Neurobiology of Disease 88 (2016) 75–84

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

The new β amyloid-derived peptide Aβ1–6$_{A2V}$-TAT(D) prevents Aβ oligomer formation and protects transgenic *C. elegans* from Aβ toxicity

Luisa Diomede [a,*], Margherita Romeo [a], Alfredo Cagnotto [a], Alessandro Rossi [a], Marten Beeg [a], Matteo Stravalaci [a], Fabrizio Tagliavini [b], Giuseppe Di Fede [b], Marco Gobbi [a], Mario Salmona [a]

[a] Department of Molecular Biochemistry and Pharmacology, IRCCS-Istituto di Ricerche Farmacologiche "Mario Negri", Via La Masa 19, 20156 Milan, Italy
[b] Division of Neurology and Neuropathology, "Carlo Besta" National Neurological Institute, 20133 Milan, Italy

ARTICLE INFO

Article history:
Received 27 May 2015
Revised 18 December 2015
Accepted 9 January 2016
Available online 11 January 2016

Keywords:
Alzheimer's disease
Amyloid β
Aβ$_{A2V}$
Oligomer
C. elegans

ABSTRACT

One attractive pharmacological strategy for Alzheimer's disease (AD) is to design small peptides to interact with amyloid-β (Aβ) protein reducing its aggregation and toxicity. Starting from clinical observations indicating that patients coding a mutated Aβ variant (Aβ$_{A2V}$) in the heterozygous state do not develop AD, we developed Aβ$_{A2V}$ synthetic peptides, as well as a small peptide homologous to residues 1–6. These hindered the amyloidogenesis of Aβ and its neurotoxicity *in vitro*, suggesting a basis for the design of a new small peptide in *D-isomeric* form, linked to the arginine-rich TAT sequence [Aβ1–6$_{A2V}$-TAT(D)], to allow translocation across biological membranes and the blood–brain barrier. Aβ1–6$_{A2V}$-TAT(D) was resistant to protease degradation, stable in serum and specifically able to interfere with Aβ aggregation *in vitro*, reducing the appearance of toxic soluble species and protecting transgenic *C. elegans* from toxicity related to the muscular expression of human Aβ. These observations offer a proof of concept for future pharmacological studies in mouse models of AD, providing a foundation for the design of Aβ$_{A2V}$-based peptidomimetic molecules for therapeutic purposes.

1. Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease (Hardy and Selkoe, 2002; Holtzman et al., 2011), with huge social and economic impacts (Holtzman et al., 2011). One of the central pathological features of AD is the accumulation in the brain of amyloid β (Aβ) peptides in the form of oligomers, protofibrils and fibrils, formed during aggregation (Haass and Selkoe, 2007; Hardy and Selkoe, 2002); these trigger a cascade of events leading to neuronal cell death and neurodegeneration (Huang and Jiang, 2009; Jin et al., 2011; Mattson, 2004; St George-Hyslop and Petit, 2005). These altered Aβ species are one of the main targets for the design of new therapeutic interventions (Hardy and Selkoe, 2002; Karran et al., 2011; Murakami, 2014; Panza et al., 2012; Rosenblum, 2014; Selkoe, 2001). No effective treatments are currently available to delay the onset, slow the progression or stop the course of AD, and the development of effective disease-modifying therapies remains a major, urgent need.

One attractive pharmacological strategy involves the design of small peptides and pseudo-peptides able to interact with Aβ, modifying its kinetics of aggregation and reducing its toxicity. Support for a peptide-based strategy comes from the identification of a novel mutation in the amyloid precursor protein (APP) gene, resulting in alanine-to-valine substitution at codon 673 (A673V), corresponding to position 2 in the N-terminal part of the Aβ sequence (Di Fede et al., 2009). This natural Aβ variant (Aβ$_{A2V}$) has a dominant-negative effect on Aβ amyloidogenesis (Di Fede et al., 2009; Giaccone et al., 2010), since humans carrying the A2V mutation in the heterozygous state do not develop AD. Although the A2V-mutation increases the propensity of Aβ to adopt a ß-sheet structure, boosts the formation of oligomers and increases neurotoxicity (Di Fede et al., 2012; Messa et al., 2014), the interaction between the A2V mutant and wild-type (WT) Aβ *in vitro* hinders amyloidogenesis and Aβ-mediated neurotoxicity, explaining the absence of disease in the A673V heterozygous carriers (Di Fede et al., 2009, 2012). Studies with a short synthetic peptide homologous to residues 1–6 of Aβ$_{A2V}$ (Aβ1–6$_{A2V}$) indicate that it binds to preformed amyloid fibrils and hinders amyloidogenesis of Aβ$_{1–40}$ peptide (Di Fede et al., 2009), providing a basis for the design of innovative Aβ$_{A2V}$-based disease-modifying peptides for AD. This prompted us to design and synthesize a new six-mer Aβ peptide, linked to the arginine-rich TAT sequence to allow translocation across the cell membranes and blood–brain barrier (Davoli et al., 2014). The corresponding

Abbreviations: (AD), Alzheimer's disease; (Aβ), amyloid-β; (APP), amyloid precursor protein; (A673V), alanine-to-valine substitution at codon 673; (Aβ$_{A2V}$), A-V mutated Aβ; (WT), wild-type; (Aβ1–6$_{A2V}$), peptide homologous to residues 1–6 of Aβ$_{A2V}$; [Aβ1–6$_{A2V}$(D)], *D-isomer* Aβ1–6$_{A2V}$; [Aβ1–6$_{A2V}$-TAT(D)], Aβ1–6$_{A2V}$(D)linked to TAT; (SPR), surface plasmon resonance; (ThT), thioflavine T; (AChRs), acetylcholinesterase receptors; (TFA), trifluoroacetic acid; (TOF), MALDI-time-of-flight; (MS), mass spectrometry; (NGM), Nematode Growth Medium; (HCCA), α-cyano-4-hydroxycinnamic acid; (DTT), dithiothreitol.

* Corresponding author.
E-mail address: luisa.diomede@marionegri.it (L. Diomede).
Available online on ScienceDirect (www.sciencedirect.com).

http://dx.doi.org/10.1016/j.nbd.2016.01.006
0969-9961/

D-isomer [Aβ1–6_{A2V}-TAT(D)] was synthesized to obtain a peptide predicted to be more resistant to degradation by endogenous proteases.

We examined the stability of this peptide in biological medium and its effects on Aβ polymerization *in vitro* and Aβ-induced toxicity *in vivo*. In particular, we investigated how the peptide counteracted the toxicity specifically due to oligomeric species, using CL4176 transgenic *Caenorhabditis elegans* worms as a simplified invertebrate model of Aβ amyloidogenesis (Diomede et al., 2010, 2013). In these transgenic animals, the inducible expression of human $Aβ_{1-42}$ and the consequent accumulation of oligomeric assemblies in the body wall muscle cells lead to paralysis (Diomede et al., 2010, 2013). We also used transgenic *C. elegans* nematodes constitutively expressing cytoplasmic human $Aβ_{3-42}$ in body wall muscle cells (CL2120) (Rebolledo et al., 2011). This strain shows an age-related progressive reduction of muscle-specific motility connected to the accumulation of both Aβ fibrils and oligomers (Rebolledo et al., 2011).

These transgenic strains have all previously been employed to demonstrate the protective effect of several pharmacological treatments against Aβ toxicity *in vivo* (Diomede et al., 2010, 2013; Rebolledo et al., 2011). The *in vitro* data indicate that Aβ1–6_{A2V}(D) inhibits the formation of toxic oligomers. However, this peptide did not counteract the toxic effects of $Aβ_{42}$ in transgenic *C. elegans in vivo* unless it was linked to the TAT protein sequence. In addition, the mutated Aβ1–6_{A2V}-TAT(D) peptide specifically interfered with Aβ aggregation, skipping the appearance of toxic soluble species.

2. Materials and methods

2.1. Synthesis and chemical characterization of peptides

Aβ1–6_{A2V}(D) and Aβ1–6_{WT}(D) were synthesized by solid-phase FMOC chemistry on an Applied Biosystems 433 A peptide synthesizer (Life Technologies, Monza, Italy) using D-amino acid derivatives and Novasyn-TGA resin on 0.1 mM scale (Di Fede et al., 2009). Aβ1–6_{A2V}-TAT(D), Aβ1–6_{A2V}-TAT(L) and Aβ1–6_{WT}-TAT(D) peptides were synthesized using D- or L-amino acid derivatives, with the Aβ1–6_{A2V} (L and D isomers) or Aβ1–6_{WT}(D) sequence, a spacer of four glycines linked to the sequence homologous to residue 48–57 of TAT (GRKKRRQRRR), using the same FMOC chemistry. Peptides were cleaved from the resin by incubation for 3 h with a trifluoroacetic acid (TFA) solution containing 5% phenol, 5% 3,6-dioxa-1,8-octanedithiol and 5% water. They were then purified by reverse-phase HPLC on a semi-preparative Jupiter C18 10 μm column (250 × 10 mm, 300 Å, Phenomenex, Torrance, CA, USA) on a System Gold HPLC (Beckman Coulter, Milan, Italy) using water: acetonitrile gradient elution. Identity and purity of the peptides were determined by MALDI-time-of-flight (TOF) mass spectrometry (MS) with a Bruker Reflex III TOF mass spectrometer operating in reflector mode (Bruker, Billerica, MA, USA). Peptide purity was also checked by reverse-phase HPLC using a System Gold HPLC equipped with a EcoCART 125-3 LiChrospher 60RP-selected B 5 μm analytics column (Merck, Darmstadt, Germany) and UV/VIS detector (214 nm). The retention time was 14 min for Aβ1–6_{A2V}-TAT (L and D isomers), 15 min for $Aβ_{1-6A2V}$ (D) in gradient solvent elution (A: H_2O + 0.1% TFA; B: acetonitrile + 0.08% TFA) following a linear gradient of B from 4.0% to 39.65% in 15 min at a flow rate of 0.3 ml/min. The purity was always higher than 95%. Peptides were lyophilized and stored at −80 °C.

2.2. Preparation of synthetic $Aβ_{1-42}$ oligomers

Synthetic $Aβ_{1-42}$ was prepared in-house using the "depsi-peptide" technique produces seed-free starting solutions (Beeg et al., 2011; Stravalaci et al., 2012). Depsi-$Aβ_{1-42}$, synthesized first, is much more soluble than $Aβ_{1-42}$ and has much less propensity to aggregate, preventing the spontaneous formation of seeds in solution. $Aβ_{1-42}$ was then obtained from the depsi-peptide by a "switching" procedure involving a change in pH (Beeg et al., 2011) and used immediately. The switched solution was diluted at 100 μM in 10 mM phosphate buffer containing 150 mM NaCl, pH 7.4 (PBS), and incubated for 5 h at 25 °C to form oligomers (Stravalaci et al., 2012).

2.3. C. elegans strains

We used ancestral Bristol N2 *C. elegans*, the temperature-inducible transgenic nematode strain CL4176 (*smg-1*(*cc546ts*)I; *dvIs27*[pAF29(*myo-3*/Aβ1–42/let UTR) + pRF4(*rol-6*(*su1006*)]X), producing human $Aβ_{1-42}$ in the body-wall muscle cells and its control CL802 (*smg-1*(*cc546ts*)I; *rol-6*(*su1006*)II) (Link et al., 2003), and the transgenic CL2120 *C. elegans* strain (*dvIs14*[*unc-54*/hAβ1–42(pCL12) + *mtl-2*::*GFP* (pCL26)]) constitutively producing human $Aβ_{3-42}$ in the body-wall muscles, with its control CL2122 (*dvIs15*[pPD30.38(*unc-54* vector) + *mtl-2*::*GFP*(pCL26)]) (Fay et al., 1998). All nematode strains were obtained from the Caenorhabditis Genetic Center (CGC, University of Minnesota, USA) and were propagated on solid Nematode Growth Medium (NGM) seeded with *Escherichia coli* (OP50, CGC, USA) for food. To prepare age-synchronized animals, the nematodes were transferred to fresh NGM plates on reaching maturity at three days of age and left to lay eggs overnight. Isolated hatchlings from the synchronized eggs were cultured on fresh NGM plates at 16 °C for CL4176 and CL802 worms or 21 °C for N2, CL2120 and CL2122 worms.

2.4. Stability of Aβ1–6_{A2V}-TAT(D) peptide

To determine the stability of the Aβ1–6_{A2V}-TAT(D) in the presence of proteinase K, the peptide (1 μg/μl, 415 μM) was incubated at 37 °C with or without 10 ng/μl of proteinase K (Sigma Aldrich, St. Louis, USA). Samples were spotted with α-cyano-4-hydroxycinnamic acid (HCCA, Sigma Aldrich) on a metal plate (MTP 384 Ground Steel, Bruker) before and 24 h after incubation, and analysed by MALDI-TOF MS. To evaluate the stability in serum, 1 mM of peptide was incubated with mouse serum at 37 °C. Different times after incubation (from 0 up to 1 h), 1 μl of the sample was spotted with HCCA matrix on a metal plate (MTP 384 Ground Steel, Bruker) and analysed by MALDI-TOF MS.

2.5. Internalization of Aβ1–6_{A2V}-TAT(D) into nematodes

Synchronized transgenic CL4176 (100 worms/plate) were grown at 16 °C for 60 h. Then the temperature was raised to 24 °C to induce $Aβ_{1-42}$ expression and 12 h later they were treated with 100 μM Aβ1–6_{A2V}-TAT(D) (100 μl/plate) freshly dissolved in water. Control worms received vehicle alone. After 36 h the worms were collected by washing the plates with M9 buffer. The suspension was transferred to tubes, centrifuged at 1100 ×*g* for 4 min and washed twice to eliminate bacteria. The worm pellet was then resuspended in lysis buffer (5.0 mM NaCl, 5.0 mM EDTA, 1.0 mM dithiothreitol (DTT) and protease inhibitor mixture in 25 mM Tris/HCl buffer, pH 7.5) and homogenized using a TeSeE homogenizer (Bio-Rad, Hercules, CA,USA) with acid-washed glass beads (Sigma Aldrich) (Diomede et al., 2010). Equal amounts of proteins from worm lysates (5–10 μg) were then spotted onto nitrocellulose membranes (Millipore) and Aβ1–6_{A2V}-TAT(D) was identified and analysed by MALDI-TOF MS.

2.6. Surface plasmon resonance (SPR)

SPR was used for the specific detection of toxic $Aβ_{1-42}$ oligomers, as previously described (Stravalaci et al., 2012), using the ProteOn XPR36 Protein Interaction Array System, (Bio-Rad). The anti-Aβ antibody 4G8 (Signet Laboratories, Emeryville, CA, USA) was immobilized on parallel strips of the same sensor chip (Bio-Rad Laboratories) by standard amine