Novel neurturin conjugates for pharmaceutical use

Description

Field of the Invention

The present invention relates to the modification of human neurturin to increase its serum half life and pharmacokinetic profile. In particular, the invention relates to novel neurturin conjugates comprising neurturin or biologically active fragments thereof covalently attached to polyol moieties, preferably polyethylene glycol. The invention further relates to pharmaceutical compositions with increased bioavailability comprising novel neurturin conjugates as active agent for therapy, treatment, prevention and/or diagnosis of diabetes and neurodegeneration.

Background

Pancreatic beta-cells secrete insulin in response to elevated blood glucose levels. Insulin amongst other hormones plays a key role in the regulation of the fuel metabolism. Insulin leads to the storage of glycogen and triglycerides and to the synthesis of proteins. The entry of glucose into muscles and adipose cells is stimulated by insulin. In patients who suffer from diabetes mellitus type I or LADA (latent autoimmue diabetes in adults, Pozzilli & Di Mario, 2001, Diabetes Care. 8:1460-1467) beta-cells are being destroyed due to autoimmune attack. The amount of insulin produced by the remaining pancreatic islet cells is too low, resulting in elevated blood glucose levels (hyperglycemia). In diabetes mellitus type II liver and muscle cells loose their ability to respond to normal blood insulin levels (insulin resistance). High blood glucose levels (and also high blood lipid levels) in turn lead to an impairment of beta-cell function and to an increase in beta-cell death. Interestingly, beta-cell regenerative processes like neogenesis and replication do not appear to

compensate for the loss of beta cell mass in type II diabetics, thus causing a reduction in total beta-cell mass over time. Eventually the application of exogenous insulin becomes necessary in type II diabetics for an adequate control of blood glucose levels.

In type I diabetics, where beta-cells are being destroyed by autoimmune attack, treatments have been devised which modulate the immune system and may be able to stop or strongly reduce islet destruction (Raz et al., 2001, Lancet 358: 1749-1753; Chatenoud et al., 2003, Nat Rev Immunol. 3: 123-132; Homann et al., Immunity. 2002, 3:403-415). However, due to the relatively slow regeneration of human beta-cells such treatments can be more successful if they are combined with treatments that can stimulate beta-cell regeneration.

Diabetes is a very disabling disease, because today's common anti-diabetic drugs do not control blood sugar levels well enough to completely prevent the occurrence of high and low blood sugar levels. Frequently elevated blood sugar levels are toxic and cause long-term complications like for example nephropathy, retinopathy, neuropathy and peripheral vascular disease. Extensive loss of beta cells also leads to deregulation of glucagon secretion from pancreatic alpha cells which contributes to an increased risk of dangerous hypoglycemic episodes. There is also a host of related conditions, such as obesity, hypertension, heart disease and hyperlipidemia, for which persons with diabetes are substantially at risk.

Apart from the impaired quality of life for the patients, the treatment of diabetes and its long term complications presents an enormous financial burden to our healthcare systems with rising tendency. Thus, for the treatment of diabetes mellitus type I and LADA, but also for the treatment of late stages of diabetes mellitus type II there is a strong need in the art to identify factors that induce regeneration of pancreatic insulin producing beta-cells. These factors could restore normal function of the endocrine pancreas once its function is impaired or event could prevent the development or progression of diabetes type I,

LADA or diabetes type II.

Neurturin is a secreted protein which is expressed in embryonic pancreas. Recombinant neurturin has been shown to stimulate the differentiation of mouse embryonic stemcells into insulin producing cells. Moreover, transgenic mice with elevated neurturin levels in the pancreas have a substantially increased pancreatic beta-cell mass. Based on these findings, the use of neurturin for the treatment of pancreatic disorders such as diabetes has been proposed (see for example WO 03/99318 and WO 2005/051415, the disclosure of which is herein incorporated by reference).

Neurturin is a member of the GDNF family of ligands (GFL) comprised of Glial cell line-derived neurotrophic factor (GDNF), Neurturin, Artemin and Persephin. Mature neurturin is a homodimer with a size of 23.6 kDa comprised of 102 amino acid monomers. Each monomer contains 3 intra-chain disulfide bonds forming a cystine knot. An additional disulfide bridge is connecting the monomers.

Neurturin had previously been proposed as a treatment for neurodegenerative diseases such as Parkinsons, Alzheimers and Huntington's disease, motor neuron disorders, spinal cord injuries or hearing disorders (WO 97/08196, WO 99/06064; Akerud et al. J Neurochem. 1999;73(1):70-78; Koeberle & Ball Neuroscience. 2002; 110(3):555-567; Bilak et al. Mol Cell Neurosci. 1999;13(5):326-336; Perez-Navarro et al. Neuroscience. 2000; 98(1):89-96; Rosenblad et al, Eur J Neurosci. 1999; 11(5):1554-1566, the disclosures of which are herein incorporated by reference).

It was found, however, that upon convection-enhanced delivery (CED) into rat brains, the distribution volume of neurturin and the related factor GDNF was limited (Hamilton et al., Exp Neurol. 2001; 168(1):155-161). Proteins must typically be administered by injection. After injection most proteins are cleared rapidly from the body, necessitating frequent injections. In our own

studies, we found that the bioavailability after subcutaneous and intravenous injection of neurturin in mammals is low. Only about 10 percent of subcutaneously injected neurturin enter the circulation. Consequently, it is difficult to achieve therapeutically useful blood levels of the proteins in patients.

Thus, there is a strong need to develop a neurturin variant with enhanced availability in patients and therewith the development of methods to prolong the circulating half-lives of protein therapeutics in the body – the bioavailability – so that neurturin as active agent does not have to be injected frequently in order to satisfy the needs of patients for "user-friendly" protein therapeutics.

Thus, the underlying problem of the present invention was to provide new variants and formulations of neurturin that enhance its bioavailability.

The problem is solved by providing the embodiments characterized in the claims.

Summary of the Invention

The present invention relates to a novel neurturin conjugate comprising a polyol moiety covalently bound to a human neurturin protein product.

The present invention further relates to a pharmaceutical composition comprising at least one neurturin protein product and/or a biologically active fragment thereof which is conjugated to at least one polyethylene glycol molecule as an active ingredient together with a pharmaceutically acceptable carrier, diluent and/or adjuvant.

In a further embodiment, the present invention relates to a method for administering a pharmaceutical composition to a subject in need thereof comprising a pharmaceutically active amount of at least one modified

neurturin protein product or a biologically active fragment thereof which is conjugated to at least one polyethylene glycol molecule as an active ingredient together with a pharmaceutically acceptable carrier, diluent and/or adjuvant.

It is a further aspect of the present invention to provide a method for administering a pharmaceutical composition to a subject in need thereof comprising a pharmaceutically active amount of at least one modified neurturin protein product or a biologically active fragment thereof which is conjugated to at least one polyethylene glycol molecule as an active ingredient together with a low-molecular weight substance together with a pharmaceutically acceptable carrier, diluent and/or adjuvant.

The compositions of the present invention are suitable for preventing or treating pancreatic disorders or neurodegenerative diseases, particularly pancreatic autoimmune disorders, e.g. autoimmune diabetes such as type I diabetes or LADA but also type II diabetes.

Detailed Description of the Invention

The present invention provides neurturin conjugates comprising covalently attached polyol moieties such as polyethylene glycol (PEG), as well as pharmaceutical compositions comprising these neurturin conjugates, as well as variants, derivatives or biologically active fragments thereof.

Unless specific definitions are provided, the nomenclature utilized in connection with, and the laboratory procedures, techniques and methods described herein are those known in the art to which they pertain. Standard chemical symbols and abbreviations are used interchangeably with the full names represented by such symbols. Standard techniques may be used for chemical syntheses, chemical analyses, pharmaceutical preparation, composition, delivery, and treatment of patients. Standard techniques may be used for recombinant DNA methodology, oligonucleotide synthesis, tissue

culture and the like. Reactions and purification techniques may be performed e.g., using kits according to manufacturer's specifications, as commonly accomplished in the art or as described herein. The foregoing techniques and procedures may be generally performed according to conventional methods well known in the art and as described in various general or more specific references that are cited and discussed throughout the present specification.

The term "analog" refers to a polypeptide which resembles neurturin in structure, having a similar function.

The term "biologically active" or "biologic activity" as used herein means that the neurturin product induces and/or stimulates the differentiation of insulin producing cells from progenitor, e.g. stem cells, and/or promotes the protection, survival, or regeneration of insulin producing cells, e. g. beta cells *in vivo* or *in vitro.* The biologic activity of the modified neurturin protein products as disclosed herein can easily be determined in standard *in vitro* assays as disclosed for the determination of GDNF activity, described in WO 93/06116 and in U.S. Patent Application No. 08/535,681 or as described in the present Examples.

As used herein, "fragments" of neurturin refer to portions of neurturin that are generated by any method, including but not limited to enzymatic digestion and chemical cleavage (e.g. CNBr) of neurturin and physical shearing of the polypeptide. Fragments of neurturin may also be generated, e.g. by recombinant DNA technology and by amino acid synthesis.

As "active fragment" or "biologically active fragment" of neurturin as used in the present invention refers to any fragment or precursor of the polypeptidic chain of neurturin, alone or in combination with related molecules or residues bound to it, for example, residues of sugars or phosphates, or aggregates of the polypeptide molecule when such fragments or precursors show the same biological activity of neurturin. The term "biologically active fragment" also

relates to a fragment of a neurturin product which induces and/or stimulates the differentiation of insulin producing cells from progenitor, e.g. stem cells, and/or promotes the protection, survival, or regeneration of insulin producing cells, e. g. beta cells *in vivo* or *in vitro*. The biologic activity of a fragment of the modified neurturin protein products as disclosed herein can easily be determined in standard *in vitro* assays as disclosed for the determination of GDNF activity, described in WO 93/06116 and in U.S. Patent Application No. 08/535,681 or as described in the present Examples.

The term "homologue" is used herein to relate to a polypeptide related to neurturin by descent from a common ancestral protein sequence. The term, homolog, may apply to the relationship between proteins separated by the event of genetic duplication.

The term "low molecular weight substance" refers to a substance with an average molecular weight of between 100 and 12000 Da, preferably of between 200 and 8000 Da and most preferably of about 500 and 7000 Da.

The low molecular weight substance may be a polyanionic polymer which is natural or synthetic and which contains a plurality of anionic groups such as carboxylate and/or sulfate groups. For example, the polyanionic polymer may be selected from low molecular weight sulphated saccharides, sulphated cyclodextrins, or sulphated synthetic polymers such as acrylic polymers, aromatic polymers, and/or polyalcohols. More particularly, the polyanionic polymer is selected from low molecular weight heparins or heparin derivatives, heparan sulphates, chondroitin sulfates, dextran sulphates, chemically modified heparin-derived oligosaccharides (list from Wang et al. (2002), supra), heparin-like oligosaccharides, dextran sulphates, sulphated low molecular weight glycosaminoglycans, dextrin-2-sulphates, cellulose sulphates and naphthalene sulfonate polymer (e.g. PRO 2000), PAVAS (a co-polymer of acrylic acid with vinyl alcohol sulphate), the sulphonated polymer PAMPS [poly(2-acryl-amido-2-methyl-1-propanesulfonic acid] (M_w e.g. approximately 7000-12000), chondroitin

sulphates, sulphated cyclodextrins, laminarin sulphate (Alban, S. in *Carbohydrates in Drug Design* (Ed. Z.J. Witczak, K.A. Nieforth) Dekker, New York, **1997**, pp 209.), polyglycerin sulphates (Türk, H., Haag, R., Alban, S. *Bioconjugate Chem.* **2004**, *15*, 162;), pentosan polysulphates (PPS) and derivatives thereof such lactose-modified pentosan polysulphates, fractionated PPS/low molecular weight PPS, fucoidan, or derivatives or combinations thereof.

Low molecular weight heparin (LMWH) analogues such as Enoxaparin, Dalteparin or Fragmin are preferred examples of suitable polymers. They are obtained by fractionation and/or limited enzymatic or chemical digestion of heparin, and have an average molecular weight of preferably about 3000 to about 7000 Dalton (Weitz 1997 supra).

The term "modified neurturin protein product" as used herein relates to a chemically modified neurturin protein conjugate as well as cell-expressed conjugate comprising a covalently attached polyol moiety.

Chemically modified neurturin conjugates may be prepared by one of skill in the art given the disclosures herein or by any of the methods known in the art.

The polyol moiety of the neurturin conjugate of the invention can be any water-soluble mono- or bifunctional poly(alkylene oxide) having a linear or branched chain. Typically, the polyol is a poly(alkylene glycol) such as poly(ethylene glycol) (PEG). However, those of skill in the art will recognize that other polyols, such as, for example poly(propylene glycol) and copolymers of polyethylene glycol and polypropylene glycol, can be suitably used. Other neurturin conjugates can be prepared by coupling neurturin to a water-soluble polymer. A non-limiting list of such polymers include other polyalkylene oxide homopolymers such as polypropylene glycols, polyoxyethylenated polyols, copolymers thereof and block copolymers thereof. As an alternative to polyalkylene oxide-based polymers, effectively

non-antigenic materials such as dextran, polyvinyl pyrrolidones, polyacrylamides, polyvinyl alcohols, carbohydrate-based polymers and the like can be used.

The protein conjugates may also be expressed in prokaryotic or eukaryotic cells well known in the art.

The term "neurturin conjugate" relates to a neurturin protein product which comprises a polyol moiety such as polyethylene glycol covalently attached to an amino acid of neurturin. The amino acid can be site-specific at the N-terminal amino acid or at any other amino acid. PEG conjugates can contain one or more PEG molecules covalently attached to any suitable site of the neurturin protein molecule.

The term "neurturin protein product", "neurturin product", "neurturin protein" or "neurturin" which are used interchangeably herein, relate to a protein or peptide having a degree of identity to the biologically active human neurturin product resulting in the cleavage of the neurturin precursor having the amino acid sequence published as GenBank Accession Number NP_004549 (SEQ ID NO: 1, Fig. 3) or to the human neurturin precursor itself, that is in excess of 70%, preferably in excess of 80%, more preferably in excess of 90% and most preferably in excess of 95%, variants or derivatives thereof. The degree of identity between the mouse and the human protein is about 91%, and it is contemplated that preferred mammalian neurturin proteins will have a similarly high degree of identity. The percentage of identity of a neurturin product and the human neurturin protein or a precursor may be determined according to standard procedures, e.g. by using the BLAST algorithm. Preferably, the percentage of identity is calculated as the percentage of amino acid residues found in the smaller of the two sequences that align with identical amino acid residues in the sequence being compared, when four gaps in a length of 100 amino acids may be introduced to assist in that alignment.

The term "ortholog" relates to polypeptides in different species that evolved from a common ancestral gene by specification. Orthologs retain the same function in the course of evolution.

The term "polyethylene glycol" or "PEG" relates to PEG itself as well as derivatives thereof. The polymer PEG is commonly used as methoxy-PEG-OH, (m-PEG), in which one terminus is the relatively inert methoxy group, while the other terminus is a hydroxyl group that is subject to chemical modification. Branched PEGs are also in common use. The number of branching arms (m) can range from three to a hundred or more. The hydroxyl groups are further subject to chemical modification. Another branched form, such as that described in PCT patent application WO 96/21469, has a single terminus that is subject to chemical modification. Yet another branched form, has reactive groups, such as carboxyl, along the PEG backbone rather than at the end of PEG chains. In addition to these forms of PEG, the polymer can also be prepared with weak or degradable linkages in the backbone. For example, Harris has shown in U.S. patent application Ser. No. 06/026,716, which is incorporated by reference herein in its entirety, that PEG can be prepared with ester linkages in the polymer backbone that are subject to hydrolysis. This hydrolysis results in cleavage of the polymer into fragments of lower molecular weight. The copolymers of ethylene oxide and propylene oxide are closely related to PEG in their chemistry, and they can be used instead of PEG in many of its applications.

The term "PEGylation" comprises covalent attachment of a polyethylene glycol molecule to a substrate, which can be a protein. PEGylation of proteins is widely known in the state of the art and has been reviewed by, for example, Veronese, F. M., Biomaterials 22 (2001) 405-417. PEG can be linked using different functional groups and polyethylene glycols with different molecular weight, linear and branched PEGs as well as different linking groups (see also Francis, G. E., et al., Int. J. Hematol. 68 (1998) 1-18; Delgado, C., et al., Crit. Rev. Ther. Drug Carrier Systems 9 (1992 (249-304).

PEGylation can be performed in aqueous solution with PEGylation reagents as described, for example, in WO 00/44785, preferably using NHS-activated linear or branched PEG molecules of a molecular weight between 5 and 40 kDa. PEGylation can also be performed at the solid phase according to Lu, Y. et al., Reactive Polymers 22 (1994) 221-229.

Selective PEGylation at the N-terminal amino acid can be performed according to Felix, A. M., et al., ACS Symp. Ser 680 (Poly(ethylene glycol)) (1997) 218-238. Selective N-terminal PEGylation is achieved during solid-phase synthesis by coupling of a N-PEGylated amino acid derivative to the N-terminal amino acid of the peptide chain. Side chain PEGylation is performed during solid-phase synthesis by coupling of N-PEGylated amino acid derivatives to the growing chain. Combined N-terminal and side chain PEGylation is processed either as described above within solid-phase synthesis or by solution phase synthesis by applying activated PEG reagents to the amino deprotected peptide .

Suitable PEG derivatives are activated PEG molecules with a preferred average molecular weight of from about 5 to about 40 kDa, more preferably from about 20 to about 40 kDa, and most preferably about 30 kDa. The PEG derivatives can be linear or branched PEGs.

Activated PEG derivatives are known in the art and described in, for example, Morpurgo, M., et al., J. Bioconj. Chem. 7 (1996) 363-368, for PEG-vinylsulfone. Linear chain and branched chain PEG species are suitable for the preparation of the PEGylated fragments. Examples of reactive PEG reagents are iodo-acetyl-methoxy-PEG and methoxy-PEG-vinylsulfone (m is preferably an integer from about 450 to about 900 and R is lower alkyl, linear or branched, having one to six carbon atoms such as methyl, ethyl, isopropyl, etc., from which methyl is preferred.

As used herein, the term "PEG" or "PEG moiety" is intended to include, but is not limited to, linear and branched PEG, methoxy PEG, hydrolytically or

enzymatically degradable PEG, pendant PEG, dendrimer PEG, copolymers of PEG and one or more polyols, and copolymers of PEG and PLGA (poly(lactic/glycolic acid)).

The term "PEGylated neurturin" or "neurturin modified by "PEGylation" or "neurturin conjugate comprising a polyethylen glycol moiety" relates to a neurturin protein product produced by a method by which a PEG moiety is covalently attached to form these PEGylated proteins.

The term "pharmaceutically acceptable carrier, diluent and/or adjuvant" relates to one and more pharmaceutically and physiologically acceptable compounds such as excipients and auxiliaries, which facilitate processing of the active ingredient(s) into preparations, which can be used pharmaceutically. Details may be found in the latest edition of Remington's Pharmaceutical Sciences (Maack Publishing Co., Easton, Pa.).

The term "unmodified control neurturin protein product" relates to a neurturin protein product of the same origin as the correlating modified neurturin protein product which is used as a control.

The term "variant" as used herein relates to a polynucleotide variant which may contain one or more substitutions, additions, deletions and/or insertions, as further described below, relative to a native polypeptide. The term "variant" also encompasses homologous polypeptides of xenogenic origin. Typically, a neurturin variant retains biological activity either completely, a substantial proportion thereof, or at least partially as, for example, can be determined using a specific assay as described below. The term "variant" as used herein also encompasses neurturin which was modified by altering the protein sequences by substitutions, additions or deletions providing functionally equivalent molecules, as is well known in the art. The latter includes altered sequences in which functionally equivalent amino acid residues are substituted by residues within the sequence resulting in a biologically silent exchange.

Neurturin variants are prepared by introducing appropriate nucleotide changes into the DNA encoding the polypeptide or by in vitro chemical synthesis of the desired polypeptide. It will be appreciated by those skilled in the art that deletions, insertions, and substitutions can be made, resulting in a protein product variant presenting neurturin biological activity.

Mutagenesis techniques for such a substitution, insertion or deletion of one or more selected amino acid residues are well known to one skilled in the art (e.g., U.S. Patent No. 4,518,584, the disclosure of which is hereby incorporated by reference.)

In a preferred embodiment, amino acid changes are introduced into the region between amino acids 47-69, preferably 51-65, of mature human neurturin (amino acids 141-164, preferably 147-160 as shown in Figure 3). These amino acid changes may comprise at least one deletion or substitution of an arginine residue in this region, e.g. arginine residues 51, 52, 54, 56, 57, 58, 60, 61 and/or 65. Preferred are substitutions of arginine by neutral or acidic amino acids, e.g. glycine, serine, alanine, aspartate or glutamate. Especially preferred is a substitution by glutamate, e.g. R51E, R52E, R54E, R56E, R57E, R58E, R60E, R61E and R65E or a neurturin variant comprising at least 2 of these substitutions.

Alternatively and/or additionally an amino acid residue of neurturin, e.g. in the above-indicated region, particularly an arginine residue, may be substituted by lysine in order to facilitate coupling to polyol groups. As there is no lysine in mature human neurturin, such a change allows site-specific modification with polyol groups. Especially preferred is a substitution by lysine in the above-indicated region, e.g. R51K, R52K, R54K, R56K, R57K, R58K, R60K, R61K and R65K or a neurturin variant comprising at least 2 of these substitutions.

Neurturin substitution variants have at least one amino acid residue of the

human or mouse neurturin amino acid sequence removed and a different residue inserted in its place. Such substitution variants include allelic variants, which are characterized by naturally occurring nucleotide sequence changes in the species population that may or may not result in an amino acid change.

Surprisingly, it was found by the present inventors that a pharmaceutical composition as disclosed has an increased bioavailability of its active ingredient, said conjugated neurturin and/or biologically active fragment thereof, compared to a pharmaceutical composition comprising no conjugated neurturin. The invention demonstrates that the conjugation of neurturin protein to a polyol such as PEG improves significantly the serum half life and bioavailability of a neurturin product. Improvement of the bioavailability of a neurturin product in turn may reduce accumulation or processing of the agent at the injection site if applied subcutaneously and may improve local tolerance this way.

The present invention provides for a method of applying the inventive neurturin conjugates as active agents to patients in a much lower dose compared to unmodified control neurturin in order to have a therapeutic effect. This complies with the need for "user-friendly protein therapeutics" and may also result in lower costs for producing active agent and/or pharmaceutical composition.

The present neurturin conjugates also have a higher solubility in aqueous solutions compared to unmodified neurturin which may result in a simplification of formulating the active agent.

As a further advantage, coupling polyols such as PEG to neurturin may reduce immunogenicity to neurturin and therefore the formation of neutralizing antibodies or allergic reactions against the active agent.

Preferably, the neurturin protein product of the present invention is a human

neurturin or a biologically active fragment thereof. Neurturin protein products are preferably produced via recombinant techniques because such methods are capable of achieving high amounts of protein at a great purity, and are not limited to products expressed in bacterial, plant, mammalian, or insect cell systems.

Recombinant neurturin protein products include glycosylated and non-glycosylated forms of the protein. In general, recombinant techniques involve isolating the genes encoding a neurturin protein product, cloning the gene in a suitable vector and/or cell type, modifying the gene if necessary to encode a desired variant, and expressing the gene in order to produce the neurturin protein product.

Alternatively, a nucleotide sequence encoding the desired neurturin product may be chemically synthesized. It is contemplated that a neurturin product may be expressed using nucleotide sequences that vary in codon usage due to the degeneration of the genetic code or allelic variations or alterations made to facilitate production of the protein product by the selected cell.

Kotzbauer et al., Nature 384:467-470, describe the identification of a mouse cDNA and amino acid sequence and a human cDNA and amino acid sequence for a neurturin protein, identified herein as SEQ ID NO: 1.

The neurturin products according to the present invention may be isolated or generated by a variety of means. Exemplary methods for producing neurturin products are described in patent application WO 97/08196, which is hereby incorporated by reference. Also described therein are a variety of vectors, host cells, and culture growth conditions for the expression of neurturin protein, as well as methods to synthesize variants of neurturin protein product. Additional vectors suitable for the expression of neurturin protein product in E. coli are disclosed in Patent No. EP 0 423 980, the disclosure of which is hereby incorporated by reference.

The biologically active form of the protein neurturin is a disulfide-bonded dimer which can be determined by the molecular weight of purified neurturin. The material isolated after expression in a bacterial system is essentially biologically inactive, and exists as a monomer. Refolding is necessary to produce the biologically active disulfide-bonded dimer. Processes suitable for the refolding and maturation of the neurturin expressed in bacterial systems are substantially similar to those described in WO 93/06116. Standard in vitro assays for the determination of neurturin activity are also substantially similar to those determining GDNF activity as described in WO 93/06116 and in U.S. Patent Application No. 08/535,681, and are hereby incorporated by reference.

Covalent attachment of the inert, non-toxic, big-degradable polymer polyethylene glycol (PEG), also known as polyethylene oxide (PEO), to neurturin has important applications in biotechnology and medicine. PEGylation of neurturin results in improved pharmacokinetics resulting in sustained duration, improve safety (e.g. lower toxicity, immunogenicity and antigenicity), increase efficacy, decrease dosing frequency, improve drug solubility and stability, reduce proteolysis, and facilitate controlled drug release.

The present invention contemplates use of neurturin protein products, e.g. derivatives which are prokaryote-expressed neurturin, or variants thereof, linked to at least one polyethylene glycol molecule, as well as use of neurturin, or variants thereof, attached to one or more polyethylene glycol molecules via an acyl or alkyl linkage. PEGylation may be carried out by any of the pegylation reactions known in the art. See, for example: Focus on Growth Factors, 3 (2):4-10, 1992; EP 0 154 316, the disclosure of which is hereby incorporated by reference; EP 0 401 384; and the other publications cited herein that relate to PEGylation.

The pharmaceutical composition of the present invention has an increased bioavailability of the active ingredient in a mammal, e.g. a human, compared

to a pharmaceutical composition which does not contain the PEGylated neurturin (see Figure 2). Preferably, the PEGylated neurturin is present in an amount to provide an at least 2-fold, preferably at least 5-fold and more preferably at least 10-fold increase in the bioavailability of the active ingredient.

The increase of bioavailability may be determined as shown in the Examples of the present application. More particularly, a composition containing the active ingredient in a given amount or dose is compared to a pharmaceutical composition containing the active ingredient in the same dose but without PEGylation. The bioavailability of both compositions may be determined from the plasma concentration after subcutaneous administration to experimental animals, e.g. mice. Preferably, the plasma-concentration is measured over a time period of at least 120 min, preferably of 240 min and most preferably of 24 h (see Figure 2).

The pharmaceutical composition of the present invention may be adapted for administration by any effective route, e.g. by oral, nasal, rectal, pulmonal, topical, transdermal or parenteral routes of administration. Thus, the composition may be a solid or liquid composition, e.g. a tablet, capsule, powder, cream, gel, ointment, solution, emulsion, suspension, lyophilisate etc. Preferably, however, the composition is administered by injection or infusion, more preferably by injection, e.g. by subcutaneous or intravenous injection. The pharmaceutical composition is preferably an aqueous solution.

In addition to the active ingredient and optionally low molecular weight substance such as a polyanionic polymer, the pharmaceutical composition may comprise a pharmaceutically acceptable carrier, diluent and/or adjuvant, such as buffers, agents for adjusting tonicity, stabilizers, fillers, disintegrants, thickeners, etc.

The pharmaceutical composition contains the active ingredient in a therapeutically effective amount or dose. The therapeutically effective dose

depends on the type of the active ingredient, the type and the variety of the disease to be treated and the type of administration. For parenteral compositions containing neurturin as an active ingredient, the therapeutically effective dose is preferably in the range of about 0.001 mg to 500 mg, more preferably from about 0.05 to about 100 mg, most preferably from about 0.01 to about 5 mg per day.

The composition is preferably administered to a mammal, particularly a human. Thus, the composition is suitable for human and veterinary medicine. The composition is particularly suitable for the prevention and/or treatment of neurodegenerative or pancreatic disorders, particularly pancreatic autoimmune disorders such as diabetes type I and LADA, or diabetes type II.

The neurturin conjugate as described herein may be administered by any suitable means, preferably enterally or parenterally or topically directly to the pancreas, as known to those skilled in the art. The specific dose may be calculated according to considerations of body weight, body surface area or organ size. Further refinement of the calculations necessary to determine the appropriate dosage for treatment involving each of the above mentioned compositions is routinely made by those of ordinary skill in the art and is within the ambit of tasks routinely performed. Appropriate dosages may be ascertained through use of the established assays for determining dosages utilized in conjunction with appropriate dose-response data. The final dosage regimen involved in a method for treating the above described conditions will be determined by the attending physician, considering various factors which modify the action of drugs, e.g., the age, condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. As studies are conducted, further information will emerge regarding the appropriate dosage levels for the treatment of various diseases and conditions.

It is envisioned that the continuous administration or sustained delivery of a

neurturin conjugate as described herein may be advantageous for a given treatment. While continuous administration may be accomplished via a mechanical means, such as with an infusion pump, it is contemplated that other modes of continuous or near continuous administration may be practiced. For example, chemical derivatization or encapsulation may result in sustained release forms of the protein having the effect of continuous presence, in predictable amounts, based on a determined dosage regimen. Thus, neurturin protein products include proteins derivatized or otherwise formulated to effectuate such continuous administration.

In a further preferred embodiment, neurturin conjugate can be delivered directly to progenitor, e.g. stem cells in order to stimulate the differentiation of insulin producing cells *in vitro* or *in vivo*. In this embodiment of the invention, neurturin conjugate may be added preferably at concentrations between 0.1 ng/ml and 500 ng/ml, more preferably between 1 ng/ml and 100 ng/ml, even more preferably between 20 and 80 ng/ml and most preferably 50 ng/ml.

The neurturin conjugate as disclosed herein may be used either in a monotherapy or in a combination therapy with other pharmaceutical agents. For example, it may be administered together with other pharmaceutical agents suitable for the treatment or prevention of pancreatic diseases and/or obesity and/or metabolic syndrome, particularly with other pharmaceutical agents suitable for stimulating and/or inducing the differentiation of insulin producing cells from progenitor cells. Further, it may be used together with pharmaceutical agents which have an immunosuppressive activity, e.g. antibodies, polypeptides and/or peptidic or non-peptidic low molecular weight substances as disclosed in WO 2005/51415.

A further embodiment of the present invention is a variant of human neurturin comprising at least one, e.g. 1, 2, 3 or 4 amino acid changes compared to wild-type human neurturin. The variant of human neurturin may contain one or more substitutions, additions, deletions and/or insertions, as

described in detail above, relative to the native human polypeptide. Neurturin variants can be prepared as described above.

In a preferred embodiment, amino acid changes are present in the region between amino acids 47-69, preferably 51-65 of mature human neurturin (amino acids 141-164, preferably 147-160 as shown in Figure 3). These amino acid changes may comprise at least one deletion or substitution of an arginine residue in this region, e.g. arginine residues 51, 52, 54, 56, 57, 58, 60, 61 and/or 65. Preferred are substitutions of arginine by neutral or acidic amino acids, e.g. glycine, serine, alanine, aspartate or glutamate. Especially preferred is a substitution by glutamate, e.g. R51E, R52E, R54E, R56E, R57E, R58E, R60E, R61E and R65E, or a neurturin variant comprising at least two of these substitutions.

Also preferred are variants of human neurturin, wherein alternatively and/or additionally an amino acid residue, e.g. in the above-indicated region, particularly an arginine residue, is substituted by lysine. Thereby, site-specific coupling of the neurturin variant to polyol groups is facilitated. Especially preferred is a substitution by lysine in the above-indicated region, e.g. R51K, R52K, R54K, R56K, R57K, R58K, R60K, R61K and R65K or a neurturin variant comprising at least 2 of these substitutions.

Neurturin substitution variants according to the present invention have at least one amino acid residue of the human neurturin amino acid sequence removed and a different residue inserted in its place. Such substitution variants include allelic variants which are characterized by naturally occurring nucleotide sequence changes in the species population that may or may not result in an amino acid change.

The present invention further provides a method for administering a pharmaceutical composition to a subject in need thereof, wherein said composition comprises a pharmaceutically active amount or dose of at least one neurturin protein product conjugated with at least one polyol such as

PEG or a biologically active fragment of such conjugated neurturin as an active ingredient together with a pharmaceutically acceptable carrier, diluent and/or adjuvant.

The invention even further provides a method for administering a pharmaceutical composition to a subject in need thereof, wherein said composition comprises a pharmaceutically active amount or dose of at least one modified neurturin protein product or a biologically active fragment thereof as an active ingredient together with a low molecular weight substance together with a pharmaceutically acceptable carrier, diluent and/or adjuvant.

Figures

The following Figures and Examples illustrate the invention and are non-limiting embodiments of the invention as claimed below. Numerous additional aspects and advantages of the invention will become apparent to those skilled in the art upon consideration of the following description of the Figures.

Figure 1: Biological activity of PEGylated neurturin in cell culture

The reporter cell line was subjected to increasing concentrations of neurturin or neurturin conjugated with PEG (PEGylated neurturin). The resulting reporter gene activity (relative light units (RLU) given on the y-axis) was plotted against the final concentration of the test agent (given on the x-axis in ng/ml; Figure 1A and B). Two independently produced batches of unmodified neurturin were used as experimental controls (PeproTech; Lot0805112 and Lot0106112) and their relative activity was compared to those of the three PEGylated conjugates (mono-mPEG-NHS-neurturin, mono-mPEG-CHO-neurturin and mono-CES0310-neurturin). Given values are means of at least 3 wells $\pm$ S.D. The calculated EC_{50} of the various tests are: Neurturin (Lot0805112, unmodified protein): EC_{50} = 2,2 ng/ml, neurturin (Lot0106112 ,

unmodified protein): EC_{50} = 1,2 ng/ml, mono-CES0310-neurturin: EC_{50} = 12 ng/ml (Figure 1A), and neurturin (Lot0106112, unmodified protein): EC_{50} = 3,8 ng/ml, mono-mPEG-NHS-neurturin: EC_{50} = 9,7 ng/ml, and mono-mPEG-CHO-neurturin: EC_{50} = 7,4 ng/ml (Figure 1B).

Figure 2: Pharmacokinetic (PK) studies after subcutaneous administration of test agents in mice

Figure 2A and B show the neurturin plasma concentrations in ng/ml at various time points after the administration of 0,050 mg/kg BW (Fig. 2A) or 0,50mg/kg BW (Fig. 2B) neurturin or PEGylated neurturin to mice. Resulting plasma levels were determined by a neurturin ELISA assay. Time point zero shows the average background signal for neurturin in mouse serum using this ELISA assay. The background values were calculated from independently measured values from sera of 12 (Fig. 2A) or from 11 (Fig. 2B) untreated mice The other indicated values are means of measured serum levels from three animals ± SD. Figure 2A shows PK data for the neurturin conjugate mono-NHS-neurturin stemming from two independent experiments (mono-NHS-neurturin_Exp.1 and -_Exp.2). Figure 2B shows data derived from yet another experiment where mice were treated with 0,50 mg/kg BW mono-NHS-neurturin.

For area under the curve (AUC) analysis covering all measured time points, the baseline was set to 0.6 ng/ml (the average background value at the time point 0): Neurturin unmodified protein: 18,8 ng*min/ml, mono-mPEG-NHS-neurturin_Exp1: 409 ng*min/ml, mono-mPEG-NHS-neurturin_Exp2: 918 ng*min/ml, mono-CES0310-neurturin: EC_{50} = 142 ng*min/ml (Figure 2A), and neurturin unmodified protein: 324 ng*min/ml, mono-mPEG-NHS-neurturin: 1157 ng*min/ml, and mono-mPEG-CHO-neurturin: 845 ng*min/ml (Figure 2B).

For the area under the curve (AUC) analysis (Figure 2A and B) of neurturin serum levels the baseline was set to 0,6 ng/ml neurturin which corresponds

to the average background level determined in the serum of untreated mice.

Figure 3:

Protein sequence of human neurturin precursor as published by the National Center for Biotechnology Information (NCBI) (accession number: NP_004549). The sequence of the mature protein which corresponds to the biologically as well as pharmacologically active form is highlighted in bold.

Figure 4:

Figure 4A. Expression of human neurturin and mutated neurturin variants. Native-PAGE/Western-blot of concentrated supernatant from HEK-293 cells expressing neurturin and neurturin variants (numbering of the positions according to the sequence of mature human neurturin, R: arginine, E: glutamic acid).

Figure 4B. Neurturin ELISA: Representative standard curve using human recombinant neurturin expressed from *E. coli*.

Figure 5: Quantification of biological activity of neurturin

Figure 5A. Cellular neurturin biological activity assay: Tyrosine hydroxylase reporter gene activity stimulated by exogenous human recombinant neurturin from *E.coli* in increasing concentrations combined with increasing serum concentrations.

Figure 5B. Biological activity of neurturin variants (n.d. : not detected)

Figure 6: Alignment of neurturin sequences from different vertebrate species

Amino acids 47-69 of mature human neurturin are shown (numbering of the

positions according to the sequence of mature human neurturin peptide, genbank accession number NP_004549, mature peptide aa 96-197). Amino acid variations different to human neurturin are printed bold and underlined (GenBank Accession Numbers in brackets).

Figure 7: DNA Sequence of a neurturin expression construct: Kr-G3-H6-G3-Xa-rhneurturin (wt)

Kr-G3-H6-G3-Xa-rhneurturin(wt) cloned into pMA vector. The DNA-sequence of the sense and the anti-sense strand are shown together with restriction sites and the resulting amino-acid sequence of the coding region.

Figure 8: Anti-neurturin antibody ELISA

Anti-neurturin antibody ELISA: left: Standard curve using anti-neurturin IgG, right: Standard curve using anti-neurturin IgG/IgM

It should be noted that all preferred embodiments discussed for one or several aspects of the invention also relate to all other aspects.

The versatility of the present invention is illustrated, but not limited, by the following Examples.

EXAMPLES

Example 1 – PEGylation of neurturin

Polyethylenglycol groups (PEG) were conjugated to neurturin through a process called PEGylation. This technology is widely used for the modification of therapeutic proteins and the procedure is familiar to anyone skilled in the art. In the present invention, mono-PEGylated neurturin conjugates were produced by linking a single PEG molecule to the N-terminal amino acid of one of the two subunits of the homodimer. Two

different conjugates of mono-PEGylated neurturin were produced that differ in size and structure of the attached PEG as well as in the method of production to conjugate the PEG to the protein. The conjugate disclosed as "mono-mPEG-NHS-neurturin" herein was produced using an about 5 kDa linear PEG-reagent (mPEG-succinimidyl succinat; NOF, Japan). This so-called "NHS-method" is the most commonly used method for PEGylation of soluble proteins. Further, a linear 5 kDa polyethylene glycol butyraldehyde (Nektar, 082M0H01) was used to generate the conjugate disclosed as "mono-mPEG-CHO-neurturin". Under specific experimental conditions NHS esters or aldehydes efficiently react with free amino groups of proteins and peptides. In yet another conjugate disclosed as "mono-CES0310-neurturin", the N-terminal amino acid of one chain of the neurturin homodimer was conjugated to a six-arm branched PEG of about 5 kDa using a PEGylation methodology known in the prior art (see, for example, but not limited to, DE 2005 100 04 157.0, EP 1 631 545 A2; WO 04/108634; WO 07/025763; CA 2528667). Neurturin does not contain lysine residues, therefore the PEGylation reactions described above are expected to primarily lead to a N-terminal PEGylation via the reactive amino groups of the N-terminal amino acids of the neurturin homodimer. The quality (size and purity) of the PEGylated products was confirmed using standard biochemical methodology known to anyone skilled in the art.

Example 2 – Biological activity of PEGylated neurturin conjugates or variants thereof

The biological activity of the neurturin conjugates was evaluated using a cell based reporter assay. It was determined how strong neurturin or its PEGylated conjugates activate a reporter gene construct in a cell line expressing neurturin receptors on the surface. In this assay the activity of both PEGylated neurturin conjugates was found to be reduced 3 to 10 fold relative to that of unmodified neurturin (Figure 1A and B).

The human neuroblastoma cell line TGW (JCRB0618) which expresses the

receptors for neurturin on the cell surface was stably transfected with a neurturin reporter gene construct. The reporter gene constructs contains a luciferase gene which is among other regulatory elements under the transcriptional control of repetitive serum response elements (SRE). In these cells the expression of luciferase is stimulated in response to the activation of the MAPK pathway, for instance through binding of neurturin to its surface receptors. The assay is carried out in 96 well plates. The luciferase activity was measured by addition of luciferin substrate to the wells and read-out was generated on a Analyst reader (Molecular Devices) operating in the luminescence mode.

Example 3 – Improved bioavailability of PEGylated neurturin conjugates or variants thereof

The relative bioavailability of the PEGylated conjugates of neurturin was estimated with the help of PK studies in mice (Fig. 2A and B). In these experiments the protein was subcutaneously injected into the neck region of mice. Following, the serum concentration of neurturin was determined at defined time points after the delivery of the protein using a specific neurturin ELISA assay (Fig. 2A and B). The sensitivity of this neurturin ELISA assay was limited because of relatively high background signals in the presence of mouse serum, as detected by the use of serum from mice that were not treated with neurturin (point zero values). After the injection of 0,050 mg/kg body weight (BW) unmodified neurturin, the neurturin serum levels did not significantly exceed this background level at any time point. On the contrary, when the same amount of PEGylated neurturin was applied a significant increase in serum levels of the injected protein was detected, irrespective of the kind of the PEG modification (Fig. 2A). When the amount of injected unmodified neurturin was increased 10 times (0,5 mg/kg BW) the protein was significantly detected in the serum at time points 60, 90, 120 and 180 minutes (Fig. 2B). Significance of this observation was confirmed using the T-test and p-values < 0,05 were calculated. Also, the serum levels of the PEGylated neurturin derivatives given at the 0,5 mg/kg BW dose were a few-

fold higher than these of the unmodified protein (Fig. 2A and B).

The neurturin conjugates presented herein consistently demonstrate higher serum levels relative to those of the unmodified protein. In conclusion, PEGylation of neurturin significantly improves the bioavailability of neurturin.

Example 4 – Neurturin variants

Basic arginine residues that are located within the basic patch of neurturin (amino acids 51-65 of the mature protein) were replaced by acidic glutamic acid residues through site directed PCR-based mutagenesis. Briefly, the coding sequence of mature human neurturin with an additional N-terminal eukaryotic secretion signal peptide was modified by PCR-based mutagenesis using mismatch primers introducing the desired mutations:

R51E, R52E, R54E, R56E, R57E, R58E, R60E, R61E, R63E, or R65E

(numbering of the positions according to the sequence of mature human neurturin peptide, Genbank Accession Number NP_004549, mature peptide aa 96-197, this sequence is also shown in Figure 3; R: arginine; E: glutamic acid).

The resulting plasmids were purified and the correctness of the coding sequence was checked by sequencing. The coding sequences were cloned via flanking HindIII and XhoI cloning sites into pCDNA3.1+ vector (Invitrogen Cat. No. V790-20) for eukaryotic expression.

For eukaryotic expression, these vectors as well as empty control vector and GFP expression vector as transfection control were transiently transfected in HEK-293 cells cultured on 175 cm^2 tissue culture vessels with 25 ml medium, each. Media harvest was performed 48 hours after transfection and the media supernatant was concentrated by the use of ultra-filtration columns to app. 0.4 ml, each.

The expression of wt human neurturin and the mutated variants was first controlled by native-PAGE/Western-blot using neurturin-specific antibodies for detection (see Figure 4). As control human recombinant neurturin expressed from *E. coli* was used.

Figure 4A shows that human wild-type neurturin (wt) as well as all modified neurturin variants except R63E were expressed in similar amounts. The apparent mass of the neurturin variants ranged between 20 and 25 kDa similar to wt neurturin.

The yield of expressed neurturin was determined by an ELISA specific for neurturin. Briefly, rabbit anti-neurturin-antibody was coated onto micro-wells for neurturin capture from the neurturin containing concentrated media supernatant. After washing, the captured neurturin was quantified by a luminescence read-out using biotin-conjugated goat anti-neurturin-antibody followed by the use of peroxidase-conjugated streptavidin. Human recombinant neurturin expressed from *E. coli* was used to establish a standard curve (see Figure 4B).

Furthermore, the biological activity of the expressed neurturin variants were quantified in a specific cellular assay. The assay principle is based on experiments by Tanaka et al., 2002, 2003 (Tanaka M, Xiao H, Kiuchi K. Heparin facilitates glial cell line-derived neurotrophic factor signal transduction. Neuroreport. 2002 Oct 28;13(15):1913-6; Tanaka M, Xiao H, Hirata Y, Kiuchi K. A rapid assay for glial cell line-derived neurotrophic factor and neurturin based on transfection of cells with tyrosine hydroxylase promoter-luciferase construct. Brain Res Brain Res Protoc. 2003 May;11(2): 119-22.) showing the induction of a tyrosine hydroxylase (TH) reporter gene construct by neurturin stably expressed in the human TGW neuroblastoma cell line. Briefly, TGW cells over-expressing the tyrosine hydroxylase (TH) reporter gene construct were treated with the neurturin containing samples leading to induction of MAPK pathway by neurturin signaling. The reporter

gene constructs contains a luciferase gene under control of repetitive serum response elements (SRE). The expression of luciferase is depending of MAPK pathway activation. The assay is carried out in 96 well plates. neurturin mediated luciferase expression is measured by addition of luciferin and analyzed with a luminescence reader.

A representative experiment demonstrating the quantification of the biological activity of neurturin using human recombinant neurturin expressed in *E. coli* as standard in combination with increasing serum concentrations is shown in Figure 5A.

Figure 5B shows that wild-type recombinant human neurturin as well as all neurturin variants except R63E were expressed in similar amounts resulting in protein concentrations between 1.2 - 1.7 g/l, and all expressed variants displayed a similar biological activity in vitro compared to wild-type recombinant human neurturin with an EC_{50} between 0.32 - 0.89 ng/ml (see Figure 5B). The human recombinant neurturin from *E. coli* used for comparison showed a weaker potency with an EC_{50} of 2.20 ng/ml probably resulting from a portion of not properly refolded neurturin in the sample (see Figure 5B).

Thus, Figure 5 shows that modifications in neurturin's "heel" region such as an introduction of a negatively charged, acidic amino acid into the basic patch does not compromise the biological activity of neurturin in general. This is surprising that the prior art shows that a modification from basic to acidic amino acids in the heel region would interfere with the biological activity of neurturin (see sequence and structure comparisons with other GFL, as discussed above).

The failure of expressing neurturin variant R63E shows that at this position a change from arginine to glutamic acid is not tolerated. Nevertheless, other modifications not resulting in a complete change of amino acid charge are functional. A sequence comparison of known neurturin homologs shows that

a R63E variant exists in the human neurturin homolog of *Ornithorhynchus anatinus*, *Monodelphis domestica*, and *Gallus gallus*. Also natural variants of the arginin residues at position 52, 57, 58, and 61 exist in different neurturin homologs (see Figure 6).

In order to create biologically active neurturin variants with improved bioavailability, the inventors replaced basic arginines in the "heel" region with Lysine. As arginine and lysine are structurally related, such exchanges are expected to affect the structure of the neurturin molecule only minimally. Such changes are enabling the site-specific directed PEGylation of neurturin via the the primary amine of the lysine side chain in a region of the protein shown to tolerate substantial amino acid changes, and which is therefore not important for biological activity. This reaction is highly specific since no lysine is present in the mature native human neurturin sequence. Reactions of activated PEG with other amines are minimized by optimizing reaction conditions, e.g. pH, temperature and reaction time. By placing a PEG modification within the basic patch of the "heel" region, two objectives are achieved: (i) the benefits of protein PEGylation (improved bioavailability via reduced renal clearance, reduced immunogenicity, improved protein stability) can be obtained while minimizing effects on protein activity and (ii) PEGylation in this region is expected to shield the basic patch by preventing interactions with negatively charged cell surface proteoglycans, which is also expected to improve protein bioavailability.

In a first step, the coding sequence of mature human neurturin which is optimized for human codon usage (with an additional N-terminal signal peptide for eukaryotic secretion) and an additional purification tag (repeat of six histidine residues with an additional site for cleavage of this purification tag with factor-Xa) is modified by PCR-based site directed mutagenesis. Here, codons are exchanged at arginine residues 51, 52, 54, 56, 57, 58, 60, 61, 63, or 65 with the codons for lysine residues.

The resulting expression construct referred to as **Kr-G3-H6-G3-Xa-**

rhneurturin in this invention containing the following features:

- Kr: DNA-sequence of mouse kringle-containing transmembrane protein 2, amino acids 1 - 26, signal peptide (GenBank Accession Number NP_082692) for cytoplasmic secretion optimized for human codon usage (amino acid sequence: MGTPHLQGFLLLFPLLLRLHGASAGS; SEQ ID NO: 2).
- G3: DNA-sequence of a glycine spacer optimized for human codon usage (amino acid sequence: GGG; SEQ ID NO: 3).
- H6: DNA-sequence of a 6-histidine tag for purification purposes optimized for human codon usage (amino acid sequence: HHHHHH; SEQ ID NO: 4).
- Xa: DNA-sequence of Factor-Xa protease recognition site optimized for human codon usage (amino acid sequence: IEGR; SEQ ID NO: 5). Factor-Xa protease cuts C-terminal after the arginine.
- rhneurturin: DNA-sequence of mature recombinant human neurturin optimized for human codon usage (amino acid sequence: ARLGARPCGLRELEVRVSELGLGYASDETVLFRYCAGACEAAARVY DLGLRRLRQRRRLRRERVRAQPCCRPTAYEDEVSFLDAHSRYHTV HELSARECACV; SEQ ID NO: 6). Single arginine codons at positions 51, 52, 54, 56, 57, 58, 60, 61, 63, or 65 are replaced by the lysine codon AAG in the respective neurturin variant expression construct.

The complete DNA sequence of Kr-G3-H6-G3-Xa-rhneurturin(wt) cloned into pMA vector is shown in Figure 7; SEQ ID NO: 7.

Example 5 – Peglyation of neurturin Variants

The resulting constructs are cloned in the eukaryotic expression vector pcDNA3.1+ which are transfected into HEK-293 cells for the expression of the modified neurturin variants as describe above.

The expressed neurturin variants are purified by ultra-filtration and by affinity

chromatography by means of the introduced purification tag.

The structure, purity and biological activity of the resulting neurturin variant preparations are checked by native-PAGE/Western-blot and neurturin activity assay as shown above.

Biological active neurturin variants which are expressed with sufficient yield and are purified to acceptable purity are used for covalent modification with different mono-disperse PEGs. This PEGs have a preferably size of 2.5 - 30 kDa, are linear or branched and activated with *N*-hydroxysuccinimide (NHS) ester. The *N*-hydroxysuccinimide (NHS) ester is spontaneously reactive with primary amines, providing for efficient PEGylation of proteins. Other PEG derivatives are for example but not limited to molecules with different sizes, not mono-disperse or with different branching patterns and coupling chemistries may be applied as well.

After the reaction, the purification tag is cleaved at the introduced proteinase site by factor-Xa and PEG-neurturin variants are purified by size exclusion and/or affinity chromatography.

A number of additional purification tags and protease site/recombinant protease combinations are well known in the art and may replace the His-Tag and/or factor-Xa site/factor Xa cleavage. Alternatively, the neurturin protein containing the introduced PEGylation site(s) may be purified from the supernatant of eukaryotic expression systems or from the lysate or supernatant of overexpressing bacteria without purification tag by chromatographic methods.

The structure, purity and biological activity of the resulting PEG-neurturin variant preparations are checked by SDS-PAGE/Western-blot, native-PAGE/ Western-blot, and neurturin activity assay as shown above.

Purified PEG-neurturin variants showing sufficient biological activity in vitro

are assayed in vivo for their pharmacokinetic behavior after subcutaneous administration of 50 µmol/kg s.c. to mice and rats. Plasma neurturin concentration is determined by neurturin ELISA at baseline, and at 0.25, 0.5, 1, 1.5, 2, 3, 4, 6 and 8 hours after administration.

Selected PEG-neurturin variants showing improved bioavailability compared to wt neurturin are tested for their efficacy in vivo by subcutaneous administration to neo STZ rats once daily 50 µmol/kg for 6 days. To assay beta-cell function, blood glucose is determined every day, and pancreatic insulin content is determined after 6 days treatment compared to vehicle control and wt neurturin groups.

Example 6 – Immunogenic potential of pegylated neurturin

Human recombinant wt neurturin has an immunogenic potential as demonstrated by the appearance of anti-neurturin antibodies after administration to adult mice for more than 2 weeks. The anti-neurturin antibodies have been detected with a specific assay. Briefly, recombinant human neurturin was coated on microplates to capture serum antibodies. After washing, the captured anti-neurturin antibodies were quantified by a luminescence read-out using biotin-conjugated goat anti-IgG antibody or goat anti-IgG/IgM antibody followed by the use of peroxidase conjugated streptavidin. Anti-neurturin IgG and anti-neurturin IgM diluted in serum was used to establish standard curves (see Figure 8).

As the PEGylation of pharmacologically active proteins reduces their immunogenic potential, selected PEG-neurturin variants with improved bioavailability and sustained potency are tested for their immunogenic potential by subcutaneous administration of once daily 50 µmol/kg for 28 days in adult mice. The appearance of anti-neurturin antibodies is determined by anti-neurturin antibody ELISA every weak compared to vehicle control and wt neurturin groups.

Claims

1. A neurturin conjugate comprising a polyol moiety covalently bound to a human neurturin protein product or a biologically active fragment thereof.

2. The neurturin conjugate according to claim 1, comprising a polypeptide encoded by the amino acid sequence set forth in SEQ ID. NO. 1, or a homologue, ortholog, variant, analog, derivative, biologically active fragment, or biologically active mutant thereof.

3. The neurturin conjugate according to claim 1 or 2, wherein the polyol moiety is a polyethylene glycol moiety.

4. The neurturin conjugate according to any one of the claims 1-3, wherein the polyol moiety is a single chain polyol moiety.

5. The neurturin conjugate according to any one of the claims 1-3, wherein the polyol moiety is a branched chain polyol moiety.

6. The neurturin conjugate according to any one of the claims 1-3 , wherein the polyol moiety is a polyalkylene glycol moiety.

7. The neurturin conjugate according to any one of the claims 1-6, having the same or a higher in vivo neurturin activity as native human neurturin.

8. The neurturin conjugate according to any one of the claims 1-7 having at least one amino acid change compared to human neurturin.

9. The neurturin conjugate according to claim 8, wherein the amino acid change is between amino acids 47-69, preferably 51-65 of mature human neurturin.

10. The neurturin conjugate according to any one of claims 8 or 9, wherein at least one of the arginine residues 51, 52, 54, 56, 57, 58, 60, 61 or 65 is deleted or substituted.

11. The neurturin conjugate according to claim 10, wherein at least one arginine residue is substituted by a neutral or acidic amino acid residue, e.g. glutamic acid.

12. The neurturin conjugate according to any one of claims 8-10, wherein at least one lysine residue is introduced.

13. A pharmaceutical composition comprising at least one neurturin protein product and/or a biologically active fragment thereof conjugated to at least one polyethylene glycol molecule as an active ingredient together with a pharmaceutically acceptable carrier, diluent and/or adjuvant.

14. The composition of claim 13, wherein said neurturin protein product is a human neurturin protein product or a biologically active fragment thereof.

15. The composition of any one of the claims 13 or 14, wherein said modified neurturin protein product is mono-PEGylated, comprising a single polyethylene glycol molecule chain.

16. The composition of any one of the claims 13 or 14, wherein said modified neurturin protein product is oligo- or poly-PEGylated, comprising two, three, four or several polyethylene glycol molecule chains.

17. The composition of any one of the claims 13-16, wherein said modified neurturin protein product comprises at least one linear polyethylene glycol molecule chain.

18. The composition of any one of the claims 13-16, wherein said modified neurturin protein product comprises at least one branched polyethylene glycol molecule chain.

19. The composition of any one of the claims 13-18, wherein said polyethylene glycol molecule has an average molecular weight of 100 to 10000 Da, preferably 200 to 8000 Da and most preferably of 1000 to 7000 Da.

20. The composition of any one of the claims 13-19, wherein said polyethylene glycol molecule is linked to the N-terminal amino acid of said modified neurturin protein product.

21. The composition of any one of the claims 13-20, wherein said polyethylene glycol molecule is linked to said modified neurturin protein product via an acyl or alkyl linkage.

22. The composition of any one of the claims 13-21, wherein said polyethylene glycol molecule is terminated by an OH-, OCH_3- or OEt-group.

23. The composition of any one of the claims 13-22, which has an increased bioavailability of the active ingredient compared to a composition with an unmodified control neurturin protein product.

24. The composition of any one of the claims 13-23, wherein said modified neurturin protein is present in an amount to provide an at least 2-fold, preferably at least 5-fold and more preferably at least 10-fold increase in the bioavailability of the active ingredient.

25. The composition of any one of the claims 13-24 for injection or infusion.

26. The composition of claim 25 for subcutaneous or intravenous injection.

27. The composition of any one of the claims 13-26 for the prevention or treatment of pancreatic or neurodegenerative disorders.

28. The composition of claim 27 for the prevention or treatment of diabetes mellitus type I, LADA and diabetes mellitus type II.

29. The composition of any one of the claims 13-28 for administration to a mammal, particularly a human.

30. A variant of human neurturin comprising at least one, e.g. 1, 2, 3 or 4 amino acid changes compared to wild-type human neurturin.

31. The variant of human neurturin of claim 30, wherein the amino acid changes are as defined in claims 9-12.

32. A method for administering a pharmaceutical composition to a subject in need thereof comprising a pharmaceutically active amount of at least one modified neurturin protein product or a biologically active fragment thereof which is conjugated to at least one polyethylene glycol molecule as an active ingredient together with a pharmaceutically acceptable carrier, diluent and/or adjuvant.

33. A method for administering a pharmaceutical composition to a subject in need thereof comprising a pharmaceutically active amount of at least one modified neurturin protein product or a biologically active fragment thereof which is conjugated to at least one polyethylene glycol molecule as an active ingredient together with a low-molecular weight substance together with a pharmaceutically acceptable carrier, diluent and/or adjuvant.