USE OF DEFENSINS AGAINST MENINGITIS

Reference to a Sequence Listing

This application contains a Sequence Listing in computer readable form. The computer readable form is incorporated herein by reference.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to the treatment of meningitis with defensin polypeptides.

Background

Meningitis is the inflammation of the protective membranes covering the central nervous system, known collectively as the meninges. Meningitis may develop in response to a number of causes, most prominently bacteria and viruses. Meningitis is a potentially serious condition owing to the proximity of the inflammation to the brain and spinal cord. The potential for serious neurological damage or even death necessitates prompt medical attention and evaluation. Bacterial meningitis is typically treated with antibiotics and requires close observation. Numerous microorganisms may cause bacterial meningitis, but *Streptococcus pneumoniae* ("pneumococcus") and *Neisseria meningitidis* ("meningococcus") are the most common pathogens in patients without immune deficiency. Bacterial meningitis is a serious threat to global health accounting for an estimated 171 000 deaths worldwide per year.

Streptococcus pneumoniae is the prevalent organism responsible for invasive bacterial infection in young children. Mortality in children with pneumococcal meningitis is at least twice as high as in meningococcal meningitis and the survivors have the highest incidence of sequelae. A recent study reports data from most European countries on the incidence of pneumococcal meningitis in children less than 5 years of age. Overall, the lowest incidence was reported in Finland (0.3/100000) and the highest in France (12.0/100000). Penicillin-resistant pneumococci are often multi-resistant, therefore posing serious problems for therapy. Resistance to macrolides is also widespread, being particularly high in the Mediterranean region.

It is an object of the present invention to provide polypeptides, which are capable of penetrating the blood-brain barrier, and methods of using these, for the treatment of meningitis.

SUMMARY OF THE INVENTION

We have now found that a synthetic defensin shows excellent activity against pneumococcal meningitis, and can be used in the treatment of bacterial meningitis.

In a first aspect, the present invention provides the use of a polypeptide having

antimicrobial activity, which comprises an amino acid sequence having at least 90% identity to the amino acid sequence of SEQ ID NO:1, for the manufacturing of a medicament for therapeutic treatment of meningitis.

In a second aspect, the present invention provides a polypeptide having antimicrobial activity, which comprises an amino acid sequence having at least 90% identity to the amino acid sequence of SEQ ID NO:1, for therapeutic treatment of meningitis.

In another aspect the present invention provides a method of treating meningitis, comprising administering to a subject in need of such treatment an effective amount, e.g. an anti-meningitis effective amount, of a polypeptide having antimicrobial activity, which comprises an amino acid sequence having at least 90% identity to the amino acid sequence of SEQ ID NO:1.

In one embodiment, the polypeptide is a defensin polypeptide, preferably a beta-defensin polypeptide. In another embodiment, the polypeptide is capable of penetrating the blood-brain barrier.

Meningitis according to the present invention may be bacterial meningitis, preferably pneumococcal meningitis, such as meningitis caused by a *Streptococcus*, preferably *Streptococcus pneumoniae*. In a preferred embodiment, the meningitis is caused by a penicillin-resistant *Streptococcus pneumoniae*.

A polypeptide for use according to the present invention or for treating meningitis according to the present invention is designated hereinafter as "polypeptide(s) of (according to) the present invention".

DETAILED DESCRIPTION OF THE INVENTION

Definitions

Antimicrobial activity: The term “antimicrobial activity” is defined herein as an activity which is capable of killing or inhibiting growth of microbial cells. In the context of the present invention the term “antimicrobial” is intended to mean that there is a bactericidal and/or a bacteriostatic and/or fungicidal and/or fungistatic effect and/or a virucidal effect, wherein the term "bactericidal" is to be understood as capable of killing bacterial cells. The term "bacteriostatic" is to be understood as capable of inhibiting bacterial growth, i.e. inhibiting growing bacterial cells. The term "fungicidal" is to be understood as capable of killing fungal cells. The term "fungistatic" is to be understood as capable of inhibiting fungal growth, i.e. inhibiting growing fungal cells. The term "virucidal" is to be understood as capable of inactivating virus. The term "microbial cells" denotes bacterial or fungal cells (including yeasts).

In the context of the present invention the term “inhibiting growth of microbial cells” is intended to mean that the cells are in the non-growing state, i.e., that they are not able to

propagate.

In a preferred embodiment, the term "antimicrobial activity" is defined as bactericidal and/or bacteriostatic activity. More preferably, "antimicrobial activity" is defined as bactericidal and/or bacteriostatic activity against Streptococci, preferably *Streptococcus pneumoniae*.

For purposes of the present invention, antimicrobial activity may be determined according to the procedure described by Lehrer *et al.*, Journal of Immunological Methods, Vol. 137 (2) pp. 167-174 (1991). Alternatively, antimicrobial activity may be determined according to the NCCLS guidelines from CLSI (Clinical and Laboratory Standards Institute; formerly known as National Committee for Clinical and Laboratory Standards).

Polypeptides having antimicrobial activity may be capable of reducing the number of living cells of *Streptococcus pneumoniae* (ATCC 49619) to 1/100 after 8 hours (preferably after 4 hours, more preferably after 2 hours, most preferably after 1 hour, and in particular after 30 minutes) incubation at 37°C in a relevant microbial growth substrate at a concentration of 500 µg/ml; preferably at a concentration of 250 µg/ml; more preferably at a concentration of 100 µg/ml; even more preferably at a concentration of 50 µg/ml; most preferably at a concentration of 25 µg/ml; and in particular at a concentration of 10 µg/ml of the polypeptides having antimicrobial activity.

Polypeptides having antimicrobial activity may also be capable of inhibiting the outgrowth of *Streptococcus pneumoniae* (ATCC 49619) for 8 hours at 37°C in a relevant microbial growth substrate, when added in a concentration of 500 µg/ml; preferably when added in a concentration of 250 µg/ml; more preferably when added in a concentration of 100 µg/ml; even more preferably when added in a concentration of 50 µg/ml; most preferably when added in a concentration of 10 µg/ml; and in particular when added in a concentration of 5 µg/ml.

The polypeptides of the present invention have at least 20%, preferably at least 40%, more preferably at least 50%, more preferably at least 60%, more preferably at least 70%, more preferably at least 80%, even more preferably at least 90%, most preferably at least 95%, and even most preferably at least 100% of the antimicrobial activity of the polypeptide consisting of the amino acid sequence of SEQ ID NO:1.

Defensin: The term "defensin" as used herein refers to polypeptides recognized by a person skilled in the art as belonging to the defensin class of antimicrobial peptides. To determine if a polypeptide is a defensin according to the invention, the amino acid sequence is preferably compared with the hidden markov model profiles (HMM profiles) of the PFAM database by using the freely available HMMER software package (see Example 1).

The PFAM defensin families include Defensin_1 or "Mammalian defensin" (accession no. PF00323), Defensin_2 or "Arthropod defensin" (accession no. PF01097), Defensin_beta or "Beta Defensin" (accession no. PF00711), Defensin_propep or "Defensin propeptide" (accession no. PF00879) and Gamma-thionin or "Gamma-thionins family" (accession no.

PF00304).

The defensins may belong to the alpha-defensin class, the beta-defensin class, the theta-defensin class, the insect or arthropod defensin classes, or the plant defensin class.

In an embodiment, the amino acid sequence of a defensin according to the invention comprises 4, 5, 6, 7, or 8 cysteine residues, preferably 4, 5, or 6 cysteine residues, more preferably 4 or 6 cysteine residues, and most preferably 6 cysteine residues.

The defensins may also be synthetic defensins sharing the characteristic features of any of the defensin classes.

Examples of such defensins include, but are not limited to, α-Defensin HNP-1 (human neutrophil peptide) HNP-2 and HNP-3; β-Defensin-12, Drosomycin, Heliomicin, γ1-purothionin, Insect defensin A, and the defensins disclosed in PCT applications WO 99/53053, WO 02/06324, WO 02/085934, WO 03/044049, WO 2006/050737 and WO 2006/053565.

Isolated polypeptide: The term "isolated variant" or "isolated polypeptide" as used herein refers to a variant or a polypeptide that is isolated from a source. In one aspect, the variant or polypeptide is at least 1% pure, preferably at least 5% pure, more preferably at least 10% pure, more preferably at least 20% pure, more preferably at least 40% pure, more preferably at least 60% pure, even more preferably at least 80% pure, and most preferably at least 90% pure, as determined by SDS-PAGE.

Substantially pure polypeptide: The term "substantially pure polypeptide" denotes herein a polypeptide preparation that contains at most 10%, preferably at most 8%, more preferably at most 6%, more preferably at most 5%, more preferably at most 4%, more preferably at most 3%, even more preferably at most 2%, most preferably at most 1%, and even most preferably at most 0.5% by weight of other polypeptide material with which it is natively or recombinantly associated. It is, therefore, preferred that the substantially pure polypeptide is at least 92% pure, preferably at least 94% pure, more preferably at least 95% pure, more preferably at least 96% pure, more preferably at least 96% pure, more preferably at least 97% pure, more preferably at least 98% pure, even more preferably at least 99%, most preferably at least 99.5% pure, and even most preferably 100% pure by weight of the total polypeptide material present in the preparation. The polypeptides of the present invention are preferably in a substantially pure form. This can be accomplished, for example, by preparing the polypeptide by well-known recombinant methods or by classical purification methods.

Identity: The relatedness between two amino acid sequences or between two nucleotide sequences is described by the parameter "identity".

For purposes of the present invention, the degree of identity between two amino acid sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000,

Trends in Genetics 16: 276-277; http://emboss.org), preferably version 3.0.0 or later. The optional parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. The output of Needle labeled "longest identity" (obtained using the –nobrief option) is used as the percent identity and is calculated as follows:

(Identical Residues x 100)/(Length of Alignment – Total Number of Gaps in Alignment)

For purposes of the present invention, the degree of identity between two deoxyribonucleotide sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *supra*) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *supra*; http://emboss.org), preferably version 3.0.0 or later. The optional parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EDNAFULL (EMBOSS version of NCBI NUC4.4) substitution matrix. The output of Needle labeled "longest identity" (obtained using the –nobrief option) is used as the percent identity and is calculated as follows: (Identical Deoxyribonucleotides x 100)/(Length of Alignment – Total Number of Gaps in Alignment).

Allelic variant: The term "allelic variant" denotes herein any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having altered amino acid sequences. An allelic variant of a polypeptide is a polypeptide encoded by an allelic variant of a gene.

Modification: The term "modification" means herein any chemical modification of the polypeptide consisting of the amino acid sequence of SEQ ID NO:1 as well as genetic manipulation of the DNA encoding that polypeptide. The modification(s) can be substitution(s), deletion(s) and/or insertions(s) of the amino acid(s) as well as replacement(s) of amino acid side chain(s); or use of unnatural amino acids with similar characteristics in the amino acid sequence. In particular the modification(s) can be amidations, such as amidation of the C-terminus.

Polypeptides Having Antimicrobial Activity

In a first aspect, the present invention relates to isolated polypeptides having an amino acid sequence which has a degree of identity to SEQ ID NO:1 (*i.e.*, the mature polypeptides) of at least 80%, preferably at least 85%, more preferably at least 90%, most preferably at least 95%, and in particular at least 97%, which have antimicrobial activity (hereinafter "homologous polypeptides"). In a preferred aspect, the homologous polypeptides have an amino acid sequence which differs by at the most six amino acids, preferably by at the most five amino

acids, more preferably by at the most four amino acids, even more preferably by at the most three amino acids, most preferably by at the most two amino acids, and in particular by one amino acid from the amino acid sequence of SEQ ID NO:1.

A polypeptide of the present invention preferably comprises the amino acid sequence of SEQ ID NO:1 or an allelic variant thereof. In a preferred aspect, a polypeptide comprises the amino acid sequence of SEQ ID NO:1. In another preferred aspect, a polypeptide consists of the amino acid sequence of SEQ ID NO:1 or an allelic variant thereof. In another preferred aspect, a polypeptide consists of the amino acid sequence of SEQ ID NO:1.

Preferably, amino acid changes are of a minor nature, that is conservative amino acid substitutions or insertions that do not significantly affect the folding and/or activity of the polypeptide; single deletions; small amino- or carboxyl-terminal extensions; a small linker peptide of up to about 20-25 residues; or a small extension that facilitates purification by changing net charge or another function, such as a poly-histidine tag, an antigenic epitope or a binding domain.

Examples of conservative substitutions are within the group of basic amino acids (arginine, lysine and histidine), acidic amino acids (glutamic acid and aspartic acid), polar amino acids (glutamine and asparagine), hydrophobic amino acids (leucine, isoleucine and valine), aromatic amino acids (phenylalanine, tryptophan and tyrosine), and small amino acids (glycine, alanine, serine, threonine and methionine). Amino acid substitutions which do not generally alter specific activity are known in the art and are described, for example, by H. Neurath and R.L. Hill, 1979, *In*, *The Proteins*, Academic Press, New York. The most commonly occurring exchanges are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, and Asp/Gly.

In addition to the 20 standard amino acids, non-standard amino acids (such as 4-hydroxyproline, 6-*N*-methyl lysine, 2-aminoisobutyric acid, isovaline, and alpha-methyl serine) may be substituted for amino acid residues of a wild-type polypeptide. A limited number of non-conservative amino acids, amino acids that are not encoded by the genetic code, and unnatural amino acids may be substituted for amino acid residues. “Unnatural amino acids” have been modified after protein synthesis, and/or have a chemical structure in their side chain(s) different from that of the standard amino acids. Unnatural amino acids can be chemically synthesized, and preferably, are commercially available, and include pipecolic acid, thiazolidine carboxylic acid, dehydroproline, 3- and 4-methylproline, and 3,3-dimethylproline.

Essential amino acids in the parent polypeptide can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, 1989, *Science* 244: 1081-1085). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological activity (*i.e.*, antimicrobial activity) to identify amino acid

residues that are critical to the activity of the molecule. See also, Hilton *et al.*, 1996, *J. Biol. Chem.* 271: 4699-4708. The biological interaction can also be determined by physical analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography, electron diffraction, or photoaffinity labeling, in conjunction with mutation of putative contact site amino acids. See, for example, de Vos *et al.*, 1992, *Science* 255: 306-312; Smith *et al.*, 1992, *J. Mol. Biol.* 224: 899-904; Wlodaver *et al.*, 1992, *FEBS Lett.* 309:59-64. The identities of essential amino acids can also be inferred from analysis of identities with polypeptides which are related to a polypeptide according to the invention.

Single or multiple amino acid substitutions can be made and tested using known methods of mutagenesis, recombination, and/or shuffling, followed by a relevant screening procedure, such as those disclosed by Reidhaar-Olson and Sauer, 1988, *Science* 241: 53-57; Bowie and Sauer, 1989, *Proc. Natl. Acad. Sci. USA* 86: 2152-2156; WO 95/17413; or WO 95/22625. Other methods that can be used include error-prone PCR, phage display (*e.g.*, Lowman *et al.*, 1991, *Biochem.* 30:10832-10837; U.S. Patent No. 5,223,409; WO 92/06204), and region-directed mutagenesis (Derbyshire *et al.*, 1986, *Gene* 46:145; Ner *et al.*, 1988, *DNA* 7:127).

Mutagenesis/shuffling methods can be combined with high-throughput, automated screening methods to detect activity of cloned, mutagenized polypeptides expressed by host cells. Mutagenized DNA molecules that encode active polypeptides can be recovered from the host cells and rapidly sequenced using standard methods in the art. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide of interest, and can be applied to polypeptides of unknown structure.

In a preferred embodiment, the polypeptides of the invention are defensin polypeptides, preferably beta-defensin polypeptides.

N-Terminal Extension

An N-terminal extension of the polypeptides of the invention may suitably consist of from 1 to 50 amino acids, preferably 2-20 amino acids, especially 3-15 amino acids. In one embodiment N-terminal peptide extension does not contain an Arg (R). In another embodiment the N-terminal extension comprises a kex2 or kex2-like cleavage site as will be defined further below. In a preferred embodiment the N-terminal extension is a peptide, comprising at least two Glu (E) and/or Asp (D) amino acid residues, such as an N-terminal extension comprising one of the following sequences: EAE, EE, DE and DD.

Kex2 Sites

Kex2 sites (see, e.g., Methods in Enzymology Vol 185, ed. D. Goeddel, Academic Press Inc. (1990), San Diego, CA, "Gene Expression Technology") and kex2-like sites are di-basic recognition sites (i.e., cleavage sites) found between the pro-peptide encoding region and the

mature region of some proteins.

Insertion of a kex2 site or a kex2-like site have in certain cases been shown to improve correct endopeptidase processing at the pro-peptide cleavage site resulting in increased protein secretion levels.

In the context of the invention insertion of a kex2 or kex2-like site result in the possibility to obtain cleavage at a certain position in the N-terminal extension resulting in an antimicrobial polypeptide being extended in comparison to the mature polypeptide shown in SEQ ID NO:1.

Fused Polypeptides

The polypeptides of the present invention also include fused polypeptides or cleavable fusion polypeptides in which another polypeptide is fused at the N-terminus or the C-terminus of the polypeptide of the invention or a fragment thereof. A fused polypeptide is produced by fusing a nucleotide sequence (or a portion thereof) encoding another polypeptide to a nucleotide sequence (or a portion thereof) of the present invention. Techniques for producing fusion polypeptides are known in the art, and include ligating the coding sequences encoding the polypeptides so that they are in frame and that expression of the fused polypeptide is under control of the same promoter(s) and terminator.

Methods and Uses

The invention relates to the use of a polypeptide of the invention for treating meningitis. Accordingly, the polypeptides of the invention may be used as an antimicrobial veterinarian or human therapeutic or prophylactic agent. Thus, defensin variants of the invention may be used in the preparation of veterinarian or human therapeutic agents or prophylactic agents for the treatment of meningitis.

The polypeptides of the invention may be used in an amount sufficient to kill or inhibit growth of Streptococcus sp., such as *Streptococcus pneumoniae*.

Formulations of the polypeptides of the invention are administered to a host suffering from or predisposed to meningitis, such as bacterial meningitis, for example pneumococcal meningitis. In an embodiment, meningitis is caused by infection with a penicilin-resistant *Streptococcus pneumoniae*.

Administration may be localized or systemic. Generally the dose of the antimicrobial polypeptides of the invention will be sufficient to decrease the microbial population by at least 1 log, and may be by 2 or more logs of killing. The polypeptides of the present invention are administered at a dosage that reduces the microbial population while minimizing any side-effects. It is contemplated that the composition will be obtained and used under the guidance of a physician for *in vivo* use.

Various methods for administration may be employed. The polypeptide formulation may

be given orally, or may be injected intravascularly, intramuscular, subcutaneously, peritoneally, by aerosol, opthalmically, intra-bladder, topically, *etc*. The dosage of the therapeutic formulation will vary widely, depending on the specific antimicrobial polypeptide to be administered, the frequency of administration, the manner of administration, the clearance of the agent from the host, and the like. The initial dose may be larger, followed by smaller maintenance doses. In many cases, oral administration will require a higher dose than if administered intravenously. The amide bonds, as well as the amino and carboxy termini, may be modified for greater stability on oral administration. For example, the carboxy terminus may be amidated.

Formulations

The polypeptides of this invention can be incorporated into a variety of formulations for therapeutic administration. More particularly, the polypeptides of the present invention can be formulated into pharmaceutical compositions by combination with appropriate, pharmaceutically acceptable carriers or diluents, and may be formulated into preparations in solid, semi-solid, liquid or gaseous forms, such as tablets, capsules, powders, granules, ointments, creams, foams, solutions, suppositories, injections, inhalants, gels, microspheres, lotions, and aerosols. As such, administration of the polypeptides can be achieved in various ways, including oral, buccal, rectal, parenteral, intraperitoneal, intradermal, transdermal, intracheal, *etc.,* administration. The antimicrobial polypeptides of the invention may be systemic after administration or may be localized.

The polypeptides of the present invention can be administered alone, in combination with each other, or they can be used in combination with other known compounds *(e.g.,* perforin, anti-inflammatory agents, antibiotics, *etc.)* In pharmaceutical dosage forms, the polypeptides may be administered in the form of their pharmaceutically acceptable salts. The following methods and excipients are merely exemplary and are in no way limiting.

For oral preparations, the polypeptides can be used alone or in combination with appropriate additives to make tablets, powders, granules or capsules, for example, with conventional additives, such as lactose, mannitol, corn starch or potato starch; with binders, such as crystalline cellulose, cellulose derivatives, acacia, corn starch or gelatins; with disintegrators, such as corn starch, potato starch or sodium carboxymethylcellulose; with lubricants, such as talc or magnesium stearate; and if desired, with diluents, buffering agents, moistening agents, preservatives and flavoring agents.

The polypeptides can be formulated into preparations for injections by dissolving, suspending or emulsifying them in an aqueous or nonaqueous solvent, such as vegetable or other similar oils, synthetic aliphatic acid glycerides, esters of higher aliphatic acids or propylene glycol; and if desired, with conventional additives such as solubilizers, isotonic agents, suspending agents, emulsifying agents, stabilizers and preservatives.

The polypeptides can be utilized in aerosol formulation to be administered via inhalation. The polypeptides of the present invention can be formulated into pressurized acceptable propellants such as dichlorodifluoromethane, propane, nitrogen and the like.

Furthermore, the polypeptides can be made into suppositories by mixing with a variety of bases such as emulsifying bases or water-soluble bases. The polypeptides of the present invention can be administered rectally via a suppository. The suppository can include vehicles such as cocoa butter, carbowaxes and polyethylene glycols, which melt at body temperature, yet are solidified at room temperature.

Unit dosage forms for oral or rectal administration such as syrups, elixirs, and suspensions may be provided wherein each dosage unit, for example, teaspoonful, tablespoonful, tablet or suppository, contains a predetermined amount of the composition containing one or more polypeptides of the present invention. Similarly, unit dosage forms for injection or intravenous administration may comprise the polypeptide of the present invention in a composition as a solution in sterile water, normal saline or another pharmaceutically acceptable carrier.

Implants for sustained release formulations are well-known in the art. Implants are formulated as microspheres, slabs, *etc.* with biodegradable or non-biodegradable polymers. For example, polymers of lactic acid and/or glycolic acid form an erodible polymer that is well-tolerated by the host. The implant containing the antimicrobial polypeptides of the invention is placed in proximity to the site of infection, so that the local concentration of active agent is increased relative to the rest of the body.

The term "unit dosage form", as used herein, refers to physically discrete units suitable as unitary dosages for human and animal subjects, each unit containing a predetermined quantity of polypeptides of the present invention calculated in an amount sufficient to produce the desired effect in association with a pharmaceutically acceptable diluent, carrier or vehicle. The specifications for the unit dosage forms of the present invention depend on the particular polypeptide employed and the effect to be achieved, and the pharmacodynamics associated with the polypeptide in the host.

The pharmaceutically acceptable excipients, such as vehicles, adjuvants, carriers or diluents, are readily available to the public. Moreover, pharmaceutically acceptable auxiliary substances, such as pH adjusting and buffering agents, tonicity adjusting agents, stabilizers, wetting agents and the like, are readily available to the public.

Typical dosages for systemic administration range from 0.1 pg to 100 milligrams per kg weight of subject per administration. A typical dosage may be one tablet taken from two to six times daily, or one time-release capsule or tablet taken once a day and containing a proportionally higher content of active ingredient. The time-release effect may be obtained by capsule materials that dissolve at different pH values, by capsules that release slowly by

osmotic pressure, or by any other known means of controlled release.

Those of skill will readily appreciate that dose levels can vary as a function of the specific polypeptide, the severity of the symptoms and the susceptibility of the subject to side effects. Some of the specific polypeptides are more potent than others. Preferred dosages for a given polypeptide are readily determinable by those of skill in the art by a variety of means. A preferred means is to measure the physiological potency of a given polypeptide.

The use of liposomes as a delivery vehicle is one method of interest. The liposomes fuse with the cells of the target site and deliver the contents of the lumen intracellularly. The liposomes are maintained in contact with the cells for sufficient time for fusion, using various means to maintain contact, such as isolation, binding agents, and the like. In one aspect of the invention, liposomes are designed to be aerosolized for pulmonary administration. Liposomes may be prepared with purified proteins or peptides that mediate fusion of membranes, such as Sendai virus or influenza virus, etc. The lipids may be any useful combination of known liposome forming lipids, including cationic or zwitterionic lipids, such as phosphatidylcholine. The remaining lipid will be normally be neutral or acidic lipids, such as cholesterol, phosphatidyl serine, phosphatidyl glycerol, and the like.

For preparing the liposomes, the procedure described by Kato *et al.* (1991) *J. Biol. Chem.* 266:3361 may be used. Briefly, the lipids and lumen composition containing peptides are combined in an appropriate aqueous medium, conveniently a saline medium where the total solids will be in the range of about 1-10 weight percent. After intense agitation for short periods of time, from about 5-60 sec., the tube is placed in a warm water bath, from about 25-40°C and this cycle repeated from about 5-10 times. The composition is then sonicated for a convenient period of time, generally from about 1-10 sec. and may be further agitated by vortexing. The volume is then expanded by adding aqueous medium, generally increasing the volume by about from 1-2 fold, followed by shaking and cooling. This method allows for the incorporation into the lumen of high molecular weight molecules.

Formulations with Other Active Agents

For use in the subject methods, the antimicrobial polypeptides of the invention may be formulated with other pharmaceutically active agents, particularly other antimicrobial agents. Other agents of interest include a wide variety of antibiotics, as known in the art. Classes of antibiotics include penicillins, e.g. penicillin G, penicillin V, methicillin, oxacillin, carbenicillin, nafcillin, ampicillin, etc.; penicillins in combination with beta-lactamase inhibitors, cephalosporins, e.g. cefaclor, cefazolin, cefuroxime, moxalactam, etc.; carbapenems; monobactams; aminoglycosides; tetracyclines; macrolides; lincomycins; polymyxins; sulfonamides; quinolones; cloramphenical; metronidazole; spectinomycin; trimethoprim; vancomycin; etc.

Anti-mycotic agents are also useful, including polyenes, e.g. amphotericin B, nystatin; 5-flucosyn; and azoles, e.g. miconazol, ketoconazol, itraconazol and fluconazol. Antituberculotic drugs include isoniazid, ethambutol, streptomycin and rifampin. Cytokines may also be included in a formulation of the antimicrobial polypeptides of the invention, e.g. interferon gamma, tumor necrosis factor alpha, interleukin 12, etc.

***In vitro* synthesis**

The polypeptides of the invention may be prepared by *in vitro* synthesis, using conventional methods as known in the art. Various commercial synthetic apparatuses are available, for example automated synthesizers by Applied Biosystems Inc., Beckman, etc. By using synthesizers, naturally occurring amino acids may be substituted with unnatural amino acids, particularly D-isomers (or D-forms) e.g. D-alanine and D-isoleucine, diastereoisomers, side chains having different lengths or functionalities, and the like. The particular sequence and the manner of preparation will be determined by convenience, economics, purity required, and the like.

Chemical linking may be provided to various peptides or proteins comprising convenient functionalities for bonding, such as amino groups for amide or substituted amine formation, e.g. reductive amination, thiol groups for thioether or disulfide formation, carboxyl groups for amide formation, and the like.

If desired, various groups may be introduced into the peptide during synthesis or during expression, which allow for linking to other molecules or to a surface. Thus cysteines can be used to make thioethers, histidines for linking to a metal ion complex, carboxyl groups for forming amides or esters, amino groups for forming amides, and the like.

The polypeptides may also be isolated and purified in accordance with conventional methods of recombinant synthesis. A lysate may be prepared of the expression host and the lysate purified using HPLC, exclusion chromatography, gel electrophoresis, affinity chromatography, or other purification technique. For the most part, the compositions which are used will comprise at least 20% by weight of the desired product, more usually at least about 75% by weight, preferably at least about 95% by weight, and for therapeutic purposes, usually at least about 99.5% by weight, in relation to contaminants related to the method of preparation of the product and its purification. Usually, the percentages will be based upon total protein

The present invention is further described by the following examples that should not be construed as limiting the scope of the invention.

EXAMPLES

EXAMPLE 1

Using the HMM files from the PFAM database to identify a defensin

Sequence analysis using hidden markov model profiles (HMM profiles) may be carried out either online on the Internet or locally on a computer using the well-known HMMER freely available software package. The current version is HMMER 2.3.2 from October 2003.

The HMM profiles may be obtained from the well-known PFAM database. The current version is PFAM 16.0 from November 2004. Both HMMER and PFAM are available for all computer platforms from e.g. Washington University in St. Louis (USA), School of Medicine (http://pfam.wustl.edu and http://hmmer.wustl.edu).

If a query amino acid sequence, or a fragment thereof, belongs to one of the following five PFAM families, the amino acid sequence is a defensin according to the present invention:

- Defensin_beta or "Beta Defensin", accession number: PF00711;
- Defensin_propep or "Defensin propeptide", accession number: PF00879;
- Defensin_1 or "Mammalian defensin", accession number: PF00323;
- Defensin_2 or "Arthropod defensin", accession number: PF01097;
- Gamma-thionin or "Gamma-thionins family", accession number: PF00304.

An amino acid sequence belongs to a PFAM family, according to the present invention, if it generates an E-value which is greater than 0.1, and a score which is larger or equal to zero, when the PFAM database is used online, or when the hmmpfam program (from the HMMER software package) is used locally.

When the sequence analysis is carried out locally using the hmmpfam program, it is necessary to obtain (download) the HMM profiles from the PFAM database. Two profiles exist for each family; **xxx_ls.hmm** for glocal searches, and **xxx_fs.hmm** for local searches ("xxx" is the name of the family). That makes a total of ten profiles for the five families mentioned above.

These ten profiles may be used individually, or joined (appended) into a single profile (using a text editor - the profiles are ASCII files) that could be named e.g. defensin.hmm. A query amino acid sequence can then be evaluated by using the following command line:

hmmpfam -E 0.1 defensin.hmm sequence_file

- wherein "sequence_file" is a file with the query amino acid sequence in any of the formats recognized by the HMMER software package.

If the score is larger or equal to zero (0.0), and the E-value is greater than 0.1, the query amino acid sequence is a defensin according to the present invention.

The PFAM database is further described in Bateman et al. (2004) "The Pfam Protein Families Database", Nucleic Acids Research, Vol. 32 (Database Issue) pp. D138-D141.

EXAMPLE 2

Investigating CSF penetration and bactericidal effect of a synthetic defensin during meningitis

An Isolate of a penicillin-resistant serotype 9V *Streptococcus pneumoniae* (1395), originally isolated from the CSF of a 78 year old woman with meningitis, was used in the meningitis model. This strain was formerly used for evaluating the effectiveness of treatment of meningitis with Moxifloxacin (see Ostergaard *et al.* "Evaluation of moxifloxacin, a new 8-methoxyquinolone, for treatment of meningitis caused by a penicillin-resistant pneumococcus in rabbits", *Antimicrob Agents Chemother*, vol. 42(7), pp. 1706-1712 (1998)).

The virulence of the strain was enhanced by passing the bacteria through a mouse peritonitis model. Bacteria from sampled peritoneal fluid were grown on blood agar plates, recovered and diluted in beef-broth with 10% glycerol added and frozen at -80°C.

The bacterial aliquots were thawn and grown on blood-agar plates, suspended in sterile beef-broth to reach an optical density of 3.5 at 540 nm, and subsequently diluted to obtain a final concentration of 1-2 x 10^6 CFU/ml.

The synthetic defensin used in the experiment is a polypeptide having the amino acid sequence shown in SEQ ID NO:1. In the Example, this synthetic defensin will be referred to as "Meningicin". The minimium inhibitory concentration (MIC) of Meningicin against *Streptococcus pneumoniae* (1395) was determined to be 0.25 µg/ml.

In the effect-study, the effectiveness of Meningicin in clearing the meningeal infection was compared to that of Ceftriaxone, which is an antibiotic frequently used for treating patients suffering from pneumococcal meningitis with penicillin resistant strains, and to the effect of giving no treatment ("vehicle"). The MIC of Ceftriaxone against *Streptococcus pneumoniae* (1395) was determined to be 0.5 µg/ml.

Meningitis model

Male New Zealand white rabbits weighing approx. 2500 g was used for the experiments. The rabbits arrived at the facilities 1-2 weeks prior to the experiment to allow for adaptation. They were kept in single cages covered with hay and offered food and water with no restrictions. Veterinarian expertise was available at the laboratory, and the experimental design was approved by the local animal legislation committee.

Preparation for fixation in a stereotactic frame

The rabbit was weighed and anaesthetized with dormicum 0.5 ml/kg (midazolam 5 mg/ml) s.c. and after 10 minutes with hypnorm 0.35 ml/kg (fentanylcitrat + fluanison) i.m.

The ears, scalp and neck of the rabbit were shaved and the skin disinfected. An incision measuring approx. 2 cm was made on the forehead of the rabbit and the scalp was exposed by blunt dissection. 4 bore holes were made demarcating a square and 4 screws were screwed up at right angles to the surface (2.5 to 3 turns). An acrylic helmet, embedding a turnbuckle, was

moulded from dental casting material directly onto the scalp of the rabbit and the helmet was cooled under running water. The rabbit was put back into its cage with free access to food and water to rest for 8-10 hours until the time of bacterial inoculation or start of pharmacokinetic treatment study on uninfected rabbits. Analgesia with buprenorfin, 0.1 mg/kg, was given.

Bacterial inoculation

In the evening, at 10 p.m., the day before the treatment experiments with Meningicin, the rabbit was re-anaesthetized with hypnorm-dormicum. The head of the rabbit was immobilised in a stereotactic frame and a spinal canula was introduced in cisterna magna for the inoculation of 1×10^5 CFU pneumococci. After the bacterial inoculation, the rabbit was put back into its cage with access to food and water for another 8 hours. Analgesia with buprenorfin, 0.1 mg/kg, was given (note: uninfected rabbits did not experience the procedure with the bacterial inoculation).

Setup for studies on pharmacokinetics and effect-studies

The rabbit was re-anaesthetized with urethan 3.5 ml/kg (dimethyl-acrylat 50%, 1.75 g/kg) s.c. at 7.30 a.m.. A venous catheter was applied in the left ear-vein and Mebumal approx. 0.5-1 ml (Pentobarbital 50 mg/ml) i.v. was infused slowly until the rabbit was asleep and deeply anaesthetized. A three-way tap was connected to the venous catheter and syringes containing pentobarbital for supplemental anaesthetics and isotonic NaCl and heparin 1 UI/ml for flushing were connected to the tap. Rabbits were observed every half hour. When the need for supplemental anaesthesia arose, additional 0.2 ml Mebumal (Pentobarbital 50 mg/ml) was given. During the experiment observations and anaesthetics were recorded in the scheme of the laboratory animal-department and in the book of the Laboratory Animal Inspection.

An arterial canula was applied in the artery of the right ear and used for blood-sampling throughout the experiment. A bolt was fastened to the turnbuckle embedded in the acrylic helmet, and the rabbit's head was fixed in a stereotactic frame. The rabbit remained fixed throughout the experiment.

Cisterna magna was punctured with a spinal-canula, fastened to the stereotactic frame. The canula was left in place throughout the experiment and used for CSF-sampling.

At the time-point relevant to the conducted experiment 1 ml of blood and 0.3 ml of CSF was aspirated from the arterial canula / the spinal needle and transferred to EDTA-tubes / Eppendorf-tubes. After the last sampling, the experiment was terminated and the rabbits were killed with an overdose of Mebumal (Pentobarbital 200 mg/ml).

Analyses

CSF and plasma concentrations of Meningicin were measured by use of HPLC. When evaluating bacterial concentrations in inocula and samples, serial 10-fold dilutions (requiring 20

µl of test-material) plated as spots of 50 µl on blood-agar plates were used for calculations of CFU/ml.

Specific to studies on pharmacokinetics

Meningicin (dosages of 20, 40 and 80 mg/kg respectively) was administered through the three-way tap as an intravenous bolus infusion over 10 minutes at the beginning of the experiment.

Rabbits used for the study of passage of Meningicin through inflamed meninges were infected as described above and awaited i.v. Meningicin administration until 10 hours after bacterial inoculation. Groups of two rabbits were used for studying the penetration (corresponding CSF and plasma levels) of Meningicin at 3 different doses through inflamed and un-inflamed meninges. Blood and CSF were sampled at 0, ¼, ½, 1, 1½, 2, 3, 4, 5 and 6 hours after administration of Meningicin to determine the Meningicin concentration (HPLC). Based on MIC and the pharmacokinetics observed for Meningicin, the dosing regimes for the treatment-trials were established.

Specific to studies on efficacy

After applying the spinal canula 0.5 ml of CSF was aspirated into a syringe. 0.1 ml was used for dissolving the bacterial inoculum before inoculation and 0.2 ml was used for flushing the syringe and canula afterwards. Serial 10-fold dilutions of the inoculum were made to verify the infectious dose.

Treatment with Meningicin or Ceftriaxone was initialized 10-11 hours after the bacterial inoculation. Sampling of blood and cerebrospinal fluid were made at 0, 1, 3, 5, 6 and 10 hours after intravenous dose of antibiotics. The concentrations of antibiotics and bacterial counts were determined.

Results of pharmacokinetics

As described above, Meningicin was administered in dosages of 20 mg/kg, 40 mg/kg and 80 mg/kg. The concentration of Meningicin in serum from infected rabbits was measured and “area under the curve” (AUC serum) was calculated. Likewise, the concentration of Meningicin in cerebrospinal fluid (CSF) was measured and “area under the curve” (AUC CSF) was calculated.

Meningicin dosage (mg/kg)	Peak concentration in serum (µg/l)	AUC serum (µg/l)	Peak concentration in CSF (µg/l)	AUC CSF (µg/l)	Meningicin penetration of the blood-brain barrier

20	31,390	40,162	2,001	8,557	21%
40	113,117	97,040	10,230	47,902	49%
80	376,140	192,990	8,040	30,476	16%

Results of efficacy studies

The efficacy studies were done in triplicate. A dosage of 40 mg/kg Meningicin was administered at time=0 hours (start of treatment), and another dosage of 20 mg/kg Meningicin was administered after 5 hours. Ceftriaxone (125 mg/kg at time=0 hours) was used as a positive control. The table below shows the bacterial burden in the cerebrospinal fluid.

Time (hours)	Meningicin treatment (CFU/ml)	Meningicin treatment (CFU/ml)	Meningicin treatment (CFU/ml)	Ceftriaxone treatment (CFU/ml)	Vehicle (CFU/ml)
-10	240,000	240,000	240,000	240,000	240,000
0	145,000	1,680,000	155,000	3,480,000	1,280,000
3	1,500	2,500	1,250	92,500	5,000,000
5	2,750	0.1	0.1	25,000	8,250,000
10	0.1	0.1	0.1	750	70,000,000

The tables below show the reduction in bacterial burden (Δ log CFU) in the cerebrospinal fluid during treatment. The data has been normalized to start from 0.00 at time=0 hours.

The results demonstrate a very high efficacy of Meningicin in treating meningitis. The efficasy of Meningicin is at least as high as the efficacy of Ceftriaxone.

Time (hours)	**Meningicin** treatment (Δ log CFU)						
	Rabbit 1	Rabbit 2	Rabbit 3	Rabbit 4	Rabbit 5	Rabbit 6	**Average**
0	0.00	0.00	0.00	0.00	0.00	0.00	**0.00**
3	-3.84	-3.28	-3.81	-1.99	-2.83	-2.09	**-2.97**
5	-5.31	-3.86	-5.01	-1.72	-5.23	-4.19	**-4.22**
10	-5.71	-4.86	-5.01	-4.16	-5.23	-4.19	**-4.86**

Time (hours)	**Ceftriaxon** treatment (Δ log CFU)				
	Rabbit 7	Rabbit 8	Rabbit 9	Rabbit 10	**Average**
0	0.00	0.00	0.00	0.00	**0.00**
3	-2.26	-2.11	-2.53	-1.58	**-2.12**
5	-3.28	-2.77	-3.72	-2.14	**-2.98**
10	-5.16	-3.57	-4.32	-3.67	**-4.18**

Time (hours)	**Vehicle** (Δ log CFU)
	Rabbit 11
0	0.00
3	0.59
5	0.81
10	1.74

CLAIMS

1. Use of a polypeptide having antimicrobial activity, which comprises an amino acid sequence having at least 90% identity to the amino acid sequence of SEQ ID NO:1, for the manufacturing of a medicament for therapeutic treatment of meningitis.

2. A polypeptide having antimicrobial activity, which comprises an amino acid sequence having at least 90% identity to the amino acid sequence of SEQ ID NO:1, for therapeutic treatment of meningitis.

3. A method of treating meningitis, comprising administering to a subject in need of such treatment an effective amount, e.g. an anti-meningitis effective amount, of a polypeptide having antimicrobial activity, which comprises an amino acid sequence having at least 90% identity to the amino acid sequence of SEQ ID NO:1.

4. The use, polypeptide or method of any of claims 1-3, wherein the polypeptide comprises an amino acid sequence having at least 95% identity to the amino acid sequence of SEQ ID NO:1, preferably wherein the polypeptide comprises or consists of the polypeptide of SEQ ID NO:1.

5. The use, variant or method of any of claims 1-3, wherein the polypeptide is a defensin polypeptide, preferably a beta-defensin polypeptide.

6. The use, variant or method of any of claims 1-3, wherein meningitis is bacterial meningitis

7. The use, variant or method of claim 6, wherein bacterial meningitis is caused by infection with a *Streptococcus* sp.

8. The use, variant or method of claim 6, wherein bacterial meningitis is pneumococcal meningitis.

9. The use, variant or method of claim 8, wherein pneumococcal meningitis is caused by infection with a penicilin-resistant *Streptococcus pneumoniae*.