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(54) Title: ERYTHROPOIETIN CONJUGATES

(57) Abstract: The invention relates to polypeptide conjugates exhibiting erythropoietin (EPO) activity, comprising at least one polymer molecule, preferably polyethylene glycol, covalently attached to an attachment site of a polypeptide, e.g. a lysine or cysteine residue or a carbohydrate chain.

ERYTHROPOIETIN CONJUGATES

FIELD OF THE INVENTION

The present invention relates to new polypeptides exhibiting erythropoietin (EPO) activity, to conjugates between a polypeptide exhibiting EPO activity and a polymer molecule, to methods for preparing such polypeptides or conjugates, and the use of such polypeptides or conjugates in therapy, in particular for the treatment of diseases or conditions involving low red blood cell levels or deficient red blood cell production.

BACKGROUND OF THE INVENTION

Naturally occurring human EPO (hEPO) is a 165-166 amino acid glycoprotein hormone produced in the kidney. The protein is the humoral plasma factor which stimulates red blood cell production. Human EPO stimulates the division and differentiation of committed erythroid progenitors in the bone marrow, exerting its biological activity by binding to receptors on erythroid precursors.

Human EPO is expressed as a 166 amino acid protein from a 193 amino acid precursor. In post-translational modification, arginine 166 is cleaved by carboxypeptidase to form the physiologically active 165 amino acid protein. The native protein is both N- and O-glycosylated, with three N-linked oligosaccharide groups (at Asn24, Asn38 and Asn83) and one O-linked oligosaccharide group at Ser126. The carbohydrate chains have been shown to be modified with terminal sialic acid residues that have been shown to be essential for *in vivo* activity. The molecular weight of the non-glycosylated protein is about 18 kDa, the oligosaccharide groups comprising about 40% of the molecular weight of the glycosylated protein. Human EPO comprises two cysteine bridges, Cys7-Cys161 and Cys29-Cys33, the latter not being necessary for biological activity. The protein has a four-helix bundle structure, with the four long alpha helices located approximately in positions 8-26 (helix A), 55-83 (helix B), 90-112 (helix C) and 138-161 (helix D) (Syed et al., *Nature* 395:511-516, 1998).

Because EPO is essential in red blood cell formation, the hormone is useful in the treatment of blood disorders characterized by low or defective red blood cell production. Clinically, EPO is used in the treatment of, for example, anemia in chronic renal failure patients as well as for other indications such as in AIDS and cancer patients undergoing chemotherapy.

A problem with EPO products such as Epogen® (epoetin alfa, recombinant hEPO), however, is that the bioavailability of hEPO is limited by a short plasma half-life and susceptibility to protease degradation. A novel erythropoiesis stimulating protein (darbopoietin alfa, referred to as "NESP" or Aranesp™) contains five N-linked carbohydrate chains, i.e. two more than human EPO, and has an increased sialic acid content. NESP has been reported to have an approximately 3-fold longer serum half-life compared to recombinant hEPO (rhEPO), allowing it to be administered less frequently than rhEPO (Egrie et al., *Br. J. Cancer* 84(8A): 3-10, 2001; Macdougall, *Neprol. Dial. Transplant* 16 Suppl. 3:14-21, 2001; Glaspy et al., *Br. J. Cancer* 84(8A):17-23, 2001). Aranesp™ has recently been approved in Europe and the United States for treatment of anemia associated with chronic renal failure and chemotherapy. Although Aranesp™ has an increased serum half-life compared to recombinant human EPO, it is nevertheless contemplated that there is still room for improvement and/or for alternative EPO products with properties that differ from those of currently available products.

The literature on altered EPO molecules deals almost exclusively with increased or otherwise altered glycosylation patterns, and similarly altered sialic acid contents, since increased glycosylation leads to increased sialic acid content. The purpose of such modifications has in particular been to increase plasma half-life and improve potency of the EPO product.

EP 428267 B1 discloses an EPO product consisting essentially of EPO molecules with a single specific number (1-14) of sialic acids per molecule, and an EPO isoform with a single isoelectric point and 1-14 sialic acids per molecule.

EP 668351 B1 discloses glycosylated EPO with one or more added N- or O-linked glycosylation sites as compared to hEPO.

US 5,614,184 and US 6,048,971 disclose human EPO proteins with one or more amino acid mutations in position 101, 103, 104, 105 or 108.

WO 95/05465 and WO 00/24893 disclose analogs of hEPO with at least one additional glycosylation site, e.g. at one or more of positions 30, 51, 57, 69, 88, 89, 136 or 138.

EP 902085 A1 discloses a glycosylated polypeptide with EPO activity comprising hEPO or a variant thereof wherein at least one N-linked glycosylation site is inactivated and wherein greater than 5% of the N-glycan structures are sulfated.

WO 99/28346 discloses glycosylated EPO comprising a defined carbohydrate structure in terms of the content of tetra-antenna structures.

An alternative strategy to altered glycosylation as a means for achieving modified proteins with e.g. an increased *in vivo* half-life is to attach one or more polymer moieties, in

particular polyethylene glycol (PEG), to a protein. Attachment of a polymer moiety such as PEG has the effect of increasing the apparent size, thereby increasing the *in vivo* half-life, the increased half-life being due a reduced renal clearance and/or a reduced receptor-mediated clearance. Furthermore, attachment of PEG groups may effectively block proteolytic enzymes from physical contact with the protein, thus preventing degradation by non-specific proteolysis.

Attachment of PEG moieties, often referred to as "PEGylation" has been reported for a number of different proteins. Possible beneficial effects of PEGylation, in addition to an increased *in vivo* half-life, include decreased immunogenicity as a result of surface epitopes of the protein being shielded by the PEG groups. On the other hand, the presence of PEG groups can also result in an undesired disruption of the structure and/or function of a protein, e.g. if they are located at or near a receptor-binding site.

US 4,904,584 discloses modified polypeptides with one or more lysines deleted or replaced with different amino acid residue, the polypeptides being conjugated to at least one polyalkylene glycol moiety.

WO 90/12874 discloses cysteine-added EPO variants comprising a human EPO polypeptide sequence modified to contain at least one non-native cysteine residue covalently attached to a polyethylene glycol moiety.

WO 99/03887 discloses cysteine variants of the GH supergene family, including EPO, wherein a cysteine residue is introduced in any of a number of different positions, the cysteine variants being PEGylated. Introduction of a cysteine residue is in particular stated to be performed in the AB loop (defined as residues 23-58), the BC loop (defined as residues 77-89), the CD loop (defined as residues 108-131), as well as the N-terminal (residues 1-8) and the C-terminal (residues 153-166).

WO 00/42175 discloses a method for obtaining a soluble protein having a free cysteine that can be modified by PEGylation.

EP 1064951 A discloses EPO conjugates comprising an EPO polypeptide linked to 1-3 20-40 kDa PEG groups, where the EPO can be wild-type EPO or a variant with 1-6 introduced glycosylation sites.

WO 01/76640 discloses attachment of water-soluble polymers such as PEG to novel erythropoietin stimulating protein (NESP) containing two additional N-linked carbohydrate chains at amino acid residues 30 and 88.

Although recombinant hEPO has generally been considered not to be immunogenic, it has been reported that administration to rhesus monkeys of a recombinant human

GM-CSF-EPO hybrid protein resulted in a severe anti-EPO antibody response (Coscarella et al., *Cytokine* 10(12):964-9, 1998). More recently, the development of anti-EPO antibodies in patients treated with rhEPO has been reported (Casadevall et al., *N. Engl. J. Med.* 2002, 346(7):469-475). On 19 Nov. 2001, the U.K. Medicines Control Agency posted a safety message regarding 40 cases of pure red cell aplasia (PRCA) in patients treated with Eprex epoetin (rhEPO). It was reported that the affected patients experienced worsening of anemia following months or years of therapy and were unresponsive to increasing doses of EPO, and that most had detectable antibodies to EPO. Further, many of the affected PRCA patients did not respond to alternative EPO treatment after discontinuing Eprex and became transfusion-dependent.

In spite of the fact that some PEGylated EPO variants have been described, no such PEGylated EPO products are yet available, and this route for providing improved or alternative EPO variants is still largely unexplored. The present invention is directed to providing novel PEGylated EPO variants with improved or alternative properties compared to currently available recombinant human EPO, e.g. an increased *in vivo* half-life, increased potency and/or reduced immunogenicity.

BRIEF DISCLOSURE OF THE INVENTION

More specifically, the present invention relates to polypeptide conjugates comprising a polypeptide exhibiting EPO activity conjugated to one or more polymer molecules, methods for preparation of the polypeptide conjugates, and use of the polypeptide conjugates in medical treatment and in the preparation of pharmaceuticals. The invention further relates to polypeptides having an amino acid sequence as defined herein, i.e. having one or more of the amino acid alterations described herein relative to human EPO. Accordingly, in a first aspect the invention relates to various conjugates comprising a polypeptide exhibiting EPO activity and having an amino acid sequence that differs from the amino acid sequence of human EPO in at least one altered amino acid residue comprising an attachment group for a polymer molecule, and having at least one polymer molecule attached to an attachment group of the polypeptide. Other aspects and particular embodiments of the invention will be apparent from the following description and claims.

DETAILED DISCLOSURE OF THE INVENTION

Definitions

In the context of the present application and invention the following definitions apply:

The term "conjugate" is intended to indicate a heterogeneous molecule formed by the covalent attachment of one or more polypeptides, typically a single polypeptide, to at least one polymer molecule. The term covalent attachment means that the polypeptide and the polymer molecule are either directly covalently joined to one another, or else are indirectly covalently joined to one another through an intervening moiety or moieties, such as a bridge, spacer, or linkage moiety or moieties. Preferably, the conjugate is soluble at relevant concentrations and conditions, i.e. soluble in physiological fluids such as blood. The term "non-conjugated polypeptide" may be used about the polypeptide part of the conjugate. Such non-conjugated polypeptides having one or more of the amino acid alterations disclosed herein relative to human EPO are also comprised by the invention.

The term "polypeptide" may be used interchangeably herein with the term "protein".

The "polymer molecule" is a molecule formed by covalent linkage of two or more monomers, wherein none of the monomers is an amino acid residue, except where the polymer is human albumin or another abundant plasma protein. The term "polymer" or "polymer moiety" may be used interchangeably with the term "polymer molecule". The term is intended to cover carbohydrate molecules, although, in the present context, the term is not intended to cover the type of carbohydrate molecule which is attached to the polypeptide by *in vivo* N- or O-glycosylation. Except where the number of polymer molecule(s) is expressly indicated every reference to "a polymer", "a polymer molecule", "the polymer" or "the polymer molecule" contained in a polypeptide of the invention or otherwise used in the present invention shall be a reference to one or more polymer molecule(s).

The term "attachment group" is intended to indicate an amino acid residue group of the polypeptide capable of coupling to the relevant polymer molecule. For instance, in particular for polymer conjugation to polyethylene glycol (PEG), a frequently used attachment group is the ε-amino group of lysine or the N-terminal amino group. Other polymer attachment groups include a free carboxylic acid group (e.g. that of the C-terminal amino acid residue or of an aspartic acid or glutamic acid residue), suitably activated carbonyl groups, oxidized carbo-

hydrate moieties and mercapto groups. Useful attachment groups and their matching non-peptide moieties are apparent from the table below.

Attachment group	Amino acid	Examples of non-peptide moiety	Conjugation method/-Activated PEG	Reference
$-NH_2$	N-terminal, Lys, His, Arg	Polymer, e.g. PEG, with amide or imine group	mPEG-SPA Tresylated mPEG	Shearwater Corp. Delgado et al., Critical reviews in Therapeutic Drug Carrier Systems 9(3,4):249-304 (1992)
-COOH	C-term, Asp, Glu	Polymer, e.g. PEG, with ester or amide group Oligosaccharide moiety	mPEG-Hz *In vitro* coupling	Shearwater Corp.
-SH	Cys	Polymer, e.g. PEG, with disulfide, maleimide or vinyl sulfone group Oligosaccharide moiety	PEG-vinylsulphone PEG-maleimide *In vitro* coupling	Shearwater Corp. Delgado et al., Critical reviews in Therapeutic Drug Carrier Systems 9(3,4):249-304 (1992)
-OH	Ser, Thr, -OH, Lys	Oligosaccharide moiety PEG with ester, ether, carbamate, carbonate	*In vivo* O-linked glycosylation	
$-CONH_2$	Asn as part of an N-glycosylation site	Oligosaccharide moiety Polymer, e.g. PEG	*In vivo N*-glycosylation	
Aromatic residue	Phe, Tyr, Trp	Oligosaccharide moiety	*In vitro* coupling	
$-CONH_2$	Gln	Oligosaccharide moiety	*In vitro* coupling	Yan and Wold, Biochemistry, 1984, Jul 31; 23(16): 3759-65
Aldehyde Ketone	Oxidized oligo-saccharide	Polymer, e.g. PEG, PEG-hydrazide	PEGylation	Andresz et al., 1978, Makromol. Chem. 179:301, WO 92/16555, WO 00/23114

Guanidino	Arg	Oligosaccharide moiety	*In vitro* coupling	Lundblad and Noyes, Chemical Reagents for Protein Modification, CRC Press Inc., Florida, USA
Imidazole ring	His	Oligosaccharide moiety	*In vitro* coupling	As for guanidine

In the present application, amino acid names and atom names (e.g. CA, CB, NZ, N, O, C, etc.) are used as defined by the Protein Data Bank (PDB) (Berman et al., "The Protein Data Bank", *Nucleic Acids Res.*, 28(1): 235-242 (2000); www.rcsb.org/pdb/), which is based on the IUPAC nomenclature (IUPAC Nomenclature and Symbolism for Amino Acids and Peptides (residue names, atom names etc.), *Eur. J. Biochem.*, 138, 9-37 (1984) together with their corrections in *Eur. J. Biochem.*, 152, 1 (1985). The term "amino acid residue" is primarily intended to indicate an amino acid residue contained in the group consisting of the 20 naturally occurring amino acids, i.e. alanine (Ala or A), cysteine (Cys or C), aspartic acid (Asp or D), glutamic acid (Glu or E), phenylalanine (Phe or F), glycine (Gly or G), histidine (His or H), isoleucine (Ile or I), lysine (Lys or K), leucine (Leu or L), methionine (Met or M), asparagine (Asn or N), proline (Pro or P), glutamine (Gln or Q), arginine (Arg or R), serine (Ser or S), threonine (Thr or T), valine (Val or V), tryptophan (Trp or W), and tyrosine (Tyr or Y) residues.

The terminology used for identifying amino acid positions/substitutions is illustrated as follows: R14 indicates position number 14 occupied by an arginine residue in the reference amino acid sequence. R14K indicates that the arginine residue of position 14 has been substituted with a lysine residue. Unless otherwise indicated, the numbering of amino acid residues made herein is made relative to the amino acid sequence of hEPO, as shown in SEQ ID NO:1 (hEPO with the C-terminal Arg in position 166). Alternative substitutions are indicated with a "/", e.g. Q65D/E means an amino acid sequence in which glutamine in position 65 is substituted with either aspartic acid or glutamic acid. Multiple substitutions are indicated with a "+", e.g. L75N+G77S/T means an amino acid sequence which comprises a substitution of the leucine residue in position 75 with an asparagine residue and a substitution of the glycine residue in position 77 with a serine or a threonine residue.

It will be understood that the term "human erythropoietin", "human EPO" or "hEPO" as used herein is intended to include both the 166 amino acid sequence containing Arg166 and the 165 amino acid sequence without the C-terminal Arg.

The term "nucleotide sequence" is intended to indicate a consecutive stretch of two or more nucleotide molecules. The nucleotide sequence may be of genomic, cDNA, RNA, semi-synthetic or synthetic origin, or any combination thereof.

"Cell", "host cell", "cell line" and "cell culture" are used interchangeably herein and all such terms should be understood to include progeny resulting from growth or culturing of a cell. "Transformation" and "transfection" are used interchangeably to refer to the process of introducing DNA into a cell.

"Operably linked" refers to the covalent joining of two or more nucleotide sequences, by means of enzymatic ligation or otherwise, in a configuration relative to one another such that the normal function of the sequences can be performed. For example, the nucleotide sequence encoding a presequence or secretory leader is operably linked to a nucleotide sequence for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide: a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the nucleotide sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, then synthetic oligonucleotide adaptors or linkers are used, in conjunction with standard recombinant DNA methods.

The term "introduce" refers to introduction of an amino acid residue comprising an attachment group for a polymer molecule, in particular by substitution of an existing amino acid residue, or alternatively by insertion of an additional amino acid residue. The term "remove" refers to removal of an amino acid residue comprising an attachment group for a polymer molecule, in particular by substitution of the amino acid residue to be removed by another amino acid residue, or alternatively by deletion (without substitution) of the amino acid residue to be removed.

When substitutions are performed in relation to a parent polypeptide, they are preferably "conservative substitutions", in other words substitutions performed within groups of amino acids with similar characteristics, e.g. small amino acids, acidic amino acids, basic amino acids, hydrophilic amino acids, polar amino acids, hydrophobic amino acids, sulfur-containing amino acids, aliphatic amino acids and aromatic amino acids. Conservative substitutions may for example be chosen from among the conservative substitution groups listed in the table below.

Conservative substitution groups:

1	Alanine (A)	Glycine (G)	Serine (S)	Threonine (T)
2	Aspartic acid (D)	Glutamic acid (E)		
3	Asparagine (N)	Glutamine (Q)		
4	Arginine (R)	Histidine (H)	Lysine (K)	
5	Isoleucine (I)	Leucine (L)	Methionine (M)	Valine (V)
6	Phenylalanine (F)	Tyrosine (Y)	Tryptophan (W)	

Additional groups of amino acids can also be formulated, e.g. based on similar function, chemical structure or composition. For example, an aliphatic grouping may comprise glycine, alanine, valine, leucine and isoleucine. Other conservative substitution groups include: hydrophilic amino acids: serine, threonine, asparagine and glutamine; hydrophobic amino acids: leucine, isoleucine and valine; sulfur-containing: cysteine and methionine.

The term "immunogenicity" as used in connection with a given substance is intended to indicate the ability of the substance to induce a response from the immune system. The immune response may be a cell or antibody mediated response (see, e.g., Roitt: Essential Immunology (8th Edition, Blackwell) for further definition of immunogenicity). Normally, reduced antibody reactivity will be an indication of reduced immunogenicity. The reduced immunogenicity may be determined by use of any suitable method known in the art, e.g. *in vivo* or *in vitro*. For example, serum may be tested for the presence of antibodies to EPO as described by Casadevall et al. in *N. Engl. J. Med.* 2002, 346(7):469-475.

The term "functional *in vivo* half-life" is used in its normal meaning, i.e. the time at which 50% of the biological activity of the polypeptide conjugate is still present in the body/target organ, or the time at which the activity of the polypeptide conjugate is 50% of the initial value. As an alternative to determining functional *in vivo* half-life, "serum half-life" may be determined, i.e. the time in which 50% of the polypeptide conjugate molecules circulate in the plasma or bloodstream prior to being cleared. Alternative terms to serum half-life include "plasma half-life", "circulating half-life", "serum clearance", "plasma clearance" and "clearance half-life". The polypeptide conjugate is cleared by the action of one or more of the reticuloendothelial systems (RES), kidney, spleen or liver, by receptor-mediated degradation, or by specific or non-specific proteolysis, in particular by the action of receptor-mediated clearance and renal clearance. Normally, clearance depends on size (relative to the cutoff for glomerular

filtration), charge, attached carbohydrate chains, and the presence of cellular receptors for the protein. The functionality to be retained is normally selected from erythropoietic or EPO receptor-binding activity. The functional *in vivo* half-life and the serum half-life may be determined by any suitable method known in the art.

The term "increased" as used about the functional *in vivo* half-life or serum half-life is used to indicate that the relevant half-life of the conjugate or polypeptide is statistically significantly increased relative to that of a reference molecule, such as a non-polymer conjugated human EPO (e.g. Epogen®) as determined under comparable conditions. For instance, the relevant half-life may increased by at least about 25%, such as by at least about 50%, e.g. by at least about 100%, 200%, 500% or 1000%.

The term "renal clearance" is used in its normal meaning to indicate any clearance taking place by the kidneys, e.g. by glomerular filtration, tubular excretion or tubular elimination. Renal clearance depends on physical characteristics of the conjugate, including size (diameter), symmetry, shape/rigidity and charge. Reduced renal clearance may be demonstrated by any suitable assay, e.g. an established *in vivo* assay. Typically, renal clearance is determined by administering a labelled (e.g. radioactive or fluorescent labelled) polypeptide conjugate to a patient and measuring the label activity in urine collected from the patient. Reduced renal clearance is determined relative to a corresponding reference polypeptide, e.g. the corresponding non-conjugated polypeptide, under comparable conditions. Preferably, the renal clearance rate of the conjugate is reduced by at least 50%, preferably by at least 75%, and most preferably by at least 90% compared to a relevant reference polypeptide.

Generally, activation of the receptor is coupled to receptor-mediated clearance (RMC) such that binding of a polypeptide to its receptor without activation does not lead to RMC, while activation of the receptor leads to RMC. The clearance is due to internalisation of the receptor-bound polypeptide with subsequent lysosomal degradation. Reduced RMC may be achieved by designing the conjugate so as to be able to bind and activate a sufficient number of receptors to obtain optimal *in vivo* biological response and avoid activation of more receptors than required for obtaining such response. This may be reflected in reduced *in vitro* activity and/or increased off-rate.

Typically, reduced *in vitro* activity reflects reduced efficacy/efficiency and/or reduced potency *in vitro* (although not necessarily *in vivo*) and may be determined by any suitable method for determining any of these properties. For instance, *in vitro* activity may be determined in a luciferase based assay; see, for example, Qureshi et al., *Proc. Natl. Acad. Sci.*

USA (1999), 96(21):12156-61. In cases where a reduced *in vitro* activity is desired, the conjugate of the invention may e.g. have an *in vitro* activity in the range of about 0.1-50% of the activity of hEPO, such as in the range of 0.2-40%, such as 0.5-30% or 1-25%.

Preferably, the off-rate between the polypeptide conjugate and its receptor is increased by a magnitude resulting in the polypeptide conjugate being released from its receptor before any substantial internalisation of the receptor-ligand complex has taken place. The off-rate may e.g. be determined using the Biacore® technology. The *in vitro* RMC may be determined by labelling (e.g. radioactive or fluorescent labelling) the polypeptide conjugate, stimulating cells comprising the receptor for the polypeptide, washing the cells, and measuring label activity. Alternatively, the conjugate may be exposed to cells expressing the relevant receptor. After an appropriate incubation time the supernatant is removed and transferred to a well containing similar cells. The biological response of these cells to the supernatant is determined relative to a non-conjugated polypeptide or another reference polypeptide, and this is a measure of the extent of the reduced RMC.

Normally, reduced *in vitro* activity of the conjugate is obtained as a consequence of its modification by a polymer molecule. However, in order to further reduce *in vitro* activity or for other reasons it may be of interest to modify the polypeptide part of the conjugate further. For instance, in one embodiment at least one amino acid residue located at or near a receptor-binding site of the polypeptide may be substituted with another amino acid residue as compared to the corresponding wild-type polypeptide so as to obtain reduced *in vitro* activity. The amino acid residue to be introduced by substitution may be any amino acid residue capable of reducing *in vitro* activity of the conjugate.

The term "exhibiting EPO activity" is intended to indicate that the polypeptide conjugate has one or more of the functions of native EPO, in particular hEPO with the amino acid sequence shown in SEQ ID NO:1, including the capability to bind to an EPO receptor and to stimulate production of red blood cells. The EPO activity is conveniently assayed using the TF-1 cell-based assay described in the Methods section below. Additional assay methods for erythropoietic activity and EPO receptor binding, including a cell proliferation assay, a cell binding assay and an ELISA binding competition assay, are described by Matthews et al., *Proc. Nat. Acad. Sci. USA* 93: 9471-9476 (1996). The polypeptide "exhibiting" EPO activity is considered to have such activity when it displays a measurable function, e.g. a measurable erythropoietic activity or receptor binding activity. The polypeptide exhibiting EPO activity

may also be termed "EPO molecule" herein for the sake of simplicity, even though such polypeptides are in fact variants of EPO.

The term "parent EPO" or "parent polypeptide" is intended to indicate the molecule to be modified in accordance with the present invention. The parent EPO is normally hEPO or a variant thereof, in particular with the amino acid sequence of SEQ ID NO:1. A "variant" is a polypeptide which differs in one or more amino acid residues from a parent polypeptide, normally in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acid residues.

Conjugate of the invention

As stated above, in a first aspect the invention relates to a conjugate comprising a polypeptide exhibiting EPO activity, which comprises an amino acid sequence that differs from the amino acid sequence of SEQ ID NO:1 in at least one amino acid residue selected from specified introduced or removed amino acid residues comprising an attachment group for a polymer molecule, and at least one polymer molecule attached to an attachment group of the polypeptide. The amino acid residues to be introduced and/or removed are described in further detail in the following sections. It will be understood that the conjugate itself also exhibits EPO activity.

By removing and/or introducing an amino acid residue comprising an attachment group for the polymer molecule it is possible to specifically adapt the polypeptide so as to make the molecule more susceptible to conjugation to the polymer molecule of choice, to optimize the conjugation pattern (e.g. to ensure an optimal distribution of polymer molecules on the surface of the EPO molecule and to ensure that only the attachment groups intended to be conjugated are present in the molecule) and thereby obtain a new conjugate molecule which has EPO activity and in addition one or more improved or alternative properties as compared to EPO molecules available today.

While the polypeptide may be of any origin, in particular mammalian origin, it is presently preferred to be of human origin.

In preferred embodiments of the present invention more than one amino acid residue of the polypeptide with EPO activity is altered. This may e.g. include removal as well as introduction of amino acid residues comprising an attachment group for the polymer molecule of choice.

In addition to the amino acid alterations disclosed herein aimed at removing and/or introducing attachment sites for the polymer molecule, it will be understood that the

amino acid sequence of the polypeptide of the invention may if desired contain other alterations that need not be related to introduction or removal of attachment sites, i.e. other substitutions, insertions or deletions. These may, for example, include truncation of the N- and/or C-terminus by one or more amino acid residues, or addition of one or more extra residues at the N- and/or C-terminus, e.g. addition of a methionine residue at the N-terminus. Another example of such additional alterations is the addition of an N-terminal sequence comprising one or more N-glycosylation sites as described in WO 02/02597.

The conjugate of the invention may have one or more of the following improved properties as compared to hEPO, in particular as compared to rhEPO (e.g. Epogen®): increased functional *in vivo* half-life, increased serum half-life, reduced renal clearance, reduced receptor-mediated clearance, increased potency and reduced immunogenicity.

It will be understood that the amino acid residue comprising an attachment group for a polymer molecule, whether it be removed or introduced, will be selected on the basis of the nature of the polymer molecule of choice and, in most instances, on the basis of the method by which conjugation between the polypeptide and the polymer molecule is to be achieved. For instance, when the polymer molecule is a polyethylene glycol or polyalkylene oxide derived molecule, amino acid residues comprising an attachment group may be selected from the group consisting of lysine, cysteine, aspartic acid, glutamic acid, histidine and arginine. When conjugation to a lysine residue is to be achieved, a suitable activated molecule is e.g. mPEG-SPA from Shearwater Corp., oxycarbonyl-oxy-N-dicarboxyimide-PEG (US 5,122,614), or PEG available from PolyMASC Pharmaceuticals plc.

In order to avoid too much disruption of the structure and function of the parent hEPO molecule, the total number of amino acid residues to be altered in accordance with the present invention, e.g. as described in the subsequent sections herein, (as compared to the amino acid sequence shown in SEQ ID NO:1) will typically not exceed 15. The exact number of amino acid residues and the type of amino acid residues to be introduced or removed depends in particular on the desired nature and degree of conjugation (e.g. the identity of the polymer molecule, how many polymer molecules it is desirable or possible to conjugate to the polypeptide, where conjugation is desired or should be avoided, etc.). Preferably, the polypeptide part of the conjugate of the invention or the polypeptide of the invention comprises an amino acid sequence which differs in 1-15 amino acid residues from the amino acid sequence shown in SEQ ID NO:1, typically in 2-10 amino acid residues, e.g. in 3-8 amino acid residues, such as 4-6 amino acid residues, from the amino acid sequence shown in SEQ ID NO:1. Thus,

normally the polypeptide part of the conjugate or the polypeptide of the invention comprises an amino acid sequence which differs from the amino acid sequence shown in SEQ ID NO:1 in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acid residues.

The polypeptide part of the conjugate will typically have an amino acid sequence with at least about 80% identity with SEQ ID NO:1, preferably at least about 90%, such as at least about 95%. Amino acid sequence homology/identity is conveniently determined from aligned sequences, using alignments obtained e.g. from the ClustalW program, version 1.8, June 1999, using default parameters (Thompson et al., 1994, ClustalW: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, Nucleic Acids Research, 22: 4673-4680) or from the PFAM families database version 4.0 (http://pfam.wustl.edu/) (*Nucleic Acids Res.* 1999 Jan 1; 27(1):260-2), where the degree of homology/identity is determined by use of GENEDOC version 2.5 (Nicholas, K.B., Nicholas H.B. Jr., and Deerfield, D.W. II. 1997 GeneDoc: Analysis and Visualization of Genetic Variation, EMBNEW.NEWS 4:14; Nicholas, K.B. and Nicholas H.B. Jr. 1997 GeneDoc: Analysis and Visualization of Genetic Variation).

In a preferred embodiment one difference between the amino acid sequence of the polypeptide and the amino acid sequence shown in SEQ ID NO:1 is that at least one and often more, e.g. 1-15, amino acid residues comprising an attachment group for the polymer molecule has been introduced, preferably by substitution, into the amino acid sequence. Thereby, the polypeptide part is altered in the content of the specific amino acid residues to which the polymer molecule of choice binds, whereby a more efficient, specific and/or extensive conjugation is achieved. For instance, when the total number of amino acid residues comprising an attachment group for the non-polypeptide of choice is altered to an optimised level, the clearance of the conjugate is typically significantly reduced, due to the altered shape, size and/or charge of the molecule achieved by the conjugation. Furthermore, when the total number of amino acid residues comprising an attachment group for the non-polypeptide of choice is increased, a greater proportion of the polypeptide molecule is shielded by the polymer molecules of choice, leading to a lower immune response.

The term "one difference" as used in the present application is intended to allow for additional differences being present. Accordingly, in addition to the specified amino acid difference, other amino acid residues than those specified may be mutated.

In a further preferred embodiment one difference between the amino acid sequence of the polypeptide and the amino acid sequence shown in SEQ ID NO:1 is that at least

one and preferably more, e.g. 1-15, amino acid residues comprising an attachment group for the polymer molecule has/have been removed, preferably by substitution, from the amino acid sequence. By removing one or more amino acid residues comprising an attachment group for the polymer molecule of choice it is possible to avoid conjugation to the polymer molecule in parts of the polypeptide in which such conjugation is disadvantageous, e.g. in amino acid residues located at or near a functional site of the polypeptide (since conjugation at such a site may result in inactivation or reduced EPO activity of the resulting conjugate due to impaired receptor recognition). In the present context the term "functional site" is intended to indicate one or more amino acid residues which is/are essential for or otherwise involved in the function or performance of hEPO. Such amino acid residues are a part of the functional site. The functional site may be determined by methods known in the art and is preferably identified by analysis of a structure of the polypeptide complexed to a relevant receptor, i.e. in this case the hEPO receptor.

In a still further preferred embodiment, the amino acid sequence of the polypeptide differs from the amino acid sequence shown in SEQ ID NO:1 in that a) at least one specified amino acid residue comprising an attachment group for the polymer molecule and present in the amino acid sequence shown in SEQ ID NO:1 has been removed, preferably by substitution, and b) at least one specified amino acid residue comprising an attachment group for the polymer molecule has been introduced into the amino acid sequence, preferably by substitution, the specified amino acid residues being any of those described in the subsequent sections herein. This embodiment is considered of particular interest in that it is possible to specifically design the polypeptide so as to obtain an optimal conjugation to the polymer molecule of choice. For instance, by introducing and removing selected amino acid residues as disclosed in the following sections it is possible to ensure an optimal distribution of attachment groups for the polymer molecule of choice, which gives rise to a conjugate in which the polymer molecules are placed so as to a) effectively shield epitopes and other surface parts of the polypeptide and b) ensure an optimal Stokes radius of the conjugate, without causing too much structural disruption and thereby impair the function of the polypeptide.

The conjugate of the invention will in general comprise a sufficient number and type of polymer molecules to provide the conjugate with an increased functional *in vivo* half-life and/or serum half-life as compared to hEPO, e.g. Epogen®. The increased functional *in vivo* half-life may be determined by known methods.

The conjugate of the invention may comprise at least one non-conjugated, conjugatable attachment group for the polymer molecule. In the present context the term "conjugatable attachment group" is intended to indicate an attachment group that is located in a position of the polypeptide where it is accessible for conjugation, and that but for special precautions is conjugated to the relevant polymer molecule when subjected to conjugation. For instance, such attachment group may be part of an amino acid residue involved in or otherwise essential for the polypeptide to exert its activity. A convenient way to avoid conjugation of an otherwise conjugatable attachment group is to shield the attachment group by means of a helper molecule, e.g. as described in the section entitled "Blocking of the functional site". It will be understood that the number of non-conjugated, conjugatable attachment groups will depend on the specific EPO polypeptide and the location of the conjugatable attachment groups.

Conjugate of the invention wherein the polymer molecule is attached to a lysine or the N-terminal amino acid residue

In one aspect the invention relates to a polypeptide conjugate comprising a polypeptide exhibiting EPO activity and having at least one polymer molecule attached to a lysine residue of the polypeptide, where the polypeptide comprises an amino acid sequence that differs from the amino acid sequence of hEPO (SEQ ID NO:1) in that at least one lysine residue has been introduced by substitution in a position selected from the group consisting of A1, P2, R4, L5, D8, S9, R10, V11, E13, R14, L17, E21, E23, N24, I25, T27, G28, A30, E31, H32, S34, N36, E37, N38, T40, N47, F48, Y49, A50, R53, M54, E55, V56, Q58, E62, Q65, A68, L69, S71, E72, L75, R76, Q78, A79, V82, N83, S84, S85, Q86, P87, E89, P90, Q92, L93, D96, S100, R103, S104, T106, T107, R110, A111, G113, Q115, E117, I119, S120, P121, P122, D123, A124, A125, S126, A127, A128, P129, L130, R131, T132, T134, D136, R139, R143, N147, R150, G151, L155, G158, E159, R162, T163, G164, D165 and R166 (residues having more than 25% of their side chain exposed to the surface, determined as described in the Examples section below). The polymer molecule may also be attached to the N-terminal residue. Among these residues, introduction of a lysine residue as an attachment site is preferably performed in positions that are not believed to lie in or near the receptor-binding site (see below), i.e. A1, P2, R4, E21, E23, N24, I25, T27, G28, A30, E31, H32, S34, N36, E37, N38, T40, A50, R53, M54, E55, V56, Q58, E62, Q65, A68, L69, S71, E72, L75, R76, A79, V82, N83, S84, S85, Q86, P87, E89, P90, Q92, T106, A111, G113, Q115, E117, I119, S120, P121, P122,

D123, A124, A125, S126, A127, A128, P129, L130, T132, T134, D136, R139, G158, E159, R162, T163, G164, D165 and R166.

Preferably, substitutions are performed in one or more positions in which the residue in hEPO has more than 50% of its side chain exposed to the surface: A1, P2, R4, L5, D8, S9, R10, R14, L17, N24, T27, G28, E31, S34, N36, N38, T40, N47, F48, Y49, A50, R53, E55, V56, Q65, A68, E72, L75, A79, V82, N83, S85, Q86, E89, Q92, L93, D96, S100, R103, T107, R110, A111, G113, I119, S120, P121, P122, D123, A124, A125, S126, A127, A128, P129, L130, R131, T132, D136, R143, R150, G158, R162, T163, G164 and D165. Among these residues, introduction of a lysine residue as an attachment site is preferably performed in positions that are not believed to lie in or near the receptor-binding site, i.e. A1, P2, R4, N24, T27, G28, E31, S34, N36, N38, T40, A50, R53, E55, V56, Q65, A68, E72, L75, A79, V82, N83, S85, Q86, E89, Q92, A111, G113, I119, S120, P121, P122, D123, A124, A125, S126, A127, A128, P129, L130, T132, D136, G158, R162, T163, G164 and D165.

In one embodiment, substitutions are performed in one or more of A1, P2, R4, L5, D8, S9, R10, L17, E21, E23, N24, I25, T27, G28, A30, E31, H32, S34, N36, E37, N38, T40, Y49, A50, R53, M54, E55, V56, Q58, E62, Q65, A68, L69, S71, E72, L75, R76, Q78, A79, V82, N83, S84, S85, Q86, P87, E89, P90, Q92, L93, T106, T107, R110, A111, G113, Q115, E117, I119, S120, P122, D123, A124, A125, S126, A127, A128, P129, L130, R131, T132, T134, D136, R139, R143, G158, E159, R162, T163, G164, D165 and R166, since these residues have been determined to have more than 25% of their side chain exposed to the surface upon binding to the EPO receptor (see the Examples section below). Among these residues, introduction of a lysine residue as an attachment site is preferably performed in positions that are not believed to lie in or near the receptor-binding site, i.e. A1, P2, R4, E21, E23, N24, I25, T27, G28, A30, E31, H32, S34, N36, E37, N38, T40, A50, R53, M54, E55, V56, Q58, E62, Q65, A68, L69, S71, E72, L75, R76, A79, V82, N83, S84, S85, Q86, P87, E89, P90, Q92, T106, A111, G113, Q115, E117, I119, S120, P122, D123, A124, A125, S126, A127, A128, P129, L130, T132, T134, D136, R139, G158, E159, R162, T163, G164, D165 and R166.

More preferably, substitutions are performed in one or more of the following residues that have more than 50% of their side chain exposed to the surface upon binding to the receptor: A1, P2, R4, L17, N24, T27, G28, E31, S34, N36, N38, T40, Y49, A50, R53, E55, V56, Q65, A68, E72, L75, A79, V82, N83, S85, Q86, E89, Q92, L93, A111, G113, I119, S120, D123, A124, A125, S126, A127, A128, P129, L130, T132, D136, G158, R162, T163, G164 and D165. Among these residues, introduction of a lysine residue as an attachment site is pref-

erably performed in positions that are not believed to lie in or near the receptor-binding site, i.e. A1, P2, R4, N24, T27, G28, E31, S34, N36, N38, T40, A50, R53, E55, V56, Q65, A68, E72, L75, A79, V82, N83, S85, Q86, E89, Q92, A111, G113, I119, S120, D123, A124, A125, S126, A127, A128, P129, L130, T132, D136, G158, R162, T163, G164 and D165.

A method for the determination of the degree of exposed surface area of the amino acid side chains, both for the EPO polypeptide alone and the polypepide:receptor complex, is explained in detail in the Examples section below, where it is also explained that the positions that are believed to lie in or near the receptor-binding site, so that conjugation to a polymer moiety at these positions is preferably avoided, are the following: L5, D8, S9, R10, V11, E13, R14, Y15, L16, L17, E18, K20, D43, T44, K45, V46, N47, F48, Y49, K52, Q78, L93, D96, K97, V99, S100, R103, S104, T107, L108, R110, R131, I133, K140, R143, V144, S146, N147, R150, G151, K154 and L155.

Human erythropoietin contains 8 lysines: K20, K45, K52, K97, K116, K140, K152 and K154, all of which are potential sites for polymer attachment by e.g. PEG. Since several of these are identified as being in the receptor-binding site, namely K20, K45, K52, K97, K140 and K154, it will typically be desirable when attaching PEG or other polymer moieties to a lysine residue to remove one or more of these potential attachment points in order to reduce the number of attached PEG groups and avoid polymer attachment in or near a receptor-binding site. The six lysines listed above as being in the receptor-binding site are therefore all potential targets for deletion, preferably by way of substitution with another amino acid residue, preferably by conservative substitution, more preferably with arginine. In a preferred embodiment, one, two, three, four, five or all of these residues are removed by substitution.

The polymer molecule used in this aspect of the invention is preferably selected from the group consisting of linear or branched polyethylene glycols and other polyalkylene oxides, e.g. mPEG-SPA from Shearwater Corp.

In one embodiment, the polypeptide conjugate of the invention may comprise a PEG molecule attached to some or, preferably, substantially all of the lysine residues in the polypeptide available for PEGylation, and in addition to the N-terminal amino acid residue of the polypeptide.

It will be understood that any of the amino acid changes, in particular substitutions, specified in this section can be combined with any of the amino acid changes, preferably substitutions, specified in the other sections herein disclosing specific amino acid modifications.

Conjugate of the invention wherein the polymer molecule is attached to a cysteine residue

In another aspect, the invention relates to a polypeptide conjugate comprising a polypeptide exhibiting EPO activity and having at least one polymer molecule attached to a cysteine residue of the polypeptide, where the polypeptide comprises an amino acid sequence that differs from the amino acid sequence of hEPO (SEQ ID NO:1) in that at least one cysteine residue has been introduced in any one or more of the positions listed above under the discussion of attachment to a lysine, i.e. in a position where the native residue has more than 25% of its side chain exposed to the surface, preferably more than 50%, and preferably when side chain exposure is determined upon binding to the EPO receptor. More preferred residues for substitution with a cysteine are those that are not located at or near the receptor-binding site, as is also discussed above.

In one embodiment of this aspect of the invention, at least one cysteine residue has been introduced by substitution in the A, B, C or D helix, in particular in a position selected from the group consisting of R10, V11, E13, R14, L17, K20, E21, E62, Q65, A68, L69, S71, E72, L75, R76, P90, Q92, L93, D96, K97, S100, R103, S104, T106, T134, D136, R139, K140, R143, N147, R150 and G151 (residues with more than 25% side chain exposure); and preferably in a position not located at or near the receptor-binding site, i.e. E21, E62, Q65, A68, L69, S71, E72, L75, R76, P90, Q92, T106, T134, D136 and R139.

Preferably, substitutions to introduce a cysteine residue are performed in one or more of the following residues, which have more than 50% of their side chain exposed to the surface: R10, R14, L17, K20, Q65, A68, E72, L75, Q92, L93, D96, K97, S100, R103, D136, R143 and R150; and preferably in a position not located at or near the receptor-binding site, i.e. Q65, A68, E72, L75, Q92 and D136.

More preferably, substitutions are performed in positions that in hEPO are occupied by residues having than 25% of their side chain exposed to the surface upon binding to the EPO receptor, i.e. one or more of R10, L17, K20, E21, E62, Q65, A68, L69, S71, E72, L75, R76, P90, Q92, L93, T106, T134, D136, R139 and R143; and preferably in a position not located at or near the receptor-binding site, i.e. E21, E62, Q65, A68, L69, S71, E72, L75, R76, P90, Q92, T106, T134, D136 and R139.

Still more preferably, substitutions are performed in one or more of the following residues that have more than 50% of their side chain exposed to the surface upon binding to the

receptor: L17, Q65, A68, E72, L75, Q92, L93 and D136; and preferably in a position not located at or near the receptor-binding site, i.e. Q65, A68, E72, L75, Q92 and D136.

If desired, free cysteines may be appropriately blocked, e.g. using a method disclosed in WO 00/42175 in which a host cell capable of expressing a soluble protein having a free cysteine residue is exposed to a cysteine blocking agent.

It will be understood that any of the amino acid modifications, in particular substitutions, specified in this section can be combined with any of the amino acid changes, in particular substitutions, specified in the other sections herein disclosing specific amino acid modifications.

Conjugate of the invention wherein the polymer molecule is attached to an acid group or the C-terminal amino acid residue

In a further aspect the invention relates to a polypeptide conjugate comprising a polypeptide exhibiting EPO activity and having at least one polymer molecule attached to an aspartic acid or glutamic acid residue of the polypeptide, and optionally to the C-terminal. The polypeptide of this aspect of the invention may comprise an amino acid sequence that differs from the amino acid sequence of hEPO (SEQ ID NO:1) in that at least one aspartic acid or glutamic acid residue has been introduced by substitution in a position selected from the group consisting of: A1, P2, R4, L5, S9, R10, V11, R14, L17, K20, N24, I25, T27, G28, A30, H32, S34, N36, N38, T40, K45, N47, F48, Y49, A50, K52, R53, M54, V56, Q58, Q65, A68, L69, S71, L75, R76, Q78, A79, V82, N83, S84, S85, Q86, P87, P90, Q92, L93, K97, S100, R103, S104, T106, T107, R110, A111, G113, Q115, K116, I119, S120, P121, P122, A124, A125, S126, A127, A128, P129, L130, R131, T132, T134, R139, K140, R143, N147, R150, G151, K154, L155, G158, R162, T163, G164 and R166 (residues with more than 25% side chain exposure) ; and preferably in a position not located at or near the receptor-binding site, i.e. A1, P2, R4, N24, I25, T27, G28, A30, H32, S34, N36, N38, T40, A50, R53, M54, V56, Q58, Q65, A68, L69, S71, L75, R76, A79, V82, N83, S84, S85, Q86, P87, P90, Q92, T106, A111, G113, Q115, K116, I119, S120, P121, P122, A124, A125, S126, A127, A128, P129, L130, T132, T134, R139, G158, R162, T163, G164 and R166.

Preferably, substitutions to introduce an aspartic acid or glutamic acid residue are performed in one or more of the following residues, which have more than 50% of their side chain exposed to the surface: A1, P2, R4, L5, S9, R10, R14, L17, K20, N24, T27, G28, S34, N36, N38, T40, K45, N47, F48, Y49, A50, K52, R53, V56, Q65, A68, L75, A79, V82, N83, S85,

Q86, Q92, L93, K97, S100, R103, T107, R110, A111, G113, K116, I119, S120, P121, P122, A124, A125, S126, A127, A128, P129, L130, R131, T132, R143, R150, G158, R162, T163 and G164; and preferably in a position not located at or near the receptor-binding site, i.e. A1, P2, R4, N24, T27, G28, S34, N36, N38, T40, A50, R53, V56, Q65, A68, L75, A79, V82, N83, S85, Q86, Q92, A111, G113, K116, I119, S120, P121, P122, A124, A125, S126, A127, A128, P129, L130, T132, G158, R162, T163 and G164.

More preferably, substitutions are performed in positions that in hEPO are occupied by residues having than 25% of their side chain exposed to the surface upon binding to the EPO receptor, i.e. one or more of A1, P2, R4, L5, S9, R10, L17, K20, E23, N24, I25, T27, G28, A30, H32, S34, N36, E37, N38, T40, K45, Y49, A50, K52, R53, M54, V56, Q58, E62, Q65, A68, L69, S71, L75, R76, Q78, A79, V82, N83, S84, S85, Q86, P87, P90, Q92, L93, T106, T107, R110, A111, G113, Q115, K116, I119, S120, P122, A124, A125, S126, A127, A128, P129, L130, R131, T132, T134, R139, R143, G158, R162, T163, G164 and R166; and preferably in a position not located at or near the receptor-binding site, i.e. A1, P2, R4, E23, N24, I25, T27, G28, A30, H32, S34, N36, E37, N38, T40, A50, R53, M54, V56, Q58, E62, Q65, A68, L69, S71, L75, R76, A79, V82, N83, S84, S85, Q86, P87, P90, Q92, T106, A111, G113, Q115, K116, I119, S120, P122, A124, A125, S126, A127, A128, P129, L130, T132, T134, R139, G158, R162, T163, G164 and R166.

Still more preferably, substitutions are performed in one or more of the following residues that have more than 50% of their side chain exposed to the surface upon binding to the receptor: A1, P2, R4, L17, N24, T27, G28, S34, N36, N38, T40, Y49, A50, K52, R53, V56, Q65, A68, L75, A79, V82, N83, S85, Q86, Q92, L93, A111, G113, K116, I119, S120, A124, A125, S126, A127, A128, P129, L130, T132, G158, R162, T163 and G164; and preferably in a position not located at or near the receptor-binding site, i.e. A1, P2, R4, N24, T27, G28, S34, N36, N38, T40, A50, R53, V56, Q65, A68, L75, A79, V82, N83, S85, Q86, Q92, A111, G113, K116, I119, S120, A124, A125, S126, A127, A128, P129, L130, T132, G158, R162, T163 and G164.

Substitutions to introduce an aspartic acid or glutamic acid residue may be for any other amino acid residue, and in particular for an asparagine or a glutamine residue. Conjugates according to this aspect of the invention may be prepared e.g. as described by Sakane and Pardridge, *Pharmaceutical Research* Vol. 14, No. 8, 1997, pp 1085-1091.

In the case of attachment of a polymer moiety to an aspartic acid or glutamic acid residue, it may be desirable to remove, preferably by substitution, one, two, three, four or all of

the aspartic acid and glutamic acid residues that are located in or near receptor-binding site, namely D8, E13, E18, D43 and D96. The substitution may be e.g. with an aspargine or glutamine residue.

It will be understood that any of the amino acid changes, in particular substitutions, specified in this section can be combined with any of the amino acid changes, in particular substitutions specified in the other sections herein disclosing specific amino acid changes.

Conjugate of the invention wherein the polymer molecule is attached to a carbohydrate chain

In a further aspect, conjugates may be formed by attachment of a polymer molecule, preferably polyethylene glycol, to a carbohydrate chain of the glycoprotein. Conjugation to a carbohydrate chain is described e.g. in EP 0 605 963 A2, which is hereby incorporated herein by reference. In one embodiment, the glycosylation pattern of the polypeptide is the same as that of native human EPO (i.e. the amino acid residues forming the native glycosylation sites are left unaltered), and a PEG or other polymer moiety is attached to one or more of the carbohydrate chains of the glycosylated protein. In another embodiment, the glycosylation pattern may be altered by introduction and/or removal of one or more glycosylation sites. In either case, conjugation to a carbohydrate chain may optionally be combined with other conjugation as described herein, e.g. to the amino group of a lysine or to a cysteine residue.

As mentioned above, there are three N-glycosylation sites in wild-type human erythropoietin at positions N24, N38 and N83. Sites with the sequence pattern N-X'-S/T/C-X'' (wherein X' is any amino acid residue except proline, X'' is any amino acid residue which may or may not be identical to X' and which preferably is different from proline, N is asparagine, and S/T/C is either serine, threonine or cysteine, preferably serine or threonine, and most preferably threonine) are potential glycosylation sites. New glycosylation sites can therefore be introduced by mutation of one or two residues in order to introduce the above sequence pattern, although it is preferred to introduce a glycosylation site in a position where only one mutation is required. Preferred sites for introduction of a glycosylation site are those where the N residue, or another residue to be substituted with an N residue, is more than 25% side chain exposed, e.g. more than 50%, in the structure of the complex between EPO and its receptor, and where none of the residues to be mutated is a Cys involved in a disulphide bridge. More preferred are sequence patterns already having an N in the first position or an S or T in the third position of the above-mentioned sequence pattern. Further, as mentioned above one or more N-

glycosylation sites may also be introduced in the form of an N-terminal peptide addition as described in WO 02/02597, which is hereby incorporated by reference.

Polymer molecule of the conjugate of the invention

Although the polypeptide part of the conjugate is normally conjugated to only one type of polymer molecule, it may in certain cases also be conjugated to two or more different types of polymer molecules. Further, it will be understood that in addition to the polymer molecules attached as described herein, the polypeptide conjugates of the invention will typically in addition comprise oligosaccharide moieties attached to one or more sites on the polypeptide, in particular by means *in vivo* N- or O-glycosylation. If desired, the glycosylation pattern of the polypeptide may be modified in relation to that of human EPO by introducing one or more non-native glycosylation sites and/or by removing one or more native glycosylation sites. Modification of the glycosylation pattern of human EPO is described e.g. in the references cited above.

Methods for preparing a conjugate of the invention

In general, a polypeptide conjugate according to the invention may be produced by culturing an appropriate host cell under conditions conducive for expression of the polypeptide, and recovering the polypeptide, followed by conjugation to a polymer molecule *in vitro*. As indicated above, the polypeptide will typically also contain one or more oligosaccharide moieties attached via *in vivo* glycosylation.

Conjugation to a polymer molecule

The polymer molecule to be coupled to the polypeptide may be any suitable polymer molecule, such as a natural or synthetic homo-polymer or heteropolymer, typically with a molecular weight in the range of about 300-100,000 Da, such as about 500-20,000 Da. Preferably, however, the molecular weight of the polymer molecule is less than about 20,000 Da, more preferably in the range of about 1000-15,000 Da, even more preferably in the range of about 2000-12,000 Da, such as about 3000-10,000. When used about polymer molecules herein, the word "about" indicates an approximate average molecular weight and reflects the fact that there will normally be a certain molecular weight distribution in a given polymer preparation.

Examples of homo-polymers include a polyol (i.e. poly-OH), a polyamine (i.e. poly-NH_2) and a polycarboxylic acid (i.e. poly-COOH). A hetero-polymer is a polymer which comprises different coupling groups, such as a hydroxyl group and an amine group.

Examples of suitable polymer molecules include polymer molecules selected from the group consisting of polyalkylene oxide (PAO), including polyalkylene glycol (PAG), such as linear or branched polyethylene glycol (PEG) and polypropylene glycol (PPG), poly-vinyl alcohol (PVA), poly-carboxylate, poly-(vinylpyrolidone), polyethylene-co-maleic acid anhydride, polystyrene-co-maleic acid anhydride, dextran, including carboxymethyl-dextran, or any other biopolymer suitable for reducing immunogenicity and/or increasing functional *in vivo* half-life and/or serum half-life. Another example of a polymer molecule is human albumin or another abundant plasma protein, although such polymers are less preferred. Generally, polyalkylene glycol-derived polymers are biocompatible, non-toxic, non-antigenic, non-immunogenic, have various water solubility properties, and are easily excreted from living organisms. Such polymers are therefore preferred.

In particular, PEG is the preferred polymer molecule, since it has only few reactive groups capable of cross-linking compared to polysaccharides such as dextran. In particular, monofunctional PEG, e.g. methoxypolyethylene glycol (mPEG), is of interest since its coupling chemistry is relatively simple (only one reactive group is available for conjugating with attachment groups on the polypeptide). Consequently, the risk of cross-linking is eliminated, the resulting polypeptide conjugates are more homogeneous and the reaction of the polymer molecules with the polypeptide is easier to control.

To effect covalent attachment of the polymer molecule(s) to the polypeptide, the hydroxyl end groups of the polymer molecule are provided in activated form, i.e. with reactive functional groups. Suitable activated polymer molecules are commercially available, e.g. from Shearwater Corp., Huntsville, AL, USA, or from PolyMASC Pharmaceuticals plc, UK. Alternatively, the polymer molecules can be activated by conventional methods known in the art, e.g. as disclosed in WO 90/13540. Specific examples of activated linear or branched polymer molecules for use in the present invention are described in the 2001 Shearwater Corporation Catalog (Polyethylene Glycol and Derivatives for Biomedical Applications, incorporated herein by reference). Specific examples of activated PEG polymers include the following linear PEGs: NHS-PEG (e.g. SPA-PEG, SSPA-PEG, SBA-PEG, SS-PEG, SSA-PEG, SC-PEG, SG-PEG, and SCM-PEG), and NOR-PEG), BTC-PEG, EPOX-PEG, NCO-PEG, NPC-PEG, CDI-PEG, ALD-PEG, TRES-PEG, VS-PEG, IODO-PEG, and MAL-PEG, and branched PEGs such

as PEG2-NHS and those disclosed in US 5,932,462 and US 5,643,575, both of which are incorporated herein by reference. Furthermore, the following publications, incorporated herein by reference, disclose useful polymer molecules and/or PEGylation chemistries: US 5,824,778, US 5,476,653, WO 97/32607, EP 229,108, EP 402,378, US 4,902,502, US 5,281,698, US 5,122,614, US 5,219,564, WO 92/16555, WO 94/04193, WO 94/14758, WO 94/17039, WO 94/18247, WO 94/28024, WO 95/00162, WO 95/11924, WO95/13090, WO 95/33490, WO 96/00080, WO 97/18832, WO 98/41562, WO 98/48837, WO 99/32134, WO 99/32139, WO 99/32140, WO 96/40791, WO 98/32466, WO 95/06058, EP 439 508, WO 97/03106, WO 96/21469, WO 95/13312, EP 921 131, US 5,736,625, WO 98/05363, EP 809 996, US 5,629,384, WO 96/41813, WO 96/07670, US 5,473,034, US 5,516,673, EP 605 963, US 5,382,657, EP 510 356, EP 400 472, EP 183 503 and EP 154 316.

The conjugation of the polypeptide and the activated polymer molecules is conducted by use of any conventional method, e.g. as described in the following references (which also describe suitable methods for activation of polymer molecules): R.F. Taylor, (1991), "Protein immobilisation. Fundamental and applications", Marcel Dekker, N.Y.; S.S. Wong, (1992), "Chemistry of Protein Conjugation and Crosslinking", CRC Press, Boca Raton; G.T. Hermanson et al., (1993), "Immobilized Affinity Ligand Techniques", Academic Press, N.Y.). The skilled person will be aware that the activation method and/or conjugation chemistry to be used depends on the attachment group(s) of the polypeptide (examples of which are given further above), as well as the functional groups of the polymer (e.g. being amine, hydroxyl, carboxyl, aldehyde, sulfydryl, succinimidyl, maleimide, vinysulfone or haloacetate). The PEGylation may be directed towards conjugation to most or substantially all available attachment groups on the polypeptide (i.e. such attachment groups that are exposed at the surface of the polypeptide) or may be directed towards one or more specific attachment groups, e.g. the N-terminal amino group (US 5,985,265). Furthermore, the conjugation may be achieved in one step or in a stepwise manner (e.g. as described in WO 99/55377).

It will be understood that the PEGylation is designed so as to produce the optimal molecule with respect to the number of PEG molecules attached, the size and form of such molecules (e.g. whether they are linear or branched), and where in the polypeptide such molecules are attached. The molecular weight of the polymer to be used will be chosen taking into consideration the desired effect to be achieved. For instance, if the primary purpose of the conjugation is to achieve a conjugate having a high molecular weight and larger size (e.g. to reduce renal clearance), one may choose to conjugate either one or a few high molecular weight

polymer molecules or a number of polymer molecules with a smaller molecular weight to obtain the desired effect. Typically, however, several polymer molecules with a smaller molecular weight will be used, e.g. 2-8, such as 3, 4, 5 or 6. When a high degree of epitope shielding is desirable, this may be obtained by use of a sufficiently high number of low molecular weight polymer molecules (e.g. with a molecular weight of about 5,000 Da) to effectively shield all or most epitopes of the polypeptide. For instance, 2-8, such as 3-6 such polymers may be used. It may be advantageous to have a larger number of polymer molecules with a lower molecular weight (e.g. 4-6 with a MW of about 5000) compared to a smaller number of polymer molecules with a higher molecular weight (e.g. 1-3 with a MW of 12,000-20,000) in terms of improving the functional *in vivo* half-life of the polypeptide conjugate, even where the total molecular weight of the attached polymer molecules in the two cases is the same. It is believed that the presence of a larger number of smaller polymer molecules provides the polypeptide with a larger diameter or "apparent size" than e.g. a single yet larger polymer molecule, at least when the polymer molecules are relatively uniformly distributed on the polypeptide surface.

It will be understood that the apparent size in kDa of a conjugate or polypeptide is not necessarily the same as the actual molecular weight of the conjugate or polypeptide. Rather, the apparent size is a reflection of both the actual molecular weight and the overall bulk. Since, in most cases, attachment of one or more PEG groups or other polymer molecules will result in a relatively large increase of the bulk of the polypeptide to which such moieties are attached, the polypeptide conjugates of the invention will normally have an apparent size that exceeds the actual molecular weight of the conjugate.

While conjugation of only a single polymer molecule to a single attachment group on the protein is not preferred, in the event that only one polymer molecule is attached, it will generally be advantageous that the polymer molecule, which may be linear or branched, has a relatively high molecular weight, e.g. from about 12 kDa to about 20 kDa.

Normally, the polymer conjugation is performed under conditions aiming at reacting as many of the available polymer attachment groups as possible with polymer molecules. This is achieved by means of a suitable molar excess of the polymer in relation to the polypeptide (number of attachment sites). Typical molar ratios of activated polymer molecules to polypeptide attachment sites are up to about 1000:1, such as up to about 200:1 or up to about 100:1. In some cases, the ratio may be somewhat lower, however, such as up to about 50:1, 10:1 or 5:1 or even approximately equimolar, e.g. if a lower degree of polymer attachment is desired.

It is also contemplated according to the invention to couple the polymer molecules to the polypeptide through a linker. Suitable linkers are well known to the skilled person. One example is cyanuric chloride (Abuchowski et al., (1977), *J. Biol. Chem.*, 252, 3578-3581; US 4,179,337; Shafer et al., (1986), *J. Polym. Sci. Polym. Chem.* Ed., 24, 375-378.

Subsequent to the conjugation residual activated polymer molecules are blocked according to methods known in the art, e.g. by addition of primary amine to the reaction mixture, and the resulting inactivated polymer molecules are removed by any suitable method.

In a preferred embodiment, the polypeptide conjugate of the invention comprises a PEG molecule attached to some, most or preferably substantially all of the residues in the polypeptide available for PEGylation (although preferably not residues located in a receptor-binding site), in particular a linear or branched PEG molecule, e.g. with a molecular weight of about 1-15 kDa, typically about 2-12 kDa, such as about 3-10 kDa, e.g. about 4, 5 or 6 kDa.

It will be understood that depending on the circumstances, e.g. the amino acid sequence of the polypeptide, the nature of the activated PEG compound being used and the specific PEGylation conditions, including the molar ratio of PEG to polypeptide, varying degrees of PEGylation may be obtained, with a higher degree of PEGylation generally being obtained with a higher ratio of PEG to polypeptide. The PEGylated polypeptides resulting from any given PEGylation process will, however, often comprise a stochastic distribution of polypeptide conjugates having slightly different degrees of PEGylation, at least when attaching PEG moieties to more than one attachment group. If desired, the PEGylated polypeptides may be subject to purification in order to obtain a more uniform degree of PEGylation.

Glycosylation of conjugates of the invention

Conjugation to an oligosaccharide moiety may take place *in vivo* or *in vitro*, although glycosylation will normally be obtained *in vivo*. In order to achieve *in vivo* glycosylation of an EPO molecule comprising one or more glycosylation sites the nucleotide sequence encoding the polypeptide must be inserted in a glycosylating, eukaryotic expression host. The expression host cell may be selected from fungal (filamentous fungal or yeast), insect or animal cells or from transgenic plant cells. In one embodiment the host cell is a mammalian cell, such as a CHO cell, a BHK or HEK cell, e.g. HEK 293, or an insect cell, such as an SF9 cell, or a yeast cell, e.g. *Saccharomyces cerevisiae* or *Pichia pastoris*, or any of the host cells mentioned hereinafter. Covalent *in vitro* coupling of glycosides (such as dextran) to amino acid residues

of the polypeptide may also be used, e.g. as described in WO 87/05330 and in Aplin et al., CRC Crit Rev. Biochem., pp. 259-306, 1981.

In vitro coupling of oligosaccharide moieties or PEG to protein- and peptide-bound Gln-residues can be carried out by transglutaminases (TG'ases). Transglutaminases catalyse the transfer of donor amine-groups to protein- and peptide-bound Gln-residues in a so-called cross-linking reaction. The donor-amine groups can be protein- or peptide-bound e.g. as the ε-amino-group in Lys-residues or can be part of a small or large organic molecule. An example of a small organic molecule functioning as an amino-donor in TG'ase-catalysed cross-linking is putrescine (1,4-diaminobutane). An example of a larger organic molecule functioning as an amino-donor in TG'ase-catalysed cross-linking is an amine-containing PEG (Sato et al., Biochemistry 35, 13072-13080).

TG'ases are in general highly specific enzymes, and not every Gln-residue exposed on the surface of a protein is accessible to TG'ase-catalysed cross-linking to amino-containing substances. On the contrary, only a few Gln-residues function naturally as TG'ase substrates, but the exact parameters governing which Gln-residues are good TG'ase substrates remain unknown. Thus, in order to render a protein susceptible to TG'ase-catalysed cross-linking reactions it is often a prerequisite to add at convenient positions stretches of amino acid sequence known to function very well as TG'ase substrates. Several amino acid sequences are known to be or to contain excellent natural TG'ase substrates e.g. substance P, elafin, fibrinogen, fibronectin, α_2-plasmin inhibitor, α-caseins, and β-caseins.

Blocking of the functional site

It has been reported that excessive polymer conjugation can lead to a loss of activity of the polypeptide to which the polymer is conjugated. This problem can be eliminated by e.g. removal of attachment groups located at the functional site or by blocking the functional site prior to conjugation so that the functional site is blocked during conjugation. The latter strategy constitutes a further embodiment of the invention (the first strategy being exemplified further above, e.g. by removal of lysine residues in or near the functional site). More specifically, conjugation between the polypeptide and the polymer molecule may in this case be conducted under conditions where the functional site of the polypeptide is blocked by a helper molecule capable of binding to the functional site of the polypeptide.

Preferably, the helper molecule is one which specifically recognizes a functional site of the polypeptide, such as a receptor, in particular the EPO receptor or a part of the EPO receptor.

Alternatively, the helper molecule may be an antibody, in particular a monoclonal antibody recognizing the polypeptide exhibiting EPO activity. In particular, the helper molecule may be a neutralizing monoclonal antibody.

The polypeptide is allowed to interact with the helper molecule before effecting conjugation. This ensures that the functional site of the polypeptide is shielded or protected and consequently unavailable for derivatization by the polymer molecule. Following its elution from the helper molecule, the conjugate between the polymer molecule and the polypeptide can be recovered with at least a partially preserved functional site.

The subsequent conjugation of the polypeptide having a blocked functional site to a polymer moiety is otherwise conducted in the normal way, e.g. as described above.

Irrespective of the nature of the helper molecule to be used to shield the functional site of the polypeptide from conjugation, it is desirable that the helper molecule is free of or comprises only a few attachment groups for the polymer molecule of choice in part(s) of the molecule where the conjugation to such groups would hamper desorption of the conjugated polypeptide from the helper molecule. Selective conjugation to attachment groups present in non-shielded parts of the polypeptide can hereby be obtained and it is possible to reuse the helper molecule for repeated cycles of conjugation. For instance, if the polymer molecule is e.g. PEG having the epsilon amino group of a lysine or N-terminal amino acid residue as an attachment group, it is desirable that the helper molecule is substantially free of conjugatable epsilon amino groups, preferably free of any epsilon amino groups. Accordingly, in a preferred embodiment the helper molecule is a protein or peptide capable of binding to the functional site of the polypeptide, which protein or peptide is free of any conjugatable attachment groups for the polymer molecule of choice.

Of particular interest in connection with an embodiment of the present invention wherein the polypeptide conjugates are prepared from a diversified population of nucleotide sequences encoding a polypeptide of interest, the blocking of the functional group may be effected in microtiter plates prior to conjugation, for instance by plating the expressed polypeptide variant in a microtiter plate containing an immobilized blocking group such as a receptor or antibody.

In a further embodiment the helper molecule is first covalently linked to a solid phase such as a column packing material, for instance Sephadex or agarose beads, or a surface, e.g. a reaction vessel. Subsequently, the polypeptide is loaded onto the column material carrying the helper molecule and conjugation is carried out according to methods known in the art, e.g. as described above. This procedure allows the polypeptide conjugate to be separated from the helper molecule by elution. The polypeptide conjugate is eluted by conventional techniques under physico-chemical conditions that do not lead to a substantive degradation of the polypeptide conjugate. The fluid phase containing the polypeptide conjugate is separated from the solid phase to which the helper molecule remains covalently linked. The separation can be achieved in other ways: For instance, the helper molecule may be derivatised with a second molecule (e.g. biotin) that can be recognized by a specific binder (e.g. streptavidin). The specific binder may be linked to a solid phase, thereby allowing the separation of the polypeptide conjugate from the helper molecule-second molecule complex through passage over a second helper-solid phase column which will retain, upon subsequent elution, the helper molecule-second molecule complex, but not the polypeptide conjugate. The polypeptide conjugate may be released from the helper molecule in any appropriate fashion. Deprotection may be achieved by providing conditions in which the helper molecule dissociates from the functional site of the EPO to which it is bound. For instance, a complex between an antibody to which a polymer is conjugated and an anti-idiotypic antibody can be dissociated by adjusting the pH appropriately.

Conjugation of a tagged polypeptide

In an alternative embodiment the polypeptide is expressed as a fusion protein with a tag, i.e. an amino acid sequence or peptide stretch made up of typically 1-30, such as 1-20 amino acid residues. Besides allowing for fast and easy purification, the tag is a convenient tool for achieving conjugation between the tagged polypeptide and the polymer molecule. In particular, the tag may be used for achieving conjugation in microtiter plates or other carriers, such as paramagnetic beads, to which the tagged polypeptide can be immobilised via the tag. The conjugation to the tagged polypeptide in e.g. microtiter plates has the advantage that the tagged polypeptide can be immobilised in the microtiter plates directly from the culture broth (in principle without any purification) and subjected to conjugation. Thereby, the total number of process steps (from expression to conjugation) can be reduced. Furthermore, the tag may function as a spacer molecule, ensuring an improved accessibility to the immobilised

polypeptide to be conjugated. The conjugation using a tagged polypeptide may be to any of the polymer molecules disclosed herein, e.g. to a polymer molecule such as PEG.

The identity of the specific tag to be used is not critical as long as the tag is capable of being expressed with the polypeptide and is capable of being immobilised on a suitable surface or carrier material. A number of suitable tags are commercially available, for example histidine tags and carboxy terminal epitope tags, as are antibodies against various tags. The subsequent cleavage of the tag from the polypeptide may be achieved by use of commercially available enzymes.

Methods for preparing a polypeptide of the invention or the polypeptide part of the conjugate of the invention

The polypeptide of the present invention or the polypeptide part of a conjugate of the invention, optionally in glycosylated form, may be produced by any suitable method known in the art. Such methods include constructing a nucleotide sequence encoding the polypeptide and expressing the sequence in a suitable transformed or transfected host. However, polypeptides of the invention may be produced, albeit less efficiently, by chemical synthesis or a combination of chemical synthesis or a combination of chemical synthesis and recombinant DNA technology.

A nucleotide sequence encoding a polypeptide or the polypeptide part of a conjugate of the invention may be constructed by isolating or synthesizing a nucleotide sequence encoding the parent EPO, such as hEPO with the amino acid sequence shown in SEQ ID NO:1, and then changing the nucleotide sequence so as to effect introduction (i.e. insertion or substitution) or deletion (i.e. removal or substitution) of the relevant amino acid residue(s).

The nucleotide sequence is conveniently modified by site-directed mutagenesis in accordance with conventional methods. Alternatively, the nucleotide sequence is prepared by chemical synthesis, e.g. by using an oligonucleotide synthesizer, wherein oligonucleotides are designed based on the amino acid sequence of the desired polypeptide, and preferably selecting those codons that are favored in the host cell in which the recombinant polypeptide will be produced. For example, several small oligonucleotides coding for portions of the desired polypeptide may be synthesized and assembled by polymerase chain reaction (PCR), ligation or ligation chain reaction (LCR) (Barany, PNAS 88:189-193, 1991). The individual oligonucleotides typically contain 5' or 3' overhangs for complementary assembly.

Alternative nucleotide sequence modification methods are available for producing polypeptide variants for high throughput screening, for instance methods which involve homologous cross-over such as disclosed in US 5,093,257, and methods which involve gene shuffling, i.e. recombination between two or more homologous nucleotide sequences resulting in new nucleotide sequences having a number of nucleotide alterations when compared to the starting nucleotide sequences. Gene shuffling (also known as DNA shuffling or recursive sequence recombination, RSR) involves, in a basic format, one or more cycles of random fragmentation and reassembly of the nucleotide sequences, followed by screening to select nucleotide sequences encoding polypeptides with desired properties. In order for homology-based nucleic acid shuffling to take place, the relevant parts of the nucleotide sequences are preferably at least 50% identical, such as at least 60% identical, more preferably at least 70% identical, such as at least 80% identical. The recombination can be performed *in vitro* or *in vivo*.

Examples of suitable *in vitro* gene shuffling methods are disclosed by Stemmer et al. (1994), Proc. Natl. Acad. Sci. USA; vol. 91, pp. 10747-10751; Stemmer (1994), Nature, vol. 370, pp. 389-391; Smith (1994), Nature vol. 370, pp. 324-325; Zhao et al., Nat. Biotechnol. 1998, Mar; 16(3): 258-61; Zhao H. and Arnold, FB, Nucleic Acids Research, 1997, Vol. 25. No. 6 pp. 1307-1308; Shao et al., Nucleic Acids Research 1998, Jan 15; 26(2): pp. 681-83; and WO 95/17413. An example of a suitable *in vivo* shuffling method is disclosed in WO 97/07205. Other techniques for mutagenesis of nucleic acid sequences by *in vitro* or *in vivo* recombination are disclosed e.g. in WO 97/20078 and US 5,837,458. Examples of specific shuffling techniques include "family shuffling", "synthetic shuffling" and "in silico shuffling". Family shuffling involves subjecting a family of homologous genes from different species to one or more cycles of shuffling and subsequent screening or selection. Family shuffling techniques are disclosed e.g. by Crameri et al. (1998), Nature, vol. 391, pp. 288-291; Christians et al. (1999), Nature Biotechnology, vol. 17, pp. 259-264; Chang et al. (1999), Nature Biotechnology, vol. 17, pp. 793-797; and Ness et al. (1999), Nature Biotechnology, vol. 17, 893-896. Synthetic shuffling involves providing libraries of overlapping synthetic oligonucleotides based e.g. on a sequence alignment of homologous genes of interest. The synthetically generated oligonucleotides are recombined, and the resulting recombinant nucleic acid sequences are screened and if desired used for further shuffling cycles. Synthetic shuffling techniques are disclosed in WO 00/42561. In silico shuffling refers to a DNA shuffling procedure which is performed or modelled using a computer system, thereby partly or entirely avoiding the need for physically manipulating nucleic acids. Techniques for in silico shuffling are disclosed in

WO 00/42560. Other useful methods for evolution of proteins by means of recursive sequence recombination are disclosed in WO 98/27230.

Once assembled (by synthesis, site-directed mutagenesis or another method), the nucleotide sequence encoding the polypeptide is inserted into a recombinant vector and operably linked to control sequences necessary for expression of the EPO in the desired transformed host cell.

Persons skilled in the art will be familiar with available vectors, expression control sequences and hosts, and will be able to select from among suitable vectors, expression control sequences and hosts without undue experimentation. For example, in selecting a vector, the host must be considered because the vector must replicate in it or be able to integrate into the chromosome. The vector's copy number, the ability to control that copy number, and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered. In selecting an expression control sequence, a variety of factors should also be considered. These include, for example, the relative strength of the sequence, its controllability, and its compatibility with the nucleotide sequence encoding the polypeptide, particularly as regards potential secondary structures. Hosts should be selected by consideration of their compatibility with the chosen vector, the toxicity of the product coded for by the nucleotide sequence, their secretion characteristics, their ability to fold the polypeptide correctly, their fermentation or culture requirements, and the ease of purification of the products coded for by the nucleotide sequence.

The recombinant vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector is one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

The vector is preferably an expression vector in which the nucleotide sequence encoding the polypeptide of the invention is operably linked to additional segments required for transcription of the nucleotide sequence. The vector is typically derived from plasmid or viral DNA. A number of suitable expression vectors for expression in the host cells mentioned herein are commercially available or described in the literature. Useful expression vectors for eukaryotic hosts include, for example, vectors comprising expression control sequences from SV40, bovine papilloma virus, adenovirus and cytomegalovirus. Specific vectors are, e.g., pCDNA3.1(+)\Hyg (Invitrogen, Carlsbad, CA, USA) and pCI-neo (Stratagene, La Jolla, CA,

USA). Useful expression vectors for yeast cells include the 2μ plasmid and derivatives thereof, the POT1 vector (US 4,931,373), the pJSO37 vector described in Okkels, Ann. New York Acad. Sci. 782, 202-207, 1996, and pPICZ A, B or C (Invitrogen). Useful vectors for insect cells include pVL941, pBG311 (Cate *et al.*, "Isolation of the Bovine and Human Genes for Mullerian Inhibiting Substance And Expression of the Human Gene In Animal Cells", Cell, 45, pp. 685-98 (1986), pBluebac 4.5 and pMelbac (both available from Invitrogen). Useful expression vectors for bacterial hosts include known bacterial plasmids, such as plasmids from *E. coli*, including pBR322, pET3a and pET12a (both from Novagen Inc., WI, USA), wider host range plasmids, such as RP4, phage DNAs, e.g. the numerous derivatives of phage lambda, e.g. NM989, and other DNA phages, such as M13 and filamentous single stranded DNA phages.

Other vectors for use in this invention include those that allow the nucleotide sequence encoding the polypeptide to be amplified in copy number. Such amplifiable vectors are well known in the art. They include, for example, vectors able to be amplified by DHFR amplification (see, e.g., Kaufman, U.S. Pat. No. 4,470,461, Kaufman and Sharp, "Construction Of A Modular Dihydrafolate Reductase cDNA Gene: Analysis Of Signals Utilized For Efficient Expression", Mol. Cell. Biol., 2, pp. 1304-19 (1982)) and glutamine synthetase ("GS") amplification (see, e.g., US 5,122,464 and EP 338,841).

The recombinant vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. An example of such a sequence (when the host cell is a mammalian cell) is the SV40 origin of replication. When the host cell is a yeast cell, suitable sequences enabling the vector to replicate are the yeast plasmid 2μ replication genes REP 1-3 and origin of replication.

The vector may also comprise a selectable marker, e.g. a gene whose product complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or the *Schizosaccharomyces pombe* TPI gene (described by P.R. Russell, Gene 40, 1985, pp. 125-130), or one which confers resistance to a drug, e.g. ampicillin, kanamycin, tetracyclin, chloramphenicol, neomycin, hygromycin or methotrexate. For *Saccharomyces cerevisiae*, selectable markers include *ura3 and leu2*. For filamentous fungi, selectable markers include *amdS*, *pyrG*, *arcB*, *niaD* and *sC*.

The term "control sequences" is defined herein to include all components which are necessary or advantageous for the expression of the polypeptide of the invention. Each control sequence may be native or foreign to the nucleic acid sequence encoding the polypeptide. Such control sequences include, but are not limited to, a leader sequence, polyadenylation se-

quence, propeptide sequence, promoter, enhancer or upstream activating sequence, signal peptide sequence, and transcription terminator. At a minimum, the control sequences include a promoter.

A wide variety of expression control sequences may be used in the present invention. Such useful expression control sequences include the expression control sequences associated with structural genes of the foregoing expression vectors as well as any sequence known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof.

Examples of suitable control sequences for directing transcription in mammalian cells include the early and late promoters of SV40 and adenovirus, e.g. the adenovirus 2 major late promoter, the MT-1 (metallothionein gene) promoter, the human cytomegalovirus immediate-early gene promoter (CMV), the human elongation factor 1α (EF-1α) promoter, the *Drosophila* minimal heat shock protein 70 promoter, the Rous Sarcoma Virus (RSV) promoter, the human ubiquitin C (UbC) promoter, the human growth hormone terminator, SV40 or adenovirus Elb region polyadenylation signals and the Kozak consensus sequence (Kozak, M. *J Mol Biol* 1987 Aug 20;196(4):947-50).

In order to improve expression in mammalian cells a synthetic intron may be inserted in the 5' untranslated region of the nucleotide sequence encoding the polypeptide. An example of a synthetic intron is the synthetic intron from the plasmid pCI-Neo (available from Promega Corporation, WI, USA).

Examples of suitable control sequences for directing transcription in insect cells include the polyhedrin promoter, the P10 promoter, the *Autographa californica* polyhedrosis virus basic protein promoter, the baculovirus immediate early gene 1 promoter, the baculovirus 39K delayed-early gene promoter, and the SV40 polyadenylation sequence. Examples of suitable control sequences for use in yeast host cells include the promoters of the yeast α-mating system, the yeast triose phosphate isomerase (TPI) promoter, promoters from yeast glycolytic genes or alcohol dehydrogenase genes, the ADH2-4c promoter, and the inducible GAL promoter. Examples of suitable control sequences for use in filamentous fungal host cells include the ADH3 promoter and terminator, a promoter derived from the genes encoding *Aspergillus oryzae* TAKA amylase triose phosphate isomerase or alkaline protease, an *A. niger* α-amylase, *A. niger* or *A. nidulans* glucoamylase, *A. nidulans* acetamidase, *Rhizomucor miehei* aspartic proteinase or lipase, the TPI1 terminator and the ADH3 terminator. Examples of suitable con-

trol sequences for use in bacterial host cells include promoters of the *lac* system, the *trp* system, the *TAC* or *TRC* system, and the major promoter regions of phage lambda.

The nucleotide sequence of the invention encoding a polypeptide exhibiting EPO activity, whether prepared by site-directed mutagenesis, synthesis, PCR or other methods, may optionally also include a nucleotide sequence that encodes a signal peptide. The signal peptide is present when the polypeptide is to be secreted from the cells in which it is expressed. Such signal peptide, if present, should be one recognized by the cell chosen for expression of the polypeptide. The signal peptide may be homologous (e.g. be that normally associated with hEPO) or heterologous (i.e. originating from another source than hEPO) to the polypeptide or may be homologous or heterologous to the host cell, i.e. be a signal peptide normally expressed from the host cell or one which is not normally expressed from the host cell. Accordingly, the signal peptide may be prokaryotic, e.g. derived from a bacterium such as *E. coli,* or eukaryotic, e.g. derived from a mammalian, or insect or yeast cell.

The presence or absence of a signal peptide will, e.g., depend on the expression host cell used for the production of the polypeptide to be expressed (whether it is an intracellular or extracellular polypeptide) and whether it is desirable to obtain secretion. For use in filamentous fungi, the signal peptide may conveniently be derived from a gene encoding an *Aspergillus* sp. amylase or glucoamylase, a gene encoding a *Rhizomucor miehei* lipase or protease or a *Humicola lanuginosa* lipase. The signal peptide is preferably derived from a gene encoding *A. oryzae* TAKA amylase, *A. niger* neutral α-amylase, *A. niger* acid-stable amylase, or *A. niger* glucoamylase. For use in insect cells, the signal peptide may conveniently be derived from an insect gene (cf. WO 90/05783), such as the *Lepidopteran manduca sexta* adipokinetic hormone precursor, (cf. US 5,023,328), the honeybee melittin (Invitrogen), ecdysteroid UDPglucosyltransferase (egt) (Murphy *et al.*, Protein Expression and Purification 4, 349-357 (1993) or human pancreatic lipase (hpl) (Methods in Enzymology 284, pp. 262-272, 1997). A preferred signal peptide for use in mammalian cells is that of hEPO or the murine Ig kappa light chain signal peptide (Coloma, M (1992) J. Imm. Methods 152:89-104). For use in yeast cells suitable signal peptides have been found to be the α-factor signal peptide from *S. cereviciae* (cf. US 4,870,008), a modified carboxypeptidase signal peptide (cf. L.A. Valls *et al.*, Cell 48, 1987, pp. 887-897), the yeast BAR1 signal peptide (cf. WO 87/02670), the yeast aspartic protease 3 (YAP3) signal peptide (cf. M. Egel-Mitani *et al.*, Yeast 6, 1990, pp. 127-137), and the synthetic leader sequence TA57 (WO98/32867). For use in *E. coli* cells a suitable signal peptide has been found to be the signal peptide *ompA*.

Any suitable host may be used to produce the polypeptide or polypeptide part of the conjugate of the invention, including bacteria, fungi (including yeasts), plant, insect, mammal, or other appropriate animal cells or cell lines, as well as transgenic animals or plants. Examples of bacterial host cells include gram-positive bacteria such as strains of *Bacillus,* e.g. *B. brevis* or *B. subtilis, Pseudomonas* or *Streptomyces*, or gram-negative bacteria, such as strains of *E. coli.* The introduction of a vector into a bacterial host cell may, for instance, be effected by protoplast transformation (see, *e.g.*, Chang and Cohen, 1979, *Molecular General Genetics* 168: 111-115), using competent cells (see, *e.g.*, Young and Spizizin, 1961, *Journal of Bacteriology* 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, *Journal of Molecular Biology* 56: 209-221), electroporation (see, *e.g.*, Shigekawa and Dower, 1988, *Biotechniques* 6: 742-751), or conjugation (see, *e.g.*, Koehler and Thorne, 1987, *Journal of Bacteriology* 169: 5771-5278).

Examples of suitable filamentous fungal host cells include strains of *Aspergillus,* e.g. *A. oryzae, A. niger,* or *A. nidulans, Fusarium* or *Trichoderma.* Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known *per se*. Suitable procedures for transformation of *Aspergillus* host cells are described in EP 238 023 and US 5,679,543. Suitable methods for transforming *Fusarium* species are described by Malardier *et al.*, 1989, *Gene* 78: 147-156 and WO 96/00787. Examples of suitable yeast host cells include strains of *Saccharomyces,* e.g. *S. cerevisiae*, *Schizosaccharomyces, Klyveromyces, Pichia,* such as *P. pastoris* or *P. methanolica, Hansenula,* such as *H. polymorpha* or *Yarrowia.* Yeast may be transformed using the procedures described by Becker and Guarente, *In* Abelson, J.N. and Simon, M.I., editors, *Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology*, Volume 194, pp 182-187, Academic Press, Inc., New York; Ito *et al.*, 1983, *Journal of Bacteriology* 153: 163; Hinnen *et al.*, 1978, *Proceedings of the National Academy of Sciences USA* 75: 1920: and as disclosed by Clontech Laboratories, Inc, Palo Alto, CA, USA (in the product protocol for the Yeastmaker™ Yeast Transformation System Kit).

Examples of suitable insect host cells include a *Lepidoptora* cell line, such as *Spodoptera frugiperda (*Sf9 or Sf21) or *Trichoplusioa ni* cells (High Five) (US 5,077,214). Transformation of insect cells and production of heterologous polypeptides therein may be performed as described by Invitrogen.

Examples of suitable mammalian host cells include Chinese hamster ovary (CHO) cell lines, (e.g. CHO-K1; ATCC CCL-61), Green Monkey cell lines (COS) (e.g. COS 1 (ATCC CRL-1650), COS 7 (ATCC CRL-1651)); mouse cells (e.g. NS/O), Baby Hamster Kid-

ney (BHK) cell lines (e.g. ATCC CRL-1632 or ATCC CCL-10), and human cells (e.g. HEK 293 (ATCC CRL-1573)), as well as plant cells in tissue culture. Additional suitable cell lines are known in the art and available from public depositories such as the American Type Culture Collection, Rockville, Maryland. Methods for introducing exogeneous DNA into mammalian host cells include calcium phosphate-mediated transfection, electroporation, DEAE-dextran mediated transfection, liposome-mediated transfection, viral vectors and the transfection method described by Life Technologies Ltd, Paisley, UK using Lipofectamin 2000. These methods are well known in the art and e.g. described by Ausbel *et al.* (eds.), 1996, Current Protocols in Molecular Biology, John Wiley & Sons, New York, USA. The cultivation of mammalian cells is conducted according to established methods, e.g. as disclosed in (Animal Cell Biotechnology, Methods and Protocols, Edited by Nigel Jenkins, 1999, Human Press Inc, Totowa, New Jersey, USA and Harrison MA and Rae IF, General Techniques of Cell Culture, Cambridge University Press 1997).

In the production methods of the present invention, the cells are cultivated in a nutrient medium suitable for production of the polypeptide using methods known in the art. For example, the cell may be cultivated by shake flask cultivation, small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermenters performed in a suitable medium and under conditions allowing the polypeptide to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are available from commercial suppliers or may be prepared according to published compositions (e.g., in catalogues of the American Type Culture Collection). If the polypeptide is secreted into the nutrient medium, the polypeptide can be recovered directly from the medium. If the polypeptide is not secreted, it can be recovered from cell lysates.

The resulting polypeptide may be recovered by methods known in the art. For example, the polypeptide may be recovered from the nutrient medium by conventional procedures including, but not limited to, centrifugation, filtration, extraction, spray drying, evaporation, or precipitation.

The polypeptides may be purified by a variety of procedures known in the art including, but not limited to, chromatography (e.g., ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (e.g., preparative isoelectric focusing), differential solubility (e.g., ammonium sulfate precipitation), SDS-PAGE, or extraction (see, e.g., ., *Protein Purification* (2^{nd} Edition), Janson and Ryden, editors, Wiley, New

York, 1998). Specific methods for purifying polypeptides exhibiting EPO activity are described by D. Metcalf and N. A. Nicola in *The hemopoietic colony-stimulating factors*, p. 50-51, Cambridge University Press (1995), by C. S. Bae *et al.*, Appl. Microbiol. Biotechnol, 52:338-344 (1999) and in US 4,810,643.

Pharmaceutical composition of the invention and its use

In a further aspect, the present invention comprises a composition comprising a polypeptide conjugate as described herein and at least one pharmaceutically acceptable carrier or excipient.

The polypeptide conjugate or the pharmaceutical composition according to the invention may be used for the manufacture of a medicament for treatment of diseases or conditions characterized by a low red blood cell level or defective production of red blood cells. Examples of such diseases or conditions include anemia associated with chronic renal failure, anemia related to therapy with e.g. AZT (zidovudine) in HIV-infected patients, anemia in patients with non-myeloid malignancies receiving chemotherapy, other cancer-related anemia, anemia associated with chronic inflammatory diseases, e.g. rheumatoid arthritis, anemia associated with other chronic diseases, senile anemia, and anemia in patients undergoing surgery to reduce the need of allogenic blood transfusions.

In another aspect the polypeptide conjugate or the pharmaceutical composition according to the invention is used in a method of treating a mammal having a disease or condition characterized by a low red blood cell level or defective production of red blood cells, comprising administering to a mammal in need thereof such a polypeptide conjugate or pharmaceutical composition.

In a further aspect, the polypeptide conjugate or pharmaceutical composition of the invention may be administered for prevention or treatment of a central nervous system (CNS) related condition. Although EPO has primarily been used in therapy and investigated in connection with its well-known erythropoietic effect, recent studies have revealed new information about a CNS-related role of EPO and the EPO receptor. It has thus been reported that the EPO receptor is expressed on brain capillaries, allowing circulating systemically administered EPO to cross the blood-brain barrier and enter the brain via specific receptor-mediated translocation, and that peripherally injected rhEPO is able to protect rodent brain tissue from ischemia/hypoxia, trauma, immune-mediated inflammation and excessive neuronal excitation (Brines et al., *PNAS USA* 97(19): 10526-31, Sep. 2000; Cerami et al., *Semin Oncol* 28 (2,

Suppl 8): 66-70, Apr. 2001). Novel therapeutic applications for recombinant human EPO in the treatment of stroke, head trauma and epilepsy have been proposed based on these results (Cerami, *Semin Hematol* 38 (3, Suppl 7): 33-39, Jul. 2001), and subsequent reports on animal studies and initial studies on human patients support the therapeutic use of EPO for such CNS-related indications. For example, Ehrenreich et al. (*Mol. Med.*; 8(8):495-505, Aug. 2002) describe the results of initial studies of the safety and efficacy of rhEPO for treatment of acute stroke. After trials that included a safety study on 13 patients who received rhEPO intravenously once daily for the first 3 days after stroke as well as a double-blind randomized proof-of-concept trial with 40 stroke patients, it was found that intravenous high-dose rhEPO was well tolerated in acute ischemic stroke and was associated with an improvement in clinical outcome after one month.

Compositions and methods for modulating excitable tissue function in mammals, e.g. neuronal or brain tissue function, are disclosed in WO 00/61164. The polypeptide conjugates of the invention are therefore contemplated for use as general neuroprotective agents, in particular for the prevention or treatment of a variety of different types of brain damage. Non-limiting examples of conditions where administration of the polypeptide conjugates of the invention may be indicated include head trauma, stroke, epilepsy, ischemia, hypoxia, immune-mediated inflammation and excessive neuronal excitation.

Since transport of EPO across the blood-brain barrier is believed to be dependent upon binding to the EPO receptor, it is important for this purpose that a sufficient binding affinity between an EPO conjugate of the invention and the EPO receptor is ensured, in particular since PEGylation or other polymer attachment to an EPO variant is likely to reduce the receptor binding affinity compared to the non-conjugated polypeptide. An increased receptor binding affinity may advantageously be obtained by use of one of the shuffling methods referred to above in combination with a suitable assay to identify recombined EPO variants having a desired binding affinity, thereby making it possible to produce EPO variants which, even when conjugated to e.g. PEG, have a sufficiently high receptor binding affinity to be able to cross the blood-brain barrier.

One embodiment of the invention thus relates to a method for producing an EPO variant with an increased receptor binding affinity compared to hEPO, so that the variant, when conjugated, is able to cross the blood-brain barrier, the method comprising

(a) providing a plurality of parental polynucleotides, each of said polynucleotides encoding a polypeptide having erythropoietic activity or binding affinity to the human EPO receptor, or fragments of such a polynucleotide; and

(b) recombining said plurality of parental polynucleotides or fragments to produce a plurality of recombinant polynucleotides, and

(c) screening or selecting the plurality of recombinant polynucleotides to identify at least one recombinant polynucleotide encoding a recombinant polypeptide having a desired binding affinity.

Although the above method is described in the context of producing an EPO variant with an increased binding affinity to the hEPO receptor, in particular for the purpose of improving blood-brain transport of variants of the invention and polypeptide conjugates thereof, the same general method can also be used for the purpose of identifying EPO variants having an increased or otherwise altered erythropoietic effect. In the latter case, the assay method used to screen or select variants of interest may be directed to erythropoietic activity rather than to receptor binding affinity.

The method may further involve the following additional steps:

(c) recombining at least one distinct or improved recombinant polynucleotide with a further polynucleotide encoding a polypeptide with hEPO receptor binding affinity, which further polynucleotide is identical to or different from one or more of said plurality of parental polynucleotides, to produce a library of recombinant polynucleotides;

(d) screening said library to identify at least one further distinct or improved recombinant polynucleotide encoding a polypeptide that binds the hEPO receptor and that exhibits a further improvement or distinct property compared to a polypeptide encoded by said plurality of parental polynucleotides; and, optionally,

(e) repeating (c) and (d) until said resulting further distinct or improved recombinant polynucleotide shows an additionally distinct or improved property.

The distinct or improved property can e.g. be one or both of increased binding affinity to the hEPO receptor and increased erythropoietic activity, for example erythropoietic activity as determined in a mammalian erythroid cell-based proliferation assay. An example of a suitable proliferation assay is the human TF-1 cell-based assay described below.

Once one or more EPO variants with improved receptor-binding affinity have been identified, whether by shuffling as outlined above, optionally using any of the various shuffling techniques discussed under the heading "Methods for preparing a polypeptide of the

invention or the polypeptide part of the conjugate of the invention", or by other mutagenesis methods such as site-directed or random mutagenesis, these can be subjected to conjugation with PEG or another non-polypeptide moiety and the performance of the resulting conjugate can be analyzed as appropriate.

Certain *in vivo* assay methods for determining whether a polypeptide conjugate of the invention is able to cross the blood-brain barrier and exert a desired neuroprotective effect are disclosed in WO 00/61164; see e.g. Examples 3, 4 and 5. Further, an *in vitro* model for evaluating drug transport across the blood-brain barrier based on a co-culture of brain capillary endothelial cells on one side of a filter and astrocytes on the other side is described by Cecchelli et al. in *Advanced Drug Discovery Reviews* 36: 165-178 (1999).

The polypeptides and conjugates of the invention will be administered to patients in a "therapeutically effective" dose, i.e. a dose that is sufficient to produced the desired effects in relation to the condition for which it is administered. The exact dose will depend on the disorder to be treated, and will be ascertainable by one skilled in the art using known techniques. The polypeptides or conjugates of the invention may e.g. be administered at a dose similar to that employed in therapy with rhEPO such as Epogen® or with Aranesp™. A suitable dose of a conjugate of the invention is contemplated to be in the range of about 5-300 microgram/kg body weight (based on the weight of the protein part of the conjugate), e.g. 10-200 microgram/kg, such as 25-100 microgram/kg. It will be apparent to those of skill in the art that an effective amount of a polypeptide conjugate or composition of the invention depends, *inter alia*, upon the disease, the dose, the administration schedule, whether the polypeptide conjugate or composition is administered alone or in conjunction with other therapeutic agents, the serum half-life of the compositions, the general health of the patient, and the frequency of administration. Preferably, the polypeptide conjugate or composition of the invention is administered in an effective dose, in particular a dose which is sufficient to normalize the number of red blood cells or otherwise raise the level of red blood cells to a level that is appropriate for the patient in question. In certain cases, higher doses than those exemplified above may be useful, in particular for systemic administration as a neuroprotective agent. This may especially be the case when a polypeptide conjugate of the invention is administered for the prevention or treatment of brain injury caused e.g. by head trauma, where it may be desirable to administer a large bolus in order to rapidly make the polypeptide available to the brain. Since rhEPO is known to be well-tolerated even in quite large doses, i.e. doses substantially larger than those

normally used to provide an erythropoietic effect, this is not contemplated to be a problem for such indications.

The polypeptide conjugate of the invention is preferably administered in a composition including one or more pharmaceutically acceptable carriers or excipients. The polypeptide conjugate can be formulated into pharmaceutical compositions in a manner known *per se* in the art to result in a polypeptide pharmaceutical that is sufficiently storage-stable and is suitable for administration to humans or animals. The pharmaceutical composition may be formulated in a variety of forms, including as a liquid or gel, or lyophilized, or any other suitable form. The preferred form will depend upon the particular indication being treated and will be apparent to one of skill in the art.

Drug form

The polypeptide conjugate of the invention can be used "as is" and/or in a salt form thereof. Suitable salts include, but are not limited to, salts with alkali metals or alkaline earth metals, such as sodium, potassium, calcium and magnesium, as well as e.g. zinc salts. These salts or complexes may by present as a crystalline and/or amorphous structure.

Excipients

"Pharmaceutically acceptable" means a carrier or excipient that at the dosages and concentrations employed does not cause any untoward effects in the patients to whom it is administered. Such pharmaceutically acceptable carriers and excipients are well known in the art (see Remington's Pharmaceutical Sciences, 19th edition, A. R. Gennaro, Ed., Mack Publishing Company [1995]; Pharmaceutical Formulation Development of Peptides and Proteins, S. Frokjaer and L. Hovgaard, Eds., Taylor & Francis [2000] ; and Handbook of Pharmaceutical Excipients, 3rd edition, A. Kibbe, Ed., Pharmaceutical Press [2000]).

Mix of drugs

The pharmaceutical composition of the invention may be administered alone or in conjunction with other therapeutic agents. These agents may be incorporated as part of the same pharmaceutical composition or may be administered separately from the polypeptide conjugate of the invention, either concurrently or in accordance with another treatment schedule. In addition, the polypeptide conjugate or pharmaceutical composition of the invention may be used as an adjuvant to other therapies.

Patients

A "patient" for the purposes of the present invention includes both humans and other mammals. Thus the methods are applicable to both human therapy and veterinary applications.

Administration route

The administration of the formulations of the present invention can be performed in a variety of ways, including, but not limited to, orally, subcutaneously, intravenously, intracerebrally, intranasally, transdermally, intraperitoneally, intramuscularly, intrapulmonary, vaginally, rectally, intraocularly, or in any other acceptable manner. The formulations can be administered continuously by infusion, although bolus injection is acceptable, using techniques well known in the art.

Parenterals

An example of a pharmaceutical composition is a solution designed for parenteral administration. Although in many cases pharmaceutical solution formulations are provided in liquid form, appropriate for immediate use, such parenteral formulations may also be provided in frozen or in lyophilized form. In the former case, the composition must be thawed prior to use. The latter form is often used to enhance the stability of the active compound contained in the composition under a wider variety of storage conditions, as it is recognized by those skilled in the art that lyophilized preparations are generally more stable than their liquid counterparts. Such lyophilized preparations are reconstituted prior to use by the addition of one or more suitable pharmaceutically acceptable diluents such as sterile water for injection or sterile physiological saline solution.

In case of parenterals, they are prepared for storage as lyophilized formulations or aqueous solutions by mixing, as appropriate, the polypeptide having the desired degree of purity with one or more pharmaceutically acceptable carriers, excipients or stabilizers typically employed in the art (all of which are termed "excipients"), for example buffering agents, stabilizing agents, preservatives, isotonifiers, non-ionic detergents, antioxidants and/or other miscellaneous additives.

Buffering agents help to maintain the pH in the range which approximates physiological conditions. They are typically present at a concentration ranging from about 2

mM to about 50 mM Suitable buffering agents for use with the present invention include both organic and inorganic acids and salts thereof such as citrate buffers (e.g., monosodium citrate-disodium citrate mixture, citric acid-trisodium citrate mixture, citric acid-monosodium citrate mixture, etc.), succinate buffers (e.g., succinic acid-monosodium succinate mixture, succinic acid-sodium hydroxide mixture, succinic acid-disodium succinate mixture, etc.), tartrate buffers (e.g., tartaric acid-sodium tartrate mixture, tartaric acid-potassium tartrate mixture, tartaric acid-sodium hydroxide mixture, etc.), fumarate buffers (e.g., fumaric acid-monosodium fumarate mixture, fumaric acid-disodium fumarate mixture, monosodium fumarate-disodium fumarate mixture, etc.), gluconate buffers (e.g., gluconic acid-sodium glyconate mixture, gluconic acid-sodium hydroxide mixture, gluconic acid-potassium glyuconate mixture, etc.), oxalate buffer (e.g., oxalic acid-sodium oxalate mixture, oxalic acid-sodium hydroxide mixture, oxalic acid-potassium oxalate mixture, etc.), lactate buffers (e.g., lactic acid-sodium lactate mixture, lactic acid-sodium hydroxide mixture, lactic acid-potassium lactate mixture, etc.) and acetate buffers (e.g., acetic acid-sodium acetate mixture, acetic acid-sodium hydroxide mixture, etc.). Additional possibilities are phosphate buffers, histidine buffers and trimethylamine salts such as Tris.

Preservatives are added to retard microbial growth, and are typically added in amounts of about 0.2%-1% (w/v). Suitable preservatives for use with the present invention include phenol, benzyl alcohol, meta-cresol, methyl paraben, propyl paraben, octadecyldimethylbenzyl ammonium chloride, benzalkonium halides (e.g. benzalkonium chloride, bromide or iodide), hexamethonium chloride, alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol and 3-pentanol.

Isotonicifiers are added to ensure isotonicity of liquid compositions and include polyhydric sugar alcohols, preferably trihydric or higher sugar alcohols, such as glycerin, erythritol, arabitol, xylitol, sorbitol and mannitol. Polyhydric alcohols can be present in an amount between 0.1% and 25% by weight, typically 1% to 5%, taking into account the relative amounts of the other ingredients.

Stabilizers refer to a broad category of excipients which can range in function from a bulking agent to an additive which solubilizes the therapeutic agent or helps to prevent denaturation or adherence to the container wall. Typical stabilizers can be polyhydric sugar alcohols (enumerated above); amino acids such as arginine, lysine, glycine, glutamine, asparagine, histidine, alanine, omithine, L-leucine, 2-phenylalanine, glutamic acid, threonine, etc., organic sugars or sugar alcohols, such as lactose, trehalose, stachyose, mannitol, sorbitol, xyli-

tol, ribitol, myoinisitol, galactitol, glycerol and the like, including cyclitols such as inositol; polyethylene glycol; amino acid polymers; sulfur-containing reducing agents, such as urea, glutathione, thioctic acid, sodium thioglycolate, thioglycerol, α-monothioglycerol and sodium thiosulfate; low molecular weight polypeptides (i.e. <10 residues); proteins such as human serum albumin, bovine serum albumin, gelatin or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; monosaccharides such as xylose, mannose, fructose and glucose; disaccharides such as lactose, maltose and sucrose; trisaccharides such as raffinose, and polysaccharides such as dextran. Stabilizers are typically present in the range of from 0.1 to 10,000 parts by weight based on the active protein weight.

Non-ionic surfactants or detergents (also known as "wetting agents") may be present to help solubilize the therapeutic agent as well as to protect the therapeutic polypeptide against agitation-induced aggregation, which also permits the formulation to be exposed to shear surface stress without causing denaturation of the polypeptide. Suitable non-ionic surfactants include polysorbates (20, 80, etc.), polyoxamers (184, 188 etc.), Pluronic® polyols, polyoxyethylene sorbitan monoethers (Tween®-20, Tween®-80, etc.).

Additional miscellaneous excipients include bulking agents or fillers (e.g. starch), chelating agents (e.g. EDTA), antioxidants (e.g., ascorbic acid, methionine, vitamin E) and cosolvents.

The active ingredient may also be entrapped in microcapsules prepared, for example, by coascervation techniques or by interfacial polymerization, for example hydroxymethylcellulose, gelatin or poly-(methylmethacylate) microcapsules, in colloidal drug delivery systems (for example liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, *supra*.

Parenteral formulations to be used for *in vivo* administration must be sterile. This is readily accomplished, for example, by filtration through sterile filtration membranes.

Sustained release preparations

Suitable examples of sustained-release preparations include semi-permeable matrices of solid hydrophobic polymers containing the polypeptide or conjugate, the matrices having a suitable form such as a film or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels, poly(vinylalcohol)), polylactides, copolymers of L-glutamic acid

and ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers, and poly-D-(-)-3-hydroxybutyric acid.

Pulmonary delivery

The polypeptide or conjugate of the invention, alone or in combination with other suitable components, can also be made into aerosol formulations (e.g., they can be "nebulized") to be administered via inhalation. Aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like. Formulations suitable for use with a nebulizer, either jet or ultrasonic, will typically comprise the polypeptide or conjugate dissolved in water at a concentration of, e.g., about 0.01 to 25 mg of conjugate per ml of solution. The aerosol formulation may also include, e.g., one or more of a buffer, a simple sugar (e.g. for protein stabilization and regulation of osmotic pressure), a sugar alcohol and a surfactant.

Formulations for powder inhalers will comprise a finely divided dry powder containing the polypeptide or conjugate and may also include a bulking agent such as lactose, sorbitol, sucrose or mannitol in an amount which facilitates dispersal of the powder from the device, e.g., 50% to 90% by weight of the formulation.

Mechanical devices designed for pulmonary delivery of therapeutic products include, but are not limited to, nebulizers, metered dose inhalers, and powder inhalers, all of which are familiar to those of skill in the art.

All references cited herein are hereby incorporated by reference in their entirety for all purposes. The invention is further described in the non-limiting methods and examples below.

EXAMPLES

Structures:

No experimental three-dimensional structures of wild type human erythropoietin have been reported. Attempts to perform structural studies on non-glycosylated bacterially expressed human sequence erythropoietin have been unsuccessful due to limited solubility as well as the low stability of the protein lacking its N-glycosylation.

Experimental three-dimensional structures of variants of human erythropoietin determined by X-ray crystallography have been reported by Syed et al. (*Nature* 395:511-516 (1998)), which reports on the structure of the N24K, N38K, N83K variant of hEPO as well as the structure of the N24K, N38K, N83K, P121N, P122S variant of hEPO, both of these structures being complexed with two copies of the extracellular domains of the EPO receptor. In both structures an additional methionine followed by a lysine was added to the N-terminal of the EPO molecule. None of these structures report on the conformation of the two N-terminally added residues (Met-Lys). The first structure also lacks information about the structure of residues Ala1, Ala124-Leu130 and Thr163-Arg166. The receptor was also mutated to remove one N-glycosylation site and to optimize expression.

The experimental three-dimensional structure of variants of hEPO determined by NMR spectroscopy have been reported by Cheetham et al. (*Nat. Struct. Biol.* 5:861-866 (1998), which reports on the structure in solution of the N24K, N38K, N83K variant with Met-Lys added to the N-terminal. In this case as well, no report on the conformation of the two N-terminally added residues (Met-Lys) exists.

METHODS

Accessible Surface Area (ASA)

The computer program Access (B. Lee and F.M. Richards, *J. Mol. Biol.* 55: 379-400 (1971), Version 2, © 1983 Yale University) is used to compute the accessible surface area (ASA) of the individual atoms in the structure. This method typically uses a probe-size of 1.4Å and defines the Accessible Surface Area (ASA) as the area formed by the centre of the probe. Prior to this calculation all water molecules and all hydrogen atoms should be removed from the coordinate set, as should other atoms not directly related to the protein.

Fractional ASA of side chain

The fractional ASA of the side chain atoms is computed by division of the sum of the ASA of the atoms in the side chain with a value representing the ASA of the side chain atoms of that residue type in an extended ALA-x-ALA tripeptide. See Hubbard et al. (1991) *J. Mol. Biol.* 220, 507-530. For this example the CA atom is regarded as a part of the side chain of glycine residues but not for the remaining residues. The values in the following table are used as standard 100% ASA for the side chain:

Ala	69.23	$Å^2$	Leu	140.76	$Å^2$
Arg	200.35	$Å^2$	Lys	162.50	$Å^2$
Asn	106.25	$Å^2$	Met	156.08	$Å^2$
Asp	102.06	$Å^2$	Phe	163.90	$Å^2$
Cys	96.69	$Å^2$	Pro	119.65	$Å^2$
Gln	140.58	$Å^2$	Ser	78.16	$Å^2$
Glu	134.61	$Å^2$	Thr	101.67	$Å^2$
Gly	32.28	$Å^2$	Trp	210.89	$Å^2$
His	147.00	$Å^2$	Tyr	176.61	$Å^2$
Ile	137.91	$Å^2$	Val	114.14	$Å^2$

Residues not detected in the structure are generally defined as having 100% exposure as they are thought to reside in flexible regions.

Determining distances between atoms:

The distance between atoms is most easily determined using molecular graphics software, e.g. InsightII® v. 98.0, MSI Inc.

Determination of receptor-binding site:

The receptor-binding site is defined as comprising all residues having their accessible surface area changed upon receptor binding. This is determined by at least two ASA calculations; one on the isolated ligand(s) in the ligand/receptor complex and one on the complete ligand/receptor complex.

Method for determining *in vivo* erythropoietic activity of conjugated and non-conjugated hEPO and variants thereof

Nucleotide sequences may be synthesized using standard procedures known in the art and then subcloned into a suitable host cell, e.g. an *E. coli* vector containing an amino terminal mammalian secretion signal and a carboxy terminal epitope tag (E-tag) (Amersham-Pharmacia). Other epitope tags (e.g., histidine (His) tag) known to those skilled in the art can also be used for purification and other purposes. Particular individual transformant colonies may then be selected, transferred into microtiter plates, and re-grown, and plasmid DNA of the selected clones prepared.

These plasmid DNA extracts may then be used to transfect e.g. COS cells. COS cells are selected to facilitate subsequent expression and purification of the encoded polypeptides, although other mammalian cell lines may also be suitable in this context. Supernatant containing expressed EPO homologue polypeptides is collected from the transfected COS cell lines and used in a proliferation screening assay in a human TF-1 cell line. The TF-1 cell line (ATCC No. CRL-2003) is an erythroleukemia-derived growth factor dependent cell line. See, e.g., Kitamura et al., *J. Cell Physiol.* 140:323-34 (1989), and Kitamura et al., *Blood* 73(2):375-380 (1989). Proliferation of TF-1 cells has been shown to be dependent on erythropoietin (EPO).

In the proliferation screening assay, library members are screened for an ability to induce proliferation of a population of human TF-1 cells. Briefly, in this assay, the TF-1 cells are first starved for hematopoietic growth factor and then exposed to EPO polypeptides or conjugates of the invention and tritiated thymidine (^{3}H-thymidine). Specifically, TF-1 cells are first rinsed 1x with RPMI medium 1640 (GibcoBRL® Life Technologies), 1% fetal bovine serum (FBS) without growth factor and incubated overnight. Cells are spun and re-suspended in original volume with RPMI, 1% FBS without growth factor. The cells are spun again, rinsed 2x with RPMI, 1% FBS without growth factor, and re-suspended in a small volume RPMI, 1% FBS without growth factor. The cells are counted and diluted to 1 x 10^4 cells/100 microliters (µl) in RPMI, 1% FBS and delivered to wells of 96-well microplates. To each well containing cell cultures, 100 µl of an EPO polypeptide or conjugate of the invention is added (dilutions generally at 2x concentration). Cells are incubated for 48 hours. 1 microCi ^{3}H-thymidine is added to each well in a 10-µl volume (dilutions made in phosphate-buffered saline). After a 24-hour incubation, the cells are harvested onto an absorbent glass paper and counted for radioactivity associated with the cells using a scintillation counter. Proliferative activity is measured by determining the amount of ^{3}H-thymidine incorporated in TF-1 cell cultures according to the method described in, e.g., Kitamura et al., *J Cell Physiol.* 140:323-34 (1989), *supra*. Recombinant human erythropoietin (rhEPO) may be utilized as a control for comparison with the proliferative inducing activities of the polypeptides and conjugates of the invention. The amount of ^{3}H-thymidine incorporated in human TF-1 cells (measured as counts per minute ("cpm") x 10^{-4}) vs. protein (polypeptide) concentration (nanograms/milliliter (ng/ml)) of each clone and rhEPO may be measured, and growth responses of TF-1 cells to each polypeptide variant or conjugate and rhEPO may be assessed. Protein concentration may be determined by standard immunoprecipitation procedures and Western blotting procedures (see, e.g., procedures out-

lined in Molecular Biomethods Handbook (Humana Press, 1998, R. Rapley and J. Walker eds.) and Upstate Biotechnology 2000 Catalog) and quantitation by standard densitometry procedures using e.g. an AlphaInnotech Corporation AlphaImager. Specific activity of each polypeptide or conjugate may be determined as the concentration (ng/ml) of polypeptide required to achieve half maximal thymidine incorporation into TF-1 cells (i.e., EC_{50}).

EXAMPLE 1

The X-ray structure of the N24K, N38K, N83K, P121N, P122S variant of hEPO complexed with two copies of the extracellular domains of the EPO receptor (Syed et al., *Nature* 395:511-516 1998) was used for this example. This structure was selected as it was the structure solved to the highest precision as well as the most complete structure. This structure is available from the Protein Data Bank (Berman et al., "The Protein Data Bank", *Nucleic Acids Res.*, 28(1): 235-242 (2000); www.rcsb.org/pdb/) under accession code 1EER.

The structure of the erythropoietin part (molecule A) containing residues Ala1-Arg166 was extracted and an accessible surface area calculation was performed. Similarly, an accessible surface area calculation was performed on the erythropoietin part (molecule A) in the context of the entire complex.

Surface exposure of the isolated erythropoietin molecule:

Performing fractional ASA calculations on the isolated erythropoietin molecule (molecule A) resulted in the following residues being determined to have more than 25% of their side chain exposed to the surface: A1, P2, R4, L5, D8, S9, R10, V11, E13, R14, L17, K20, E21, E23, K24, I25, T27, G28, A30, E31, H32, S34, N36, E37, K38, T40, K45, N47, F48, Y49, A50, K52, R53, M54, E55, V56, Q58, E62, Q65, A68, L69, S71, E72, L75, R76, Q78, A79, V82, K83, S84, S85, Q86, P87, E89, P90, Q92, L93, D96, K97, S100, R103, S104, T106, T107, R110, A111, G113, Q115, K116, E117, I119, S120, S122, D123, A124, A125, S126, A127, A128, P129, L130, R131, T132, T134, D136, R139, K140, R143, N147, R150, G151, K154, L155, G158, E159, R162, T163, G164, D165, R166.

The following residues were determined to have more than 50% of their side chain exposed to the surface: A1, P2, R4, L5, D8, S9, R10, R14, L17, K20, K24, T27, G28, E31, S34, N36, K38, T40, K45, N47, F48, Y49, A50, K52, R53, E55, V56, Q65, A68, E72, L75, A79, V82, K83, S85, Q86, E89, Q92, L93, D96, K97, S100, R103, T107, R110, A111, G113, K116,

I119, S120, D123, A124, A125, S126, A127, A128, P129, L130, R131, T132, D136, R143, R150, G158, R162, T163, G164, D165.

Since the surface area calculations above were performed on a variant of human erythropoietin that included the substitutions P121N and P122S, a comparison with a surface area calculation on the X-ray structure of the N24K, N38K, N83K variant of human erythropoietin was made. In this latter structure, both P121 and P122 have more than 50% side chain ASA, whereas it is smaller in the calculations above. Therefore, P121 and P122 are both expected to have more than 50% side chain ASA in wild-type hEPO.

Surface exposure of the erythropoietin molecule in the complex:

Performing fractional ASA calculations on molecule A, including all residues of molecules B and C (the receptor molecules) having at least one residue at a distance of less than 10Å from an atom in molecule A, resulted in the following residues of molecule A being determined to have more than 25% of their side chain exposed to the surface: A1, P2, R4, L5, D8, S9, R10, L17, K20, E21, E23, K24, I25, T27, G28, A30, E31, H32, S34, N36, E37, K38, T40, K45, Y49, A50, K52, R53, M54, E55, V56, Q58, E62, Q65, A68, L69, S71, E72, L75, R76, Q78, A79, V82, K83, S84, S85, Q86, P87, E89, P90, Q92, L93, T106, T107, R110, A111, G113, Q115, K116, E117, I119, S120, S122, D123, A124, A125, S126, A127, A128, P129, L130, R131, T132, T134, D136, R139, R143, G158, E159, R162, T163, G164, D165, R166.

The following residues were determined to have more than 50% of their side chain exposed to the surface: A1, P2, R4, L17, K24, T27, G28, E31, S34, N36, K38, T40, Y49, A50, K52, R53, E55, V56, Q65, A68, E72, L75, A79, V82, K83, S85, Q86, E89, Q92, L93, A111, G113, K116, I119, S120, D123, A124, A125, S126, A127, A128, P129, L130, T132, D136, G158, R162, T163, G164, D165.

Comparing the two set of lists results in V11, E13, R14, N47, F48, D96, K97, S100, R103, S104, K140, N147, R150, G151, K154 and L155 being removed from the list of residues having more than 25% side chain ASA in the isolated EPO molecule, since these residues were not found to have more than 25% side chain ASA upon receptor binding. Similarly, L5, D8, S9, R10, R14, K20, K45, N47, F48, D96, K97, S100, R103, T107 and R110, which were found to have more than 50% side chain ASA in the isolated EPO molecule, do not have more than 50% side chain ASA upon receptor binding. The ASA of amino acid residues in these positions is thus influenced by receptor binding, and it is therefore preferred in the context of the present invention not to modify these positions by conjugation to e.g. PEG. Alternatively, it

may be desirable, where appropriate, to carry out conservative amino acid substitutions in one or more of these positions in order to avoid conjugation that may adversely effect receptor binding, for example to avoid conjugation to certain lysine residues in the case of attachment of PEG moieties to lysine amino groups.

Receptor-binding site:

By performing ASA calculations as described above it was determined that the following residues of the erythropoietin variant molecule have some degree of reduced ASA in the complex as compared to the calculation of ASA on the isolated molecule: L5, D8, S9, R10, V11, E13, R14, Y15, L16, L17, E18, K20, D43, T44, K45, V46, N47, F48, Y49, K52, Q78, L93, D96, K97, V99, S100, R103, S104, T107, L108, R110, R131, I133, K140, R143, V144, S146, N147, R150, G151, K154, L155. These positions are therefore believed to lie in or sufficiently near the receptor-binding site that conjugation to residues in these positions is preferably avoided.

Lysines: substitution to remove attachment site

Human erythropoietin contains 8 lysines: K20, K45, K52, K97, K116, K140, K152 and K154, all of which are potential sites for polymer attachment, in particular by PEGylation using e.g. SPA-PEG (Shearwater Corp.). In order to remove an attachment point with the purpose of reducing the number of attached PEG groups or avoiding polymer attachment in or near a receptor-binding site, these lysines are all potential targets for deletion, preferably by way of substitution with another amino acid residue, preferably arginine. Preferably, the lysines to be removed are selected from those identified as being in the receptor-binding site: K20, K45, K52, K97, K140 and K154. In a preferred embodiment, one, two, three, four, five or all of these residues is removed by way of conservative substitution, preferably by substitution with an arginine residue.

Introduction of free cysteines

Human EPO contains cysteine residues in positions 7, 29, 33 and 161 that form two disulfide bridges between positions 7-161 and 29-33. Attachment sites for polymer moieties such as PEG can advantageously be introduced into an EPO variant by introducing free cysteines, i.e. cysteines that are not involved in formation of disulfide bonds. In one embodiment, one or more cysteine residues are introduced, preferably by substitution, within one or more of the four helices of hEPO. By comparing the ASA information provided above with information

on the location of the alpha helices in hEPO, it may be seen that several amino acid residues located in the helices have a sufficient degree of surface exposure to qualify them as potential sites for introduction of a residue forming an attachment site for a non-polypeptide moiety, in this case introduction of a cysteine residue.

As explained above, substitutions are preferably performed in residues with more than 25% of their side chain exposed to the surface, in particular those that have more than 50% side chain exposure, and preferably positions having at least 25 or 50% side chain exposure in the EPO:receptor complex. Preferred positions for possible introduction of one or more cysteine residues in this embodiment thus include S9, R10, V11, E13, R14, L17, K20, E21, E62, Q65, A68, L69, S71, E72, L75, R76, P90, Q92, L93, D96, K97, S100, R103, S104, T106, T107, T132, T134, D136, R139, K140, R143, N147, R150 and G151 (more than 25% ASA), and more preferably one or more of S9, R10, R14, L17, K20, Q65, A68, E72, L75, Q92, L93, D96, K97, S100, R103, T107, T132, D136, R143 and R150 (more than 50% ASA).

Still more preferably, possible introduction of a cysteine residue is performed in one or more positions having more than 25% ASA in the EPO:receptor complex, i.e. positions S9, R10, L17, K20, E21, E62, Q65, A68, L69, S71, E72, L75, R76, P90, Q92, L93, T106, T107, T132, T134, D136, R139 and R143, and most preferably residues with more than 50% side chain exposure in the EPO:receptor complex, i.e. positions L17, Q65, A68, E72, L75, Q92, L93, T132 and D136.

As explained elsewhere herein (see e.g. the paragraph under the heading "Receptor-binding site" above), it is preferred that amino acid residues functioning as attachment sites for a polymer molecule, in this case cysteine residues, are not introduced in positions located in or near the receptor-binding site.

CLAIMS

1. A polypeptide conjugate exhibiting erythropoietin (EPO) activity, comprising at least one polymer molecule covalently attached to an attachment site of a polypeptide, wherein said polypeptide comprises at least one removed and/or introduced lysine, cysteine, aspartic acid or glutamic acid residue compared to the amino acid sequence of human EPO (hEPO).

2. The polypeptide conjugate of claim 1, wherein at least one amino acid residue is altered in at least one position selected from the group consisting of: A1, P2, R4, L5, D8, S9, R10, V11, E13, R14, L17, K20, E21, E23, N24, I25, T27, G28, A30, E31, H32, S34, N36, E37, N38, T40, K45, N47, F48, Y49, A50, K52, R53, M54, E55, V56, Q58, E62, Q65, A68, L69, S71, E72, L75, R76, Q78, A79, V82, N83, S84, S85, Q86, P87, E89, P90, Q92, L93, D96, K97, S100, R103, S104, T106, T107, R110, A111, G113, Q115, K116, E117, I119, S120, P122, D123, A124, A125, S126, A127, A128, P129, L130, R131, T132, T134, D136, R139, K140, R143, N147, R150, G151, K154, L155, G158, E159, R162, T163, G164, D165, R166.

3. The polypeptide conjugate of claim 1, wherein at least one amino acid residue is altered in at least one position selected from the group consisting of: A1, P2, R4, L5, D8, S9, R10, R14, L17, K20, N24, T27, G28, E31, S34, N36, N38, T40, K45, N47, F48, Y49, A50, K52, R53, E55, V56, Q65, A68, E72, L75, A79, V82, N83, S85, Q86, E89, Q92, L93, D96, K97, S100, R103, T107, R110, A111, G113, K116, I119, S120, D123, A124, A125, S126, A127, A128, P129, L130, R131, T132, D136, R143, R150, G158, R162, T163, G164, D165.

4. The polypeptide conjugate of claim 1, wherein at least one amino acid residue is altered in at least one position selected from the group consisting of: A1, P2, R4, L5, D8, S9, R10, L17, K20, E21, E23, N24, I25, T27, G28, A30, E31, H32, S34, N36, E37, N38, T40, K45, Y49, A50, K52, R53, M54, E55, V56, Q58, E62, Q65, A68, L69, S71, E72, L75, R76, Q78, A79, V82, N83, S84, S85, Q86, P87, E89, P90, Q92, L93, T106, T107, R110, A111, G113, Q115, K116, E117, I119, S120, P122, D123, A124, A125, S126, A127, A128, P129, L130, R131, T132, T134, D136, R139, R143, G158, E159, R162, T163, G164, D165, R166.

5. The polypeptide conjugate of claim 4, wherein at least one amino acid residue is altered in at least one position selected from the group consisting of: A1, P2, R4, E21, E23, N24, I25, T27, G28, A30, E31, H32, S34, N36, E37, N38, T40, A50, R53, M54, E55, V56, Q58, E62, Q65, A68, L69, S71, E72, L75, R76, A79, V82, N83, S84, S85, Q86, P87, E89, P90, Q92, T106, A111, G113, Q115, K116, E117, I119, S120, P122, D123, A124, A125,

S126, A127, A128, P129, L130, T132, T134, D136, R139, G158, E159, R162, T163, G164, D165, R166.

6. The polypeptide conjugate of claim 1, wherein at least one amino acid residue is altered in at least one position selected from the group consisting of: A1, P2, R4, L17, N24, T27, G28, E31, S34, N36, N38, T40, Y49, A50, K52, R53, E55, V56, Q65, A68, E72, L75, A79, V82, N83, S85, Q86, E89, Q92, L93, A111, G113, K116, I119, S120, D123, A124, A125, S126, A127, A128, P129, L130, T132, D136, G158, R162, T163, G164, D165.

7. The polypeptide conjugate of claim 6, wherein at least one amino acid residue is altered in at least one position selected from the group consisting of: A1, P2, R4, N24, T27, G28, E31, S34, N36, N38, T40, A50, R53, E55, V56, Q65, A68, E72, L75, A79, V82, N83, S85, Q86, E89, Q92, A111, G113, K116, I119, S120, D123, A124, A125, S126, A127, A128, P129, L130, T132, D136, G158, R162, T163, G164, D165.

8. The polypeptide conjugate of any of claims 1-7, wherein the polypeptide comprises at least one lysine, cysteine, aspartic acid or glutamic acid residue introduced by way of substitution.

9. The polypeptide conjugate of claim 8, wherein the polymer molecule is attached to a lysine residue.

10. The polypeptide conjugate of claim 9, wherein the polypeptide comprises, relative to hEPO, at least one lysine residue that has been introduced by substitution in a position selected from the group consisting of A1, P2, R4, E21, E23, N24, I25, T27, G28, A30, E31, H32, S34, N36, E37, N38, T40, A50, R53, M54, E55, V56, Q58, E62, Q65, A68, L69, S71, E72, L75, R76, A79, V82, N83, S84, S85, Q86, P87, E89, P90, Q92, T106, A111, G113, Q115, E117, I119, S120, P121, P122, D123, A124, A125, S126, A127, A128, P129, L130, T132, T134, D136, R139, G158, E159, R162, T163, G164, D165 and R166.

11. The polypeptide conjugate of claim 9, wherein the polypeptide comprises, relative to hEPO, at least one lysine residue that has been introduced by substitution in a position selected from the group consisting of A1, P2, R4, N24, T27, G28, E31, S34, N36, N38, T40, A50, R53, E55, V56, Q65, A68, E72, L75, A79, V82, N83, S85, Q86, E89, Q92, A111, G113, I119, S120, P121, P122, D123, A124, A125, S126, A127, A128, P129, L130, T132, D136, G158, R162, T163, G164 and D165.

12. The polypeptide conjugate of claim 1, wherein the polypeptide comprises at least one non-lysine residue in a position that is occupied by a lysine residue in hEPO.

13. The polypeptide conjugate of claim 12, wherein the polypeptide comprises, relative to hEPO, at least one non-lysine residue in a position selected from the group consisting of K20, K45, K52, K97, K140 and K154.

14. The polypeptide conjugate of claim 8, wherein the polymer molecule is attached to a cysteine residue.

15. The polypeptide conjugate of claim 14, wherein at least one cysteine residue has been introduced in the A, B, C or D helix.

16. The polypeptide conjugate of claim 15, wherein the polypeptide comprises, relative to hEPO, at least one cysteine residue that has been introduced by substitution in a position selected from the group consisting of R10, V11, E13, R14, L17, K20, E21, E62, Q65, A68, L69, S71, E72, L75, R76, P90, Q92, L93, D96, K97, S100, R103, S104, T106, T134, D136, R139, K140, R143, N147, R150 and G151.

17. The polypeptide conjugate of claim 15, wherein the polypeptide comprises, relative to hEPO, at least one cysteine residue that has been introduced by substitution in a position selected from the group consisting of R10, R14, L17, K20, Q65, A68, E72, L75, Q92, L93, D96, K97, S100, R103, D136, R143 and R150.

18. The polypeptide conjugate of claim 15, wherein the polypeptide comprises, relative to hEPO, at least one cysteine residue that has been introduced by substitution in a position selected from the group consisting of R10, L17, K20, E21, E62, Q65, A68, L69, S71, E72, L75, R76, P90, Q92, L93, T106, T134, D136, R139 and R143.

19. The polypeptide conjugate of claim 18, wherein the polypeptide comprises, relative to hEPO, at least one cysteine residue that has been introduced by substitution in a position selected from the group consisting of E21, E62, Q65, A68, L69, S71, E72, L75, R76, P90, Q92, T106, T134, D136 and R139.

20. The polypeptide conjugate of claim 15, wherein the polypeptide comprises, relative to hEPO, at least one cysteine residue that has been introduced by substitution in a position selected from the group consisting of L17, Q65, A68, E72, L75, Q92, L93 and D136.

21. The polypeptide conjugate of claim 20, wherein the polypeptide comprises, relative to hEPO, at least one cysteine residue that has been introduced by substitution in a position selected from the group consisting of Q65, A68, E72, L75, Q92 and D136.

22. The polypeptide conjugate of any of the preceding claims, wherein the polymer molecule is a polyalkylene glycol molecule.

23. The polypeptide conjugate of claim 22, wherein the polymer molecule is polyethylene glycol (PEG).

24. The polypeptide conjugate of claim 23, wherein the PEG has a molecular weight of less than about 20,000, preferably about 1000-15,000, such as about 2000-12,000, e.g. about 3000-10,000.

25. The polypeptide conjugate of any of the preceding claims, comprising 2-8 attached polymer molecules.

26. A polypeptide comprising an amino acid sequence as defined in any of the preceding claims.

27. A composition comprising a polypeptide conjugate according to any of claims 1-25 and a pharmaceutically acceptable carrier or excipient.

28. Use of a polypeptide conjugate according to any of claims 1-25 as a pharmaceutical.

29. Use of a polypeptide conjugate according to any of claims 1-25 for the preparation of a medicament for the prevention or treatment of disorders characterized by low or defective red blood cell production.

30. Use of a polypeptide conjugate according to any of claims 1-25 for the preparation of a medicament for the prevention or treatment of head trauma, stroke, epilepsy, ischemia, hypoxia, immune-mediated inflammation, excessive neuronal excitation and other central nervous system (CNS) related conditions.

31. A method for preventing or treating a disorder characterized by low or defective red blood cell production in a mammal, comprising administering to a mammal in need thereof an effective amount of a polypeptide conjugate according to any of claims 1-25.

32. A method for preventing or treating head trauma, stroke, epilepsy, ischemia, hypoxia, immune-mediated inflammation, excessive neuronal excitation and other central nerv-

ous system (CNS) related conditions in a mammal, comprising administering to a mammal in need thereof an effective amount of a polypeptide conjugate according to any of claims 1-25.

SEQUENCE LISTING

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Maxygen ApS

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