## Anti-amyloidogenic, α-helix breaking ultra-small peptide therapeutic

The invention provides ultra-small inhibitory peptides that are capable of inhibiting amyloid formation/amyloidosis.

The inventors have recently found that a class of systematically designed ultra-small peptides is able to form amyloid structures by a stepwise formation. The initiation step occurs via crucial α-helical intermediate structures that are established before the final β-type amyloid structure is formed. The rationale for the development of anti-amyloidogenic peptide therapeutics is based on the idea of using inhibitory peptides that prevent the formation of α-helical intermediate structures.

Amyloids are tissue deposits of insoluble, proteinaceous fibrils that are rich in cross β-pleated sheet structure. The process of amyloid fibril formation is a key event in diverse and structurally unrelated pathological processes. These include many chronic, debilitating and increasingly prevalent diseases that can be broadly classified as: 1) neuro-degenerative, e.g. Alzheimer's, Parkinson's, Huntington's, 2) non-neuropathic localized amyloidosis such as in Type II Diabetes, and 3) systemic amyloidosis that occurs in multiple tissues. Despite differences in symptoms and protein monomers associated with these protein misfolding disorders (PMDs), there seems to be a common mechanism underlying protein aggregation at the molecular level. The formation of amyloid fibrils is through a molecular recognition and self-assembly process that typically starts with a thermodynamically unfavourable lag phase for the formation of a 'nucleus or seed'. This is followed by a thermodynamically favourable exponential growth phase, where monomers/oligomers are added to the growing nucleus. The conformational transition of the protein from a random-coiled soluble form via an α-helical intermediate into insoluble, cross β-pleated fiber aggregates is thought to be a key event in amyloidogenesis.

While recent scientific research has focussed on gaining more insight into the mechanism of molecular recognition and self-assembly in amyloidosis for inhibiting this process, there are still no effective preventions or treatments for any of these diseases. Small molecules that safely antagonize and prevent amyloidogenesis are desperately needed as therapeutics. The

anti-amyloidogenic candidates should also be able to fulfil stringent requirements, such as efficient and easy uptake, sufficient half-life and circulation time in vivo, non-toxicity, and permeability through the blood-brain barrier (BBB), which is needed for treating neuro-degenerative conditions. Furthermore, since there is increasing evidence that pre-fibril oligomer intermediates may be even more toxic than mature amyloid fibers, it is important to arrest or reverse the self-assembly process at an early stage.

The objects of the present invention are solved by an isolated alpha-helix breaking peptide having the general formula

$$Z\text{-}(X)_m\text{-Proline-}(X)_n,$$

wherein

*Z* is an N-terminal protecting group;

X is, at each occurrence, independently selected from amino acids and amino acid derivatives; and

m and n indicate the number of amino acids and amino acid derivatives and are integers independently selected from 1 to 5, with m + n being $\leq 6$.

Proline is abbreviated using either the three-letter code (Pro) or one-letter code (P).

In one embodiment, m + n is $\leq 5$, preferably $\leq 4$, more preferably $\leq 3$.

"Amino acids and amino acid derivatives" include naturally and non-naturally occurring L- and D-amino acids and amino acid derivatives, peptidomimetic amino acids and non-standard amino acids that are not made by a standard cellular machinery or are only found in proteins after post-translational modification or as metabolic intermediates, such as hydroxyproline, selenomethionine, carnitine, 2-aminoisobutyric acid, dehydroalanine, lanthionine, GABA and beta-alanine.

In one embodiment, X is, at each occurrence, independently selected from naturally occurring amino acids.

In one embodiment, at least one X is a D-amino acid or a peptidomimetic amino acid.

In one embodiment, X is, at each occurrence, independently selected from non-aromatic amino acids and amino acid derivatives.

In one embodiment, said non-aromatic amino acids are selected from the group consisting of isoleucine (Ile, I), leucine (Leu, L), valine (Val, V), alanine (Ala, A), glycine (Gly, G), aspartic acid (Asp, D), asparagine (Asn, N), glutamic acid (Glu, E), glutamine (Gln, Q), serine (Ser, S), threonine (Thr, T) and lysine (Lys, K).

In one embodiment, the N-terminal amino acid of said peptide is more hydrophobic than the C-terminal amino acid of said peptide. In one embodiment, the hydrophobicity decreases from the N-terminus to the C-terminus.

In one embodiment, m and n are 1, i.e. said peptide has the general formula

*Z*-X-Proline-X.

In one embodiment, said N-terminal protecting group has the general formula –C(O)–R, wherein R is selected from the group consisting of H, alkyl and substituted alkyl. In one embodiment, R is selected from the group consisting of methyl, ethyl, propyl, isopropyl, butyl and isobutyl.

In a preferred embodiment, said N-terminal protecting group is an acetyl group (R = methyl).

In one embodiment, the C-terminus of said peptide is amidated, wherein, preferably, the C-terminus has the formula -CONHR, with R being selected from the group consisting of H, alkyl and substituted alkyl.

In one embodiment, said peptide is provided in an aqueous solution, optionally comprising a physiological buffer.

In one embodiment, said peptide is based on a peptide selected from the group consisting of *Z*-LIVAGDD, *Z*-LIVAGEE, *Z*-LIVAGD, *Z*-ILVAGD, *Z*-LIVAAD, *Z*-LAVAGD, *Z*-AIVAGD, *Z*-LIVAGE, *Z*-LIVAGK, *Z*-LIVAGS, *Z*-ILVAGS, *Z*-AIVAGS, *Z*-LIVAGT, *Z*-AIVAGT, *Z*-LIVAD, *Z*-LIVGD, *Z*-IVAD, *Z*-IIID, *Z*-IIIK and *Z*-IVD, wherein one amino acid except the N-terminal and C-terminal amino acid is replaced with a Proline (Pro, P). For

example, the isolated alpha-helix breaking peptide according to the present invention may be based on the peptide Z-LIVAGD of the above list, and thus be selected from Z-LPVAGD, Z-LIPAGD, Z-LIVPGD and Z-LIVAPD.

In one embodiment, said peptide is selected from the group consisting of Z-IPD, Z-NPI, Z-IPN, Z-IPI, Z-APA, Z-LPI, Z-IPL, Z-APF, Z-KPA-$CONH_2$, Z-LPD, Z-LPE, Z-IPK-$CONH_2$, Z-APD, Z-IPF, Z-IPS, Z-KPA-$CONH_2$, Z-IPW, Z-APS, Z-NPK-$CONH_2$ and Z-LPG.

The objects of the present invention are also solved by an isolated alpha-helix breaking peptide as defined above for use as a medicament.

The objects of the present invention are further solved by an isolated alpha-helix breaking peptide as defined above for use in the treatment of a disease associated with amyloidosis.

In one embodiment, said disease associated with amyloidosis is selected from the group consisting of Neuro-degenerative diseases, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, Amyotrophic lateral sclerosis (ALS) and Prion-related or Spongiform encephalopathies, such as Creutzfeld-Jacob, Dementia with Lewy bodies, Frontotemporal dementia with Parkinsonism, Spinocerebellar ataxias, Spinocerebellar ataxia 17, Spinal and bulbar muscular atrophy, Hereditary dentatorubral-pallidoluysian atrophy, Familial British dementia, Familial Danish dementia, Non-neuropathic localized diseases, such as in Type II diabetes mellitus, Medullary carcinoma of the thyroid, Atrial amyloidosis, Hereditary cerebral haemorrhage with amyloidosis, Pituitary prolactinoma, Injection-localized amyloidosis, Aortic medial amyloidosis, Hereditary lattice corneal dystrophy, Corneal amyloidosis associated with trichiasis, Cataract, Calcifying epithelial odontogenic tumors, Pulmonary alveolar proteinosis, Inclusion-body myositis, Cutaneous lichen amyloidosis, and Non-neuropathic systemic amyloidosis, such as AL amyloidosis, AA amyloidosis, Familial Mediterranean fever, Senile systemic amyloidosis, Familial amyloidotic polyneuropathy, Hemodialysis-related amyloidosis, ApoAI amyloidosis, ApoAII amyloidosis, ApoAIV amyloidosis, Finnish hereditary amyloidosis, Lysozyme amyloidosis, Fibrinogen amyloidosis, Icelandic hereditary cerebral amyloid angiopathy, familial amyloidosis, and systemic amyloidosis which occurs in multiple tissues, such as light-chain amyloidosis.

In one embodiment, said peptide is administered orally.

The objects of the present invention are also solved by a pharmaceutical composition comprising an isolated alpha-helix breaking peptide as defined above.

In one embodiment, said pharmaceutical composition further comprises at least one pharmaceutically acceptable carrier, diluent and/or excipient.

In one embodiment, said pharmaceutical composition further comprises a monovalent or divalent metal salt, preferably a divalent metal salt.

In one embodiment, said metal is selected from the group consisting of sodium, magnesium, calcium and zinc.

The objects of the present invention are further solved by the use of an isolated alpha-helix breaking peptide as defined above in the manufacture of a medicament for the treatment of a disease associated with amyloidosis.

The objects of the present invention are also solved by a method of treatment of a disease associated with amyloidosis, said method comprising the step of administering an isolated alpha-helix breaking peptide as defined above or a pharmaceutical composition as defined above to a person in need thereof. Preferably, said isolated alpha-helix breaking peptide or said pharmaceutical composition are administered orally.

The objects of the present invention are further solved by a method of disaggregating an amyloid plaque or preventing formation thereof, said method comprising contacting an isolated alpha-helix breaking peptide as defined above with said amyloid plaque, thereby disaggregating said amyloid plaque or preventing formation thereof.

In one embodiment, the method further comprises contacting said amyloid plaque with a monovalent or divalent metal salt, preferably a divalent metal salt, wherein, preferably, said metal is selected from the group consisting of sodium, magnesium, calcium and zinc.

The inventors have surprisingly found that specific rationally designed ultra-small peptides can inhibit amyloidogenesis. These inhibitor peptides consist of 3–7 natural amino acids that

are capable of interfering with and preventing α-helical intermediate structures. These intermediate structures are thought to be important conformational transition states that drive the formation of amyloid aggregates, hence directing amyloidogenesis. Specifically, the inventors believe that the conformational change of the protein from random coiled to α-helix plays an important part in molecular self-recognition. By breaking or inhibiting the transition to α-helix, one can stop/reverse the amyloid aggregation process at a very early stage (i.e. immediately or within a few minutes). Thus, these anti-amyloidogenic peptides can also be considered as α-helical breakers. They are capable of recognizing and interacting with the short, amyloidogenic recognition motifs of misfolded proteins, while themselves having a very poor propensity to self-assemble into ordered supramolecular structures. By virtue of their short length, especially for the 3-mers, they have the potential to evade protease recognition and degradation, while facilitating easy oral delivery and BBB permeability. In addition, these ultra-small peptides are non-toxic, since they are made of non-toxic amino acids and amino acid derivatives, preferably naturally occurring amino acids. Furthermore, in order to assist disaggregation of already existing amyloids, the presence of mono- or divalent metal salts is preferred.

In summary, the small size of these ultra-short peptides enables easy and effective oral uptake, which is a key advantage in drug delivery. Furthermore, the small size of the ultra-short peptides guarantees simple batch synthesis at low costs. Because, in a preferred embodiment, the peptide-based therapeutics are made of naturally occurring amino acids they are non-toxic, non-immunogenic and biocompatible. Ultra-short peptides that are just three amino acids in length are also able to cross the blood-brain barrier, which is a key requirement for therapeutics targeted against neuro-degenerative disorders. Finally, the short peptide motif is likely to evade recognition and degradation by endogenous peptidases, hence providing greater half-life and bio-availability in biological fluids and tissues.

Development of an effective, small molecule drug that inhibits amyloidogenesis has huge and attractive market potential, especially since there is extensive evidence indicating the common molecular basis of amyloid aggregation in widespread diseases. For instance, Alzheimer's, just one of the many diseases involving amyloidosis, is the most frequent cause of late-life dementia (50–70%) and a leading cause of death in the developed world. In 2010, there was an estimated 35.6 million people with dementia, with the number expected to almost double to 65.7 million by 2030 and reach 115.4 million by 2050. Another example is Type II

diabetes, which accounts for 90–95% of all diagnosed cases of diabetes in adults. As currently available oral drugs are targeted at maintaining good control of blood glucose, there is a need to develop oral therapeutics that prevent formation of islet amyloid that is responsible for pancreatic cell death. Several of the current drugs also have serious side-effects that will be overcome by the use of natural occurring biological macromolecules. In this regard, the ultra-small inhibitor peptides according to the present invention have promising potential to address current therapeutic needs, while simultaneously trying to overcome the problems and limitations of existing drugs.

The present invention is now further described by means of the following examples, which are meant to illustrate, but not to limit the present invention.

Experimental Procedures

Peptide-based hydrogel preparation. All peptides (GL Biochem, Shanghai, China, ≥ 98% purity) were freshly prepared in order to avoid premature peptide aggregation. The peptides were dissolved in water and left at room temperature to form hydrogels. Depending on the peptide concentration, the self-assembly process occurred immediately, within hours or even within days (experimental time frame for gelation). For higher peptide concentrations peptides were dissolved in milliQ water by vortexing. If a forced and accelerated hydrogel preparation was needed, the peptide solution was subjected to sonication in a water bath (Barnstead Labline 9319 UltrasonicLC60H). No significant structural differences were observed between hydrogels produced via self-assembly and those whose assembly was facilitated by sonication.

CD studies. CD spectra were collected with an Aviv 410 CD spectrophotometer fitted with a Peltier temperature controller, using a rectangular quartz cuvette with a fitted cap and an optical path length of 1 mm. For higher peptide concentration (10-20 mg/ml), quartz cuvettes with optical path lengths of 0.01 mm were used. Data acquisition was performed in steps of 0.5 nm at a wavelength range from 180-260 nm with a spectral bandwidth of 1.0 nm. To ensure reproducibility of the CD spectra, 3 samples of each peptide were individually measured, but the spectra were not averaged. All spectra were baseline-corrected with milliQ water as the reference.

FESEM studies. Hydrogel samples were frozen at -20°C or better at -80°C. Frozen samples were then freeze-dried. Freeze-dried samples were fixed onto a sample holder using conductive tape and sputtered with platinum from both the top and the sides in a JEOL JFC-1600 High Resolution Sputter Coater. The coating current used was 30 mA and the process lasted for 60 sec. The surface of interest was then examined with a JEOL JSM-7400F Field Emission Scanning Electron Microscopy system using an accelerating voltage of 5-10 kV.

Rheology. To determine the viscoelastic properties peptide-based hydrogels were subjected to dynamic time, strain and frequency sweep experiments using the ARES-G2 rheometer (TA Instruments, Piscataway, NJ) with a 25.0 mm diameter titanium parallel plate geometry and a 0.8 mm gap distance. We compared the strength of the hydrogels at varying peptide concentrations, performing oscillatory frequency sweep studies at 0.1-100 rad/s frequency and 0.1% strain at 25°C.

Results

CD spectra and Rheology data of hydrogel-forming tri- and hexapeptides

See pages 1 and 2 of the enclosed Figures.

Ac-$LD_6$ refers to the hexapeptide Ac-LIVAGD, Ac-$NL_6$ refers to the hexapeptide Ac-NFGAIL, and Ac-$ID_3$ refers to the tripeptide Ac-IVD.

Anti-amyloid peptide therapeutics (ultra-small peptide inhibitors for oral uptake)

See pages 3 to 27 of the enclosed Figures.

Inhibitors 1 to 16 correspond to the following SEQ ID NOs: 1 to 16:

SEQ ID NO: 1
Ac-Leu Ile Pro Ala Gly Asp
LENGTH: 6
ORGANISM: Artificial Sequence
OTHER INFORMATION: Synthetic chemical peptide

SEQ ID NO: 2
Ac-Leu Pro Val Ala Gly Asp
LENGTH: 6
ORGANISM: Artificial Sequence
OTHER INFORMATION: Synthetic chemical peptide

SEQ ID NO: 3
Ac-Leu Pro Glu
LENGTH: 3
ORGANISM: Artificial Sequence
OTHER INFORMATION: Synthetic chemical peptide

SEQ ID NO: 4
Ac-Leu Pro Asp
LENGTH: 3
ORGANISM: Artificial Sequence
OTHER INFORMATION: Synthetic chemical peptide

SEQ ID NO: 5
Ac-Leu Pro Gly
LENGTH: 3
ORGANISM: Artificial Sequence
OTHER INFORMATION: Synthetic chemical peptide

SEQ ID NO: 6
Ac-Leu Pro Leu
LENGTH: 3
ORGANISM: Artificial Sequence
OTHER INFORMATION: Synthetic chemical peptide

SEQ ID NO: 7
Ac-Ile Pro Ile
LENGTH: 3
ORGANISM: Artificial Sequence
OTHER INFORMATION: Synthetic chemical peptide

SEQ ID NO: 8
Ac-Ile Pro Asp
LENGTH: 3
ORGANISM: Artificial Sequence
OTHER INFORMATION: Synthetic chemical peptide

SEQ ID NO: 9
Ac-Ile Pro Ser
LENGTH: 3
ORGANISM: Artificial Sequence
OTHER INFORMATION: Synthetic chemical peptide

SEQ ID NO: 10

Ac-Ile Pro Trp
LENGTH: 3
ORGANISM: Artificial Sequence
OTHER INFORMATION: Synthetic chemical peptide

SEQ ID NO: 11
Ac-Ile Pro Phe
LENGTH: 3
ORGANISM: Artificial Sequence
OTHER INFORMATION: Synthetic chemical peptide

SEQ ID NO: 12
Ac-Ile Pro Lys-$CONH_2$ (C-terminus is amidated)
LENGTH: 3
ORGANISM: Artificial Sequence
OTHER INFORMATION: Synthetic chemical peptide

SEQ ID NO: 13
Ac-Ala Pro Phe
LENGTH: 3
ORGANISM: Artificial Sequence

SEQ ID NO: 14
Ac-Ala Pro Asp
LENGTH: 3
ORGANISM: Artificial Sequence
OTHER INFORMATION: Synthetic chemical peptide

SEQ ID NO: 15
Ac-Ala Pro Ser
LENGTH: 3
ORGANISM: Artificial Sequence
OTHER INFORMATION: Synthetic chemical peptide

SEQ ID NO: 16
Ac-Asn Pro Lys-$CONH_2$ (C-terminus is amidated)
LENGTH: 3
ORGANISM: Artificial Sequence
OTHER INFORMATION: Synthetic chemical peptide

Influence of salt concentration on the mechanical stability of peptide-derived hydrogels

See page 28 of the enclosed Figures.

## Claims

1. An isolated alpha-helix breaking peptide having the general formula

$$Z\text{-}(X)_m\text{-Proline-}(X)_n,$$

wherein

*Z* is an N-terminal protecting group;

X is, at each occurrence, independently selected from amino acids and amino acid derivatives; and

m and n indicate the number of amino acids and amino acid derivatives and are integers independently selected from 1 to 5, with m + n being $\leq 6$.

2. The isolated alpha-helix breaking peptide according to claim 1, wherein X is, at each occurrence, independently selected from naturally occurring amino acids.

3. The isolated alpha-helix breaking peptide according to claim 1 or 2, wherein at least one X is a D-amino acid or a peptidomimetic amino acid.

4. The isolated alpha-helix breaking peptide according to any of claims 1 to 3, wherein X is, at each occurrence, independently selected from non-aromatic amino acids and amino acid derivatives.

5. The isolated alpha-helix breaking peptide according to claim 4, wherein said non-aromatic amino acids are selected from the group consisting of isoleucine, leucine, valine, alanine, glycine, aspartic acid, asparagine, glutamic acid, glutamine, serine, threonine and lysine.

6. The isolated alpha-helix breaking peptide according to any of the foregoing claims, wherein the N-terminal amino acid of said peptide is more hydrophobic than the C-terminal amino acid of said peptide.

7. The isolated alpha-helix breaking peptide according to any of the foregoing claims, wherein m and n are 1.

8. The isolated alpha-helix breaking peptide according to any of the foregoing claims, wherein said N-terminal protecting group has the general formula –C(O)–R, wherein R is selected from the group consisting of H, alkyl and substituted alkyl.

9. The isolated alpha-helix breaking peptide according to claim 8, wherein said N-terminal protecting group is an acetyl group.

10. The isolated alpha-helix breaking peptide according to any of the foregoing claims, wherein the C-terminus of said peptide is amidated, wherein, preferably, the C-terminus has the formula -CONHR, with R being selected from the group consisting of H, alkyl and substituted alkyl.

11. The isolated alpha-helix breaking peptide according to any of claims 4 to 10, wherein said peptide is provided in an aqueous solution, optionally comprising a physiological buffer.

12. An isolated alpha-helix breaking peptide according to any of claims 1 to 11 for use as a medicament.

13. An isolated alpha-helix breaking peptide according to any of claims 1 to 11 for use in the treatment of a disease associated with amyloidosis.

14. The isolated alpha-helix breaking peptide according to claim 13, wherein said disease associated with amyloidosis is selected from the group consisting of Neuro-degenerative diseases, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, Amyotrophic lateral sclerosis (ALS) and Prion-related or Spongiform encephalopathies, such as Creutzfeld-Jacob, Dementia with Lewy bodies, Frontotemporal dementia with Parkinsonism, Spinocerebellar ataxias, Spinocerebellar ataxia 17, Spinal and bulbar muscular atrophy, Hereditary dentatorubral-pallidoluysian atrophy, Familial British dementia, Familial Danish dementia, Non-neuropathic localized diseases, such as in Type II diabetes mellitus, Medullary carcinoma of the thyroid, Atrial amyloidosis, Hereditary cerebral haemorrhage with amyloidosis, Pituitary prolactinoma, Injection-localized amyloidosis, Aortic medial amyloidosis, Hereditary lattice corneal dystrophy, Corneal amyloidosis associated with trichiasis,

Cataract, Calcifying epithelial odontogenic tumors, Pulmonary alveolar proteinosis, Inclusion-body myositis, Cutaneous lichen amyloidosis, and Non-neuropathic systemic amyloidosis, such as AL amyloidosis, AA amyloidosis, Familial Mediterranean fever, Senile systemic amyloidosis, Familial amyloidotic polyneuropathy, Hemodialysis-related amyloidosis, ApoAI amyloidosis, ApoAII amyloidosis, ApoAIV amyloidosis, Finnish hereditary amyloidosis, Lysozyme amyloidosis, Fibrinogen amyloidosis, Icelandic hereditary cerebral amyloid angiopathy, familial amyloidosis, and systemic amyloidosis which occurs in multiple tissues, such as light-chain amyloidosis.

15. The isolated alpha-helix breaking peptide according to any of claims 12 to 14, wherein said peptide is administered orally.

16. A pharmaceutical composition comprising an isolated alpha-helix breaking peptide according to any of claims 1 to 11.

17. The pharmaceutical composition according to claim 16, further comprising at least one pharmaceutically acceptable carrier, diluent and/or excipient.

18. The pharmaceutical composition according to claim 16 or 17, further comprising a monovalent or divalent metal salt.

19. The pharmaceutical composition according to claim 18, wherein said metal is selected from the group consisting of sodium, magnesium, calcium and zinc.