	Ile	Phe	GIVEQCCT-SICSLEQLE-NYCN	35	FVNQHLCGSHLVEALIL-VCGERGFFYTPK	36
19	Glu(A14)Val(B16)Des(B30)-Insulin	Glu	Val	Phe	GIVEQCCT-SICSLEQLE-NYCN	37	FVNQHLCGSHLVEAL-VLVCGERGFFYTPK	38
20	Glu(A14)Glu(B3)Val(B16)Des(B30)-Insulin	Glu	Val	Phe	GIVEQCCT-SICSLEQLE-NYCN	39	FVEQHLCGSHLVEAL-VLVCGERGFFYTPK	40
21	Glu(A14)His(B25)Des(B30)-Insulin	Glu	Tyr	His	GIVEQCCT-SICSLEQLE-NYCN	41	FVNQHLCGSHLVEA-LYLVCGERGFHYTPK	42
22	Glu(A14)Ile(B25)Des(B30)-Insulin	Glu	Tyr	Ile	GIVEQCCT-SICSLEQLE-NYCN	43	FVNQHLCGSHLVEA-LYLVCGERGFIYTPK	44
23	Glu(A14)Gly(A21)Trp(B25)Des(B30)-Insulin	Glu	Tyr	Trp	GIVEQCCT-SICSLEQLE-NYCG	45	FVNQHLCGSHLVEA-LYLVCGERGFWYTPK	46
24	Glu(A14)Val(B25)Des(B30)-Insulin	Glu	Tyr	Val	GIVEQCCT-SICSLEQLE-NYCN	47	FVNQHLCGSHLVEA-LYLVCGERGFVYTPK	48

25	Glu(A14)Gly(A21)Glu(B3)Val(B25)Des(B30)-Insulin	Glu	Tyr	Val	GIVEQCCT-SICSLEQLE-NYCG	49	FVEQHLCGSHLVEA-LYLVCGERGFVYTPK	50
26	Glu(A14)Glu(B16)His(B25)Des(B30)-Insulin	Glu	Glu	His	GIVEQCCT-SICSLEQLE-NYCN	51	FVNQHLCGSHLVEALEL-VCGERGFHYTPK	52
27	Glu(A14)His(B16)Ala(B25)Des(B30)-Insulin	Glu	His	Ala	GIVEQCCT-SICSLEQLE-NYCN	53	FVNQHLCGSHLVEALHL-VCGERGFAYTPK	54
28	Glu(A14)His(B16)His(B25)Des(B30)-Insulin	Glu	His	His	GIVEQCCT-SICSLEQLE-NYCN	55	FVNQHLCGSHLVEALHL-VCGERGFHYTPK	56
29	Glu(A14)Ile(B16)Ile(B25)Des(B30)-Insulin	Glu	Ile	Ile	GIVEQCCT-SICSLEQLE-NYCN	57	FVNQHLCGSHLVEALIL-VCGERGFIYTPK	58
30	Glu(A14)Glu(B3)Ile(B16)Ile(B25)Des(B30)-Insulin	Glu	Ile	Ile	GIVEQCCT-SICSLEQLE-NYCN	59	FVEQHLCGSHLVEALIL-VCGERGFIYTPK	60
31	Glu(A14)Gly(A21)Glu(B3)Ile(B16)Trp(B25)Des(B30)-Insulin	Glu	Ile	Trp	GIVEQCCT-SICSLEQLE-NYCG	61	FVEQHLCGSHLVEALIL-VCGERGFWYTPK	62
32	Glu(A14)Ile(B16)Val(B25)Des(B30)-Insulin	Glu	Ile	Val	GIVEQCCT-SICSLEQLE-NYCN	63	FVNQHLCGSHLVEALIL-VCGERGFVYTPK	64
33	Glu(A14)Gly(A21)Glu(B3)Il	Glu	Ile	Val	GIVEQCCT-	65	FVEQHLCGSHLVEALIL-	66

	e(B16)Val(B25)Des(B30)-Insulin				SICSLEQLE-NYCG		VCGERGFVYTPK	
34	Glu(A14)Leu(B16)Ala(B25)Des(B30)-Insulin	Glu	Leu	Ala	GIVEQCCT-SICSLEQLE-NYCN	67	FVNQHLCGSHLVEALLL-VCGERGFAYTPK	68
35	Glu(A14)Val(B16)Ile(B25)Des(B30)-Insulin	Glu	Val	Ile	GIVEQCCT-SICSLEQLE-NYCN	69	FVNQHLCGSHLVEAL-VLVCGERGFIYTPK	70
36	Glu(A14)Gly(A21)Val(B16)Trp(B25)Des(B30)-Insulin	Glu	Val	Trp	GIVEQCCT-SICSLEQLE-NYCG	71	FVNQHLCGSHLVEAL-VLVCGERGFWYTPK	72
37	Glu(A14)Gly(A21)Glu(B3)Val(B16)Trp(B25)Des(B30)-Insulin	Glu	Val	Trp	GIVEQCCT-SICSLEQLE-NYCG	73	FVEQHLCGSHLVEAL-VLVCGERGFWYTPK	74
38	Glu(A14)Val(B16)Val(B25)Des(B30)-Insulin	Glu	Val	Val	GIVEQCCT-SICSLEQLE-NYCN	75	FVNQHLCGSHLVEAL-VLVCGERGFVYTPK	76
39	Glu(A14)Glu(B3)Val(B16)Val(B25)Des(B30)-Insulin	Glu	Val	Val	GIVEQCCT-SICSLEQLE-NYCN	77	FVEQHLCGSHLVEAL-VLVCGERGFVYTPK	78
40	Glu(A14)Gly(A21)Glu(B3)Val(B16)Val(B25)Des(B30)-Insulin	Glu	Val	Val	GIVEQCCT-SICSLEQLE-NYCG	79	FVEQHLCGSHLVEAL-VLVCGERGFVYTPK	80

Example 2: Insulin receptor binding affinity assays / Insulin receptor autophosphorylation assays

Insulin binding and signal transduction of various generated insulin analogs were determined by a binding assay and a receptor autophosphorylation assay.

A) Insulin receptor binding affinity assay

Insulin receptor binding affinity for the analogs listed in Table 1 was determined as described in Hartmann *et al.* (Effect of the long-acting insulin analogs glargine and degludec on cardiomyocyte cell signaling and function. Cardiovasc Diabetol. 2016;15:96): Isolation of insulin receptor embedded plasma membranes (M-IR) and competition binding experiments were performed as previously described (Sommerfeld *et al.*, PLoS One. 2010; 5(3): e9540). Briefly, CHO-cells overexpressing the IR were collected and re-suspended in ice-cold 2.25 STM buffer (2.25 M sucrose, 5 mM Tris–HCl pH 7.4, 5 mM $MgCl_2$, complete protease inhibitor) and disrupted using a Dounce homogenizer followed by sonication. The homogenate was overlaid with 0.8 STM buffer (0.8 M sucrose, 5 mM Tris–HCl pH 7.4, 5 mM $MgCl_2$, complete protease inhibitor) and ultra-centrifuged for 90 min at 100,000g. Plasma membranes at the interface were collected and washed twice with phosphate buffered saline (PBS). The final pellet was re-suspended in dilution buffer (50 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, complete protease inhibitor) and again homogenized with a Dounce homogenizer. Competition binding experiments were performed in a binding buffer (50 mM Tris–HCl, 150 mM NaCl, 0.1 % BSA, complete protease inhibitor, adjusted to pH 7.8) in 96-well microplates. In each well 2 µg isolated membrane was incubated with 0.25 mg wheat germ agglutinin polyvinyltoluene polyethylenimine scintillation proximity assay (SPA) beads. Constant concentrations of [125I]-labelled human insulin (100 pM) and various concentrations of respective unlabelled insulin (0.001–1000 nM) were added for 12 h at room temperature (23 °C). The radioactivity was measured at equilibrium in a microplate scintillation counter (Wallac Microbeta, Freiburg, Germany)."

The results of the insulin receptor binding affinity assays for the tested analogs relative to human insulin are shown in Table 2.

B) Insulin receptor autophosphorylation assays (as a measure for signal transduction)

In order to determine signal transduction of an insulin analog binding to insulin receptor B, autophosphorylation was measured *in vitro*.
CHO cells expressing human insulin receptor isoform B (IR-B) were used for IR autophosphorylation assays using In-Cell Western technology as previously described (Sommerfeld *et al.*,

PLoS One. 2010; 5(3): e9540). For the analysis of IGF1R autophosphorylation, the receptor was overexpressed in a mouse embryo fibroblast 3T3 Tet off cell line (BD Bioscience, Heidelberg, Germany) that was stably transfected with IGF1R tetracycline-regulatable expression plasmid. In order to determine the receptor tyrosine phosphorylation level, cells were seeded into 96-well plates and grown for 44 h. Cells were serum starved with serum-free medium Ham's F12 medium (Life Technologies, Darmstadt, Germany) for 2 h. The cells were subsequently treated with increasing concentrations of either human insulin or the insulin analog for 20 min at 37°C. After incubation the medium was discarded and the cells fixed in 3.75% freshly prepared para-formaldehyde for 20 min. Cells were permeabilised with 0.1% Triton X-100 in PBS for 20 min. Blocking was performed with Odyssey blocking buffer (LICOR, Bad Homburg, Germany) for 1 hour at room temperature. Anti-pTyr 4G10 (Millipore, Schwalbach, Germany) was incubated for 2 h at room temperature. After incubation of the primary antibody, cells were washed with PBS + 0.1% Tween 20 (Sigma-Aldrich, St Louis, MO, USA). The secondary anti-mouse-IgG-800-CW antibody (LICOR, Bad Homburg, Germany) was incubated for 1 h. Results were normalized by the quantification of DNA with TO-PRO3 dye (Invitrogen, Karlsruhe, Germany). Data were obtained as relative units (RU).

The results of the insulin receptor autophosphorylation assays for the tested analogs relative to human insulin are shown in Table 2.

Table 2: Relative insulin receptor binding affinities and autophosphorylation activities of tested analogs of human insulin (for the sequences, please see Table 1).

Analog	Backbone	A14	B16	B25	Insulin-receptor binding affinity*	auto-phospho-rylation activity*
WT	Human Insulin (wild-type)	Tyr	Tyr	Phe	1	1
2	Glu(A14)Des(B30)-Insulin	Glu	Tyr	Phe	1.05	0.87
3	Leu(B16)Des(B30)-Insulin	Tyr	Leu	Phe	0.24	0.34
4	Gly(A21)Trp(B16)Des(B30)-Insulin	Tyr	Trp	Phe	0.57	0.4
5	His(B16)Des(B30)-Insulin	Tyr	His	Phe	nd	nd
6	Val(B16)Des(B30)-Insulin	Tyr	Val	Phe	nd	0.32
7	Ala(B25)-Insulin	Tyr	Tyr	Ala	0.05	0.2
8	Ala(B25)Des(B30)-Insulin	Tyr	Tyr	Ala	nd	0.17
9	Glu(B25)Des(B30)-Insulin	Tyr	Tyr	Glu	nd	nd
10	His(B25)Des(B30)-Insulin	Tyr	Tyr	His	0.37	0.31
11	Leu(B25)Des(B30)-Insulin	Tyr	Tyr	Leu	0.01	0.06

12	Val(B25)Des(B30)-Insulin	Tyr	Tyr	Val	0.01	0.06
13	His(B16)His(B25)Des(B30)-Insulin	Tyr	His	His	0.11	0.1
14	Gly(A21)Trp(B16)His(B25)Des(B30)-Insulin	Tyr	Trp	His	0.4	0.35
15	Gly(A21)Trp(B16)Trp(B25)Des(B30)-Insulin	Tyr	Trp	Trp	0.43	0.38
16	Glu(A14)His(B16)Des(B30)-Insulin	Glu	His	Phe	0.36	0.29
17	Glu(A14)Gly(A21)Trp(B16)Des(B30)-Insulin	Glu	Trp	Phe	0.63	0.38
18	Glu(A14)Ile(B16)Des(B30)-Insulin	Glu	Ile	Phe	0.23	0.18
19	Glu(A14)Val(B16)Des(B30)-Insulin	Glu	Val	Phe	nd	0.32
20	Glu(A14)Glu(B3)Val(B16)Des(B30)-Insulin	Glu	Val	Phe	0.4	0.28
21	Glu(A14)His(B25)Des(B30)-Insulin	Glu	Tyr	His	nd	nd
22	Glu(A14)Ile(B25)Des(B30)-Insulin	Glu	Tyr	Ile	0.01	0.04
23	Glu(A14)Gly(A21)Trp(B25)Des(B30)-Insulin	Glu	Tyr	Trp	0.56	0.37
24	Glu(A14)Val(B25)Des(B30)-Insulin	Glu	Tyr	Val	0.01	0.04
25	Glu(A14)Gly(A21)Glu(B3)Val(B25)Des(B30)-Insulin	Glu	Tyr	Val	0.02	0.03
26	Glu(A14)Glu(B16)His(B25)Des(B30)-Insulin	Glu	Glu	His	0.01	0.07
27	Glu(A14)His(B16)Ala(B25)Des(B30)-Insulin	Glu	His	Ala		0.11
28	Glu(A14)His(B16)His(B25)Des(B30)-Insulin	Glu	His	His	0.12	0.11
29	Glu(A14)Ile(B16)Ile(B25)Des(B30)-Insulin	Glu	Ile	Ile		0.01
30	Glu(A14)Glu(B3)Ile(B16)Ile(B25)Des(B30)-Insulin	Glu	Ile	Ile	0**	0.01
31	Glu(A14)Gly(A21)Glu(B3)Ile(B16)Trp(B25)Des(B30)-Insulin	Glu	Ile	Trp	0.12	0.13
32	Glu(A14)Ile(B16)Val(B25)Des(B30)-Insulin	Glu	Ile	Val	0	0.04
33	Glu(A14)Gly(A21)Glu(B3)Ile(B16)Val(B25)Des(B30)-Insulin	Glu	Ile	Val	0.01	0.02
34	Glu(A14)Leu(B16)Ala(B25)Des(B30)-	Glu	Leu	Ala	0.01	0.04

	Insulin					
35	Glu(A14)Val(B16)Ile(B25)Des(B30)-Insulin	Glu	Val	Ile	0	0.01
36	Glu(A14)Gly(A21)Val(B16)Trp(B25)Des(B30)-Insulin	Glu	Val	Trp	0.17	0.23
37	Glu(A14)Gly(A21)Glu(B3)Val(B16)Trp(B25)Des(B30)-Insulin	Glu	Val	Trp	0.21	0.19
38	Glu(A14)Val(B16)Val(B25)Des(B30)-Insulin	Glu	Val	Val	0	0.03
39	Glu(A14)Glu(B3)Val(B16)Val(B25)Des(B30)-Insulin	Glu	Val	Val	0	0.02
40	Glu(A14)Gly(A21)Glu(B3)Val(B16)Val(B25)Des(B30)-Insulin	Glu	Val	Val	0.01	0.01

* relative to human insulin, *nd*: not determined

** a value of 0 means that the binding affinity was below the detection limit

C) Conclusions

As it can be derived from Table 2, various hydrophobic substitutions at positions B16 and/or B25 were tested (tryptophan, alanine, valine, leucine and isoleucine). Albeit to a different extent, all tested insulin analogs with hydrophobic substitutions at these positions showed a decrease of insulin receptor binding activity. As compared to Trp substitutions (see e.g. Analogs 4, 15 and 23), substitutions with aliphatic amino acids such as alanine, valine, leucine and isoleucine had a stronger impact on insulin receptor binding activity. The strongest effects were observed for valine, leucine and isoleucine which are branched-chain amino acids. Substitutions with isoleucine, valine and leucine resulted in a significant decrease of insulin receptor binding activity. Interestingly, insulin analogs with such substitutions at position B25 (such as valine, leucine or isoleucine substitution at B25, Analogs 11, 12, 22, 24, 25, 29, 30, 32, 33, 35 38, 39, 40) showed up to 6-fold enhancement in signal transduction than expected based on their IR-B binding affinities. Specifically, Leu(B25)Des(B30)-Insulin and Val(B25)Des(B30)-Insulin (Analogs 11 and 12, respectively) showed only 1% binding to insulin receptor B and 6% auto phosphorylation relative to human insulin. Similarly, a single leucine substitution at position B16 (Analog 3) also showed a similar enhancement in signal transduction albeit to a slightly lower extent. By comparison, with the exception of Analog 26, analogs bearing a histidine B25 substitution (Analogs 10, 13, 14, 21, 28) also showed reduced receptor binding, however a concomitant reduction in auto phosphorylation.

In some cases (Analogs 30, 32, 35, 38, 39), insulin receptor binding was 0% whilst still showing activity in the auto phosphorylation assay. All of these analogs have combinations of valine and/or isoleucine substitutions at positions B16 and B25 in common, suggesting that the combination is responsible for the further drop in insulin receptor binding. Insulins with no substitution at position B25 but with exchanges at position B16 exhibited slightly higher binding affinities in comparison to their autophosphorylation values (Analogs 3, 4, 16, 17, 18, 19, 20).

Alanine in position B25 shows similar effects as valine, leucine or isoleucine substitution (Analogs 11, 12, 22), although to a lower extent. The receptor binding affinity and autophosphorylation activity of analogs with valine, leucine or isoleucine substitution is lower than analogs with an alanine substitution.

Example 3: Determination of in vitro stability in different recombinant proteases and gastric simulated fluid

The insulin analogs were tested for proteolytic stability (α-chymotrypsin, cathepsin D, insulin degrading enzyme (IDE) and simulated gastric fluid.

A) Assay conditions

Protease	Final Protease concentration in the assay [µg/ml]	Used puffer system
Trypsin	2	0.1 M Ammonium bicarbonate, pH 8.3
α-Chymotrypsin	0.5	0.1 M Ammonium bicarbonate, pH 8.3
Carboxypeptidase A	0.1	Pre-activation (60 min) with Trypsin in TCNB-buffer, Assay Tris-Puffer 7.5
Carboxypeptidase B	0.1	Pre-activation (30 min) with Trypsin in TCNB-buffer, Assay Tris-Buffer 7.5
Cathepsin D	2	Acetate Buffer, pH 4.5
IDE	2	Tris-Buffer pH 7.5

B) Preparation of simulated gastric fluid

Two grams of sodium chloride and 3.2 g of purified pepsin (from porcine stomach mucosa, with an activity of 800 to 2500 units per mg of protein) were dissolved in 7.0 ml of hydrochloric acid. The volume was adjusted with water up to 1000 ml. The resulting solution was mixed and adjusted with either 0.2 N sodium hydroxide or 0.2 N hydrochloric acid to a pH of 1.2 ± 0.1.

C) General assay procedure

The stability determination was done using appropriate time points (for SIF and SGF 15, 30, 60, 120 and 240 minutes; for proteases 15, 30, 60 and 120 minutes). The incubation was done at 37°C and the % of remaining parent compound was calculated in reference to a T0 time point. For the determination of the parent compound an appropriate bioanalytical LC-MS/MS or LC-HRMS method was used, using the supernatant, after protein precipitation with ethanol (1 eq. v/v) and a centrifugation step.

D) Preparation of samples

Compounds were dissolved in diluted hydrochloric acid at a final concentration of 40 µM. The compound concentration in the assay was 2 µM. A 1:20 dilution of the working solution was done into the protease buffer and samples are then incubated at 37°C, under stirring. At the appropriate time point and aliquot was taken, the reaction was quenched ethanol (1 eq. v/v), than centrifuged. The supernatant was analyzed.

E) Conclusions

In particular, lipophilic amino acid substitutions such as valine and isoleucine at positions 16 and 25 in the B-chain were investigated. Of the tested Analogs (2, 7, 11, 12, 14, 16, 19, 22, 23, 24 and 38), only minor differences in stability were observed against the proteases trypsin, carboxypeptidase A and carboxypeptidase B relative to human insulin (data not shown). In general, all analogs bearing A14 and B25 (Analogs 22, 24, 38) substitution showed improved proteolytic stability against α-chymotrypsin, cathepsin D and insulin degrading enzyme (IDE). For example, in the case of α-chymotrypsin, human insulin was completely degraded within 2 hours whereas Analog 22 was almost completely resistant. Similarly, all B25-substituted analogs tested showed improved stability against cathepsin D, albeit with Analog 38 (a B16/B25 variant) showing superior stability compared to the other B25 variants.

One notable exception was observed in the case of IDE, in which Analog 19 with A14/B16 substitutions showed improved performance compared to the B25 variants. The data suggests however, that a substitution at A14, tested here with glutamic acid, is important for increased

stability. Other substitutions were also shown to be beneficial for increased stability: such as substitutions at position B16 and at position B25. For example, Analog 7 with an amino acid exchange in position B25, lead to increased instability.

Table 3: Percent remaining insulin analog after incubation of different insulin analogs for 30 or 120 minutes with four different proteases (for the sequences of the tested analogs, see Table 1).

Ana-log	Backbone	A14	B16	B25	Gastric fluid (30 minutes) [%]	α-Chy-motrypsin (120 minutes) [%]	Cathepsin D (30 minutes) [%]	Insulin degrading enzyme, IDE (30 minutes) [%]
WT	Insulin	Tyr	Tyr	Phe	0	5	0	38
2	Glu(A14)Des(B30)-Insulin	Glu	Tyr	Phe	0	35	0	81
7	Ala(B25)-Insulin	Tyr	Tyr	Ala	30	58	52	2
11	Leu(B25)Des(B30)-Insulin	Tyr	Tyr	Leu	nd	nd	nd	12
12	Val(B25)Des(B30)-Insulin	Tyr	Tyr	Val	44	31	43	11
14	Gly(A21)Trp(B16)His(B25)Des(B30)-Insulin	Tyr	Trp	His	nd	nd	nd	41
16	Glu(A14)His(B16)Des(B30)-Insulin	Glu	His	Phe	nd	nd	nd	88
19	Glu(A14)Val(B16)Des(B30)-Insulin	Glu	Val	Phe	0	30	0	98
22	Glu(A14)Ile(B25)Des(B30)-Insulin	Glu	Tyr	Ile	52	99	20	77
23	Glu(A14)Gly(A21)Trp(B25)Des(B30)-Insulin	Glu	Tyr	Trp	nd	nd	nd	80
24	Glu(A14)Val(B25)Des(B30)-Insulin	Glu	Tyr	Val	52	40	59	86
38	Glu(A14)Val(B16)Val(B25)Des(B30)-Insulin	Glu	Val	Val	36	34	62	86

nd: not determined

CLAUSES

The following clauses are part of the description:

1. An insulin analog comprising at least one mutation relative to the parent insulin, wherein the insulin analog comprises a mutation at position B16 which is substituted with a branched-chain amino acid and/or a mutation at position B25 which is substituted with a branched-chain amino acid.

2. The insulin analog of clause 1, wherein the parent insulin is human insulin, porcine insulin, or bovine insulin.

3. The insulin analog of clauses 1 and 2, wherein the branched-chain amino acid is selected from the group consisting of valine (Val), isoleucine (Ile), and leucine (Leu).

4. The insulin analog of any one of clauses 1 to 3, wherein said insulin analog further comprises a mutation at position A14 which is substituted with an amino acid selected from the group consisting of glutamic acid (Glu), aspartic acid (Asp) and histidine (His).

5. The insulin analog of any one of clauses 1 to 4, wherein said insulin analog further comprises a mutation at position B30.

6. The insulin analog of clause 5, wherein the mutation at position B30 is the deletion of the amino acid at position B30 of the parent insulin (Des(B30)-mutation).

7. The insulin analog of any one of clauses 1 to 6, wherein said insulin analog further comprises a mutation at position B3 which is substituted with a glutamic acid (Glu).

8. The insulin analog of any one of clauses 1 to 7, wherein said insulin further comprises a mutation at position A21 which is substituted with glycine (Gly).

9. The insulin analog of any one of clauses 1 to 8, wherein the B chain of the insulin analog comprises or consists of an amino acid sequence selected from the group consisting of
FVNQHLCGSHLVEALYLVCGERGFLYTPK (SEQ ID NO: 22)
FVNQHLCGSHLVEALYLVCGERGFIYTPK (SEQ ID NO: 44)
FVNQHLCGSHLVEALYLVCGERGFVYTPK (SEQ ID NO: 48)
FVEQHLCGSHLVEALYLVCGERGFVYTPK (SEQ ID NO: 50)
FVNQHLCGSHLVEALILVCGERGFIYTPK (SEQ ID NO: 58)
FVEQHLCGSHLVEALILVCGERGFIYTPK (SEQ ID NO: 60)
FVNQHLCGSHLVEALILVCGERGFVYTPK (SEQ ID NO: 64)

FVEQHLCGSHLVEALILVCGERGFVYTPK (SEQ ID NO: 66)
FVNQHLCGSHLVEALVLVCGERGFIYTPK (SEQ ID NO: 70)
FVNQHLCGSHLVEALVLVCGERGFVYTPK (SEQ ID NO: 76)
FVEQHLCGSHLVEALVLVCGERGFVYTPK (SEQ ID NO: 78)
FVEQHLCGSHLVEALVLVCGERGFVYTPK (SEQ ID NO: 80)
FVNQHLCGSHLVEALYLVCGERGFLYTPKT (SEQ ID NO: 85)
FVNQHLCGSHLVEALYLVCGERGFVYTPKT (SEQ ID NO: 86)
FVNQHLCGSHLVEALYLVCGERGFIYTPKT (SEQ ID NO: 87)
FVNQHLCGSHLVEALYLVCGERGFVYTPKT (SEQ ID NO: 88)
FVEQHLCGSHLVEALYLVCGERGFVYTPKT (SEQ ID NO: 89)
FVNQHLCGSHLVEALILVCGERGFIYTPKT (SEQ ID NO: 90)
FVEQHLCGSHLVEALILVCGERGFIYTPKT (SEQ ID NO: 91)
FVNQHLCGSHLVEALILVCGERGFVYTPKT (SEQ ID NO: 92)
FVEQHLCGSHLVEALILVCGERGFVYTPKT (SEQ ID NO: 93)
FVNQHLCGSHLVEALVLVCGERGFIYTPKT (SEQ ID NO: 94)
FVNQHLCGSHLVEALVLVCGERGFVYTPKT (SEQ ID NO: 95)
FVEQHLCGSHLVEALVLVCGERGFVYTPKT (SEQ ID NO: 96), and
FVEQHLCGSHLVEALVLVCGERGFVYTPKT (SEQ ID NO: 97).

10. The insulin analog of any one of clauses 1 to 9, comprising
(a) an A chain having an amino acid sequence as shown in SEQ ID NO: 43 (GIVEQCCTSICSLEQLENYCN) and a B chain having an amino acid sequence as shown in SEQ ID NO: 44 (FVNQHLCGSHLVEALYLVCGERGFIYTPK),
(b) an A chain having an amino acid sequence as shown in SEQ ID NO: 47 (GIVEQCCTSICSLEQLENYCN) and a B chain having an amino acid sequence as shown in SEQ ID NO: 48 (FVNQHLCGSHLVEALYLVCGERGFVYTPK), or
(c) an A chain having an amino acid sequence as shown in SEQ ID NO: 77 (GIVEQCCTSICSLEQLENYCN) and a B chain having an amino acid sequence as shown in SEQ ID NO: 78 (FVEQHLCGSHLVEALVLVCGERGFVYTPK).

11. An insulin analog selected from the group consisting of
Leu(B16)-human insulin,
Val(B16)-human insulin,
Ile(B16)-human insulin,
Leu(B16)Des(B30)-human insulin,
Val(B16)Des(B30)-human insulin,
Ile(B16)Des(B30)-human insulin,
Leu(B25)-human insulin,
Val(B25)-human insulin,

Ile(B25)-human insulin,
Leu(B25)Des(B30)-human insulin,
Val(B25)Des(B30)-human insulin,
Ile(B25)Des(B30)-human insulin,
Glu(A14)Leu(B16)Des(B30)-human insulin,
Glu(A14)Ile(B16)Des(B30)-human insulin,
Glu(A14)Val(B16)Des(B30)-human insulin,
Glu(A14)Leu(B16)-human insulin,
Glu(A14)Ile(B16)-human insulin,
Glu(A14)Val(B16)-human insulin,
Glu(A14)Leu(B25)Des(B30)-human insulin,
Glu(A14)Ile(B25)Des(B30)-human insulin,
Glu(A14)Val(B25)Des(B30)-human insulin,
Glu(A14)Leu(B25)-human insulin,
Glu(A14)Ile(B25)-human insulin,
Glu(A14)Val(B25)-human insulin,
Glu(A14)Gly(A21)Glu(B3)Val(B25)Des(B30)-human insulin,
Glu(A14)Ile(B16)Ile(B25)Des(B30)-human insulin,
Glu(A14)Glu(B3)Ile(B16)Ile(B25)Des(B30)-human insulin,
Glu(A14)Ile(B16)Val(B25)Des(B30)-human insulin,
Glu(A14)Gly(A21)Glu(B3)Ile(B16)Val(B25)Des(B30)-human insulin,
Glu(A14)Val(B16)Ile(B25)Des(B30)-human insulin,
Glu(A14)Val(B16)Val(B25)Des(B30)-human insulin,
Glu(A14)Glu(B3)Val(B16)Val(B25)Des(B30)-human insulin,
Glu(A14)Gly(A21)Glu(B3)Val(B16)Val(B25)Des(B30)-human insulin,
Glu(A14)Gly(A21)Glu(B3)Val(B25)-human insulin,
Glu(A14)Ile(B16)Ile(B25)-human insulin,
Glu(A14)Glu(B3)Ile(B16)Ile(B25)-human insulin,
Glu(A14)Ile(B16)Val(B25)-human insulin,
Glu(A14)Gly(A21)Glu(B3)Ile(B16)Val(B25)-human insulin,
Glu(A14)Val(B16)Ile(B25)-human insulin,
Glu(A14)Val(B16)Val(B25)-human insulin,
Glu(A14)Glu(B3)Val(B16)Val(B25)-human insulin, and
Glu(A14)Gly(A21)Glu(B3)Val(B16)Val(B25)-human insulin.

12. An insulin B chain comprising at least one mutation relative to the B chain of the parent insulin, wherein the B chain comprises a mutation at position B16 which is substituted with a branched-chain amino acid, and/or a mutation at position B25 which is substituted with a branched-chain amino acid, and optionally wherein said insulin B chain further

comprises a mutation at position B30, wherein the mutation at position B30 is the deletion of threonine at position B30 of the parent insulin (Des(B30)-mutation).

13. A proinsulin comprising an insulin A chain and an insulin B chain, wherein the insulin B chain comprises at least one mutation relative to B chain of a parent insulin, wherein the B chain comprises a mutation at position B16 which is substituted with a branched-chain amino acid and/or a mutation at position B25 which is substituted with a branched-chain amino acid, and optionally wherein the insulin A chain of said proinsulin comprises a mutation at position A14 which is substituted with an amino acid selected from glutamic acid (Glu), aspartic acid (Asp) and histidine (His).

14. A polynucleotide encoding the insulin analog of any one of clauses 1 to 11, the insulin B chain of clause 12, and/or the proinsulin of clause 13.

15. A host cell comprising insulin analog of any one of clauses 1 to 11, the insulin B chain of clauses 12, the proinsulin of clause 13, and/or the polynucleotide of clause 14.

Claims

1. An insulin analog comprising at least one mutation relative to the parent insulin, wherein the insulin analog comprises a mutation at position B16 which is substituted with a branched-chain amino acid and/or a mutation at position B25 which is substituted with a branched-chain amino acid.

2. The insulin analog of claim 1, wherein the parent insulin is human insulin, porcine insulin, or bovine insulin.

3. The insulin analog of claims 1 and 2, wherein the branched-chain amino acid is selected from the group consisting of valine (Val), isoleucine (Ile), and leucine (Leu).

4. The insulin analog of any one of claims 1 to 3, wherein said insulin analog further comprises a mutation at position A14 which is substituted with an amino acid selected from the group consisting of glutamic acid (Glu), aspartic acid (Asp) and histidine (His).

5. The insulin analog of any one of claims 1 to 4, wherein said insulin analog further comprises a mutation at position B30.

6. The insulin analog of claim 5, wherein the mutation at position B30 is the deletion of the amino acid at position B30 of the parent insulin (Des(B30)-mutation).

7. The insulin analog of any one of claims 1 to 6, wherein said insulin analog further comprises a mutation at position B3 which is substituted with a glutamic acid (Glu).

8. The insulin analog of any one of claims 1 to 7, wherein said insulin further comprises a mutation at position A21 which is substituted with glycine (Gly).

9. The insulin analog of any one of claims 1 to 8, wherein the B chain of the insulin analog comprises or consists of an amino acid sequence selected from the group consisting of
FVNQHLCGSHLVEALYLVCGERGFLYTPK (SEQ ID NO: 22)
FVNQHLCGSHLVEALYLVCGERGFIYTPK (SEQ ID NO: 44)
FVNQHLCGSHLVEALYLVCGERGFVYTPK (SEQ ID NO: 48)
FVEQHLCGSHLVEALYLVCGERGFVYTPK (SEQ ID NO: 50)
FVNQHLCGSHLVEALILVCGERGFIYTPK (SEQ ID NO: 58)

FVEQHLCGSHLVEALILVCGERGFIYTPK (SEQ ID NO: 60)
FVNQHLCGSHLVEALILVCGERGFVYTPK (SEQ ID NO: 64)
FVEQHLCGSHLVEALILVCGERGFVYTPK (SEQ ID NO: 66)
FVNQHLCGSHLVEALVLVCGERGFIYTPK (SEQ ID NO: 70)
FVNQHLCGSHLVEALVLVCGERGFVYTPK (SEQ ID NO: 76)
FVEQHLCGSHLVEALVLVCGERGFVYTPK (SEQ ID NO: 78)
FVEQHLCGSHLVEALVLVCGERGFVYTPK (SEQ ID NO: 80)
FVNQHLCGSHLVEALYLVCGERGFLYTPKT (SEQ ID NO: 85)
FVNQHLCGSHLVEALYLVCGERGFVYTPKT (SEQ ID NO: 86)
FVNQHLCGSHLVEALYLVCGERGFIYTPKT (SEQ ID NO: 87)
FVNQHLCGSHLVEALYLVCGERGFVYTPKT (SEQ ID NO: 88)
FVEQHLCGSHLVEALYLVCGERGFVYTPKT (SEQ ID NO: 89)
FVNQHLCGSHLVEALILVCGERGFIYTPKT (SEQ ID NO: 90)
FVEQHLCGSHLVEALILVCGERGFIYTPKT (SEQ ID NO: 91)
FVNQHLCGSHLVEALILVCGERGFVYTPKT (SEQ ID NO: 92)
FVEQHLCGSHLVEALILVCGERGFVYTPKT (SEQ ID NO: 93)
FVNQHLCGSHLVEALVLVCGERGFIYTPKT (SEQ ID NO: 94)
FVNQHLCGSHLVEALVLVCGERGFVYTPKT (SEQ ID NO: 95)
FVEQHLCGSHLVEALVLVCGERGFVYTPKT (SEQ ID NO: 96), and
FVEQHLCGSHLVEALVLVCGERGFVYTPKT (SEQ ID NO: 97).

10. The insulin analog of any one of claims 1 to 9, comprising

(a) an A chain having an amino acid sequence as shown in SEQ ID NO: 43 (GIVEQCCTSICSLEQLENYCN) and a B chain having an amino acid sequence as shown in SEQ ID NO: 44 (FVNQHLCGSHLVEALYLVCGERGFIYTPK),

(b) an A chain having an amino acid sequence as shown in SEQ ID NO: 47 (GIVEQCCTSICSLEQLENYCN) and a B chain having an amino acid sequence as shown in SEQ ID NO: 48 (FVNQHLCGSHLVEALYLVCGERGFVYTPK), or

(c) an A chain having an amino acid sequence as shown in SEQ ID NO: 77 (GIVEQCCTSICSLEQLENYCN) and a B chain having an amino acid sequence as shown in SEQ ID NO: 78 (FVEQHLCGSHLVEALVLVCGERGFVYTPK).

11. An insulin analog selected from the group consisting of
Leu(B16)-human insulin,
Val(B16)-human insulin,
Ile(B16)-human insulin,
Leu(B16)Des(B30)-human insulin,
Val(B16)Des(B30)-human insulin,
Ile(B16)Des(B30)-human insulin,

Leu(B25)-human insulin,
Val(B25)-human insulin,
Ile(B25)-human insulin,
Leu(B25)Des(B30)-human insulin,
Val(B25)Des(B30)-human insulin,
Ile(B25)Des(B30)-human insulin,
Glu(A14)Leu(B16)Des(B30)-human insulin,
Glu(A14)Ile(B16)Des(B30)-human insulin,
Glu(A14)Val(B16)Des(B30)-human insulin,
Glu(A14)Leu(B16)-human insulin,
Glu(A14)Ile(B16)-human insulin,
Glu(A14)Val(B16)-human insulin,
Glu(A14)Leu(B25)Des(B30)-human insulin,
Glu(A14)Ile(B25)Des(B30)-human insulin,
Glu(A14)Val(B25)Des(B30)-human insulin,
Glu(A14)Leu(B25)-human insulin,
Glu(A14)Ile(B25)-human insulin,
Glu(A14)Val(B25)-human insulin,
Glu(A14)Gly(A21)Glu(B3)Val(B25)Des(B30)-human insulin,
Glu(A14)Ile(B16)Ile(B25)Des(B30)-human insulin,
Glu(A14)Glu(B3)Ile(B16)Ile(B25)Des(B30)-human insulin,
Glu(A14)Ile(B16)Val(B25)Des(B30)-human insulin,
Glu(A14)Gly(A21)Glu(B3)Ile(B16)Val(B25)Des(B30)-human insulin,
Glu(A14)Val(B16)Ile(B25)Des(B30)-human insulin,
Glu(A14)Val(B16)Val(B25)Des(B30)-human insulin,
Glu(A14)Glu(B3)Val(B16)Val(B25)Des(B30)-human insulin,
Glu(A14)Gly(A21)Glu(B3)Val(B16)Val(B25)Des(B30)-human insulin,
Glu(A14)Gly(A21)Glu(B3)Val(B25)-human insulin,
Glu(A14)Ile(B16)Ile(B25)-human insulin,
Glu(A14)Glu(B3)Ile(B16)Ile(B25)-human insulin,
Glu(A14)Ile(B16)Val(B25)-human insulin,
Glu(A14)Gly(A21)Glu(B3)Ile(B16)Val(B25)-human insulin,
Glu(A14)Val(B16)Ile(B25)-human insulin,
Glu(A14)Val(B16)Val(B25)-human insulin,
Glu(A14)Glu(B3)Val(B16)Val(B25)-human insulin, and
Glu(A14)Gly(A21)Glu(B3)Val(B16)Val(B25)-human insulin.

12. An insulin B chain comprising at least one mutation relative to the B chain of the parent insulin, wherein the B chain comprises a mutation at position B16 which is substituted

with a branched-chain amino acid, and/or a mutation at position B25 which is substituted with a branched-chain amino acid, and optionally wherein said insulin B chain further comprises a mutation at position B30, wherein the mutation at position B30 is the deletion of threonine at position B30 of the parent insulin (Des(B30)-mutation).

13. A proinsulin comprising an insulin A chain and an insulin B chain, wherein the insulin B chain comprises at least one mutation relative to B chain of a parent insulin, wherein the B chain comprises a mutation at position B16 which is substituted with a branched-chain amino acid and/or a mutation at position B25 which is substituted with a branched-chain amino acid, and optionally wherein the insulin A chain of said proinsulin comprises a mutation at position A14 which is substituted with an amino acid selected from glutamic acid (Glu), aspartic acid (Asp) and histidine (His).

14. A polynucleotide encoding the insulin analog of any one of claims 1 to 11, the insulin B chain of claim 12, and/or the proinsulin of claim 13.

15. A host cell comprising insulin analog of any one of claims 1 to 11, the insulin B chain of claims 12, the proinsulin of claim 13, and/or the polynucleotide of claim 14.