Short Communication

Peripherally Applied Synthetic Tetrapeptides HAEE and RADD Slow Down the Development of Cerebral β -Amyloidosis in A β PP/PS1 Transgenic Mice

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Abstract. Two tetrapeptides, HAEE and RADD, which are ionic-complementary to the primary zinc recognition site of amyloid- β (A β), have been reported to inhibit zinc-induced dimerization of the A β metal-binding domain and slow A β aggregation *in vitro*. In the present study, we investigate the impact of HAEE and RADD on the development of cerebral β -amyloidosis in a mouse model of Alzheimer's disease. We have found chronic intravenous administration of each peptide results in significant decrease of amyloid plaque burden in the treated mice.

Keywords: Alzheimer's disease, amyloid- β , mouse models, protein aggregation *in vivo*

INTRODUCTION

Pathological conversion of amyloid- β peptide (A β) from its benign mostly monomeric state through neurotoxic oligomers into insoluble amyloid plaques is a hallmark of Alzheimer's disease (AD) [1]. According to the amyloid hypothesis, the A β aggregation initiates a sequence of events that lead to AD dementia [2]. AD drug development is driven mainly by this hypothesis, and most randomized clinical trials are designed

to target A β [3, 4]. Several therapeutics that were purported to reduce A β production or aggregation have failed in Phase III clinical testing, and many others are in various stages of development [5].

In vivo studies evidenced that zinc ions are markedly enriched in A β plaques and are critical for pathological A β aggregation [6]. Experimental structural data for A β oligomers complexed with zinc ions are unavailable; however, the role of the N-terminal residues 1–16 as the A β metal-binding domain has been established [7, 8], and the region 11–14 (EVHH) was determined as the primary zinc recognition site of A β [9]. This site also constitutes intermolecular interface within

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the zinc-induced dimers and oligomers of A β isoforms [10–14]. Recently, two tetrapeptides, HAEE and RADD, which are ionic-complementary to ¹¹EVHH¹⁴ region of A β , have been reported to block zinc-induced dimerization of the A β metal-binding domain and slow A β aggregation *in vitro* [15]. In the present study we investigate the impact of these two tetrapeptides on the development of cerebral β -amyloidosis in a mouse model of AD. We have found chronic intravenous administration of each peptide results in significant decrease of amyloid plaque burden in the treated mice.

MATERIALS AND METHODS

Host mice

Animals of mouse strain B6C3-Tg(APPswe, PSEN1dE9)85Dbo/j (stock number 004462), received from the Jackson Laboratory (JAX, Maine, USA), were employed in the study. Laboratory animals were produced and housed under specific pathogen-free conditions at the AAALAC-accredited Animal Breeding Facility, Branch of Shemyakin & Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences (Pushchino, Moscow Region, Russia). In the course of experiments, the animals were kept under the standard housing conditions in the barrier area according to the Institutional Animal Care and Use Program and the IACUC-approved Study Protocol. The experimental procedures were approved by the local Animal Care and Use Committee.

Reagents

All chemicals and solvents used throughout this study were of HPLC-grade or better and were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Synthetic peptides (purity >98% checked by RP-HPLC) HAEE (Ac-HAEE-NH₂) and

RADD (Ac-RADD-NH₂) were purchased from Verta Ltd. (St. Petersburg, Russia).

Synthetic HAEE and RADD preparations for injections

Respective lyophilized peptide was dissolved in sterile water, and the solution was brought to neutral pH with NaOH. This solution was further diluted with sterile 10 $\times$ phosphate-buffered saline (PBS) to reach a final peptide concentration of 4.5 mg/mL (stock HAEE/RADD preparation). Then stock solution was sterilized by steam in the autoclave at 112°C for 10 min. Sterilized stock solutions of the peptides, which were intended for all subsequent experiments, were prepared simultaneously in an identical manner and stored in 60 μ L aliquots at –20°C until usage. To prepare injection sample, an aliquot of the stock solution was diluted by sterile saline to obtain peptide concentration 180 μ g/mL. Each injection sample was prepared immediately before its introduction to the animals.

Intravenous injections

Retro-orbital injections in mice were performed according to Yardeni et al. [16]. Mice received one intravenous injection with 12–16 days intervals between injections. The contents of injections for each group of mice are described in Table 1. The mice were assigned to the various groups randomly.

Histology

Euthanasia procedures was applied for 7-month-old mice. Mice for studies were anesthetized and sacrificed by lethal dose of pentobarbital. Mouse brains were fixed in 10% formalin. Process for paraffin embedding was scheduled as follow: 75% ethanol overnight,

Table 1
Suppression of congophilic amyloid plaques formation in the brain of B6C3-Tg(APPswe,PSEN1dE9)85Dbo/J transgenic mice by intravenous injections of RADD and HAEE

Transgenic Mice				Injection	Brain Sections	Number of Congophilic Amyloid Plaques per Section	Statistical Significance
Group name	Number of animals	Age at first injection (months)	Age at sacrifice (months)	Administered compound/total number of injections	Total number	In hippocampus	Versus Intact
Intact	6	–	7	– (not injected)	60	14.2 $\pm$ 3.1	–
RADD	5	2	7	Synthetic Ac-RADD-NH ₂ (22.5 μ g in 125 μ L of PBS)/9	50	5.2 $\pm$ 1.6	$p < 0.001$
HAEE	7	2	7	Synthetic Ac-HAEE-NH ₂ (22.5 μ g in 125 μ L of PBS)/9	70	5.8 $\pm$ 2.1	$p < 0.001$

96% ethanol 5 min, 96% ethanol 10 min, 100% ethanol 10 min (two changes), ethanol-chloroform (1:1) 30 min, chloroform overnight. Paraffin embedding was performed at 60°C for 3 h (three changes). The embedding of tissues into paraffin blocks was done using Leica EG1160 device. Serial brain sections (8 μ m) were cut using micritome Leica RM2265 and mounted onto slides. For deparaffinization, hydration and staining of the sections the next steps were done: slides were consistently put in xylene three changes (10 min each), 96% ethanol (5 min), 90% ethanol (2 min), 75% ethanol (2 min), H₂O three changes (5 min each), Congo Red solution (5 min), potassium alkali solution, and water. The ImMu-Mount medium (Thermo Scientific) was used for mounting.

Quantitative assessment of cerebral β -amyloidosis

The sections spanning brain from 0.48 to 1.92 mm relative to the midline in lateral stereotaxic coordinates were used to quantify congophilic amyloid plaques in hippocampus. Every 15th section was analyzed, yielding 10 sections per animal. Amyloid plaques were identified by Congo Red staining and manually counted as described previously [17–19]. The fluorescent micrographs were captured with a digital camera Leica DFS490 (Leica Microsystems, Wetzlar, Germany) using Leica Application Suite 2.8.1 software. Amyloid plaques of all sizes were accepted for counting if they were visible. Analyses were independently undertaken by two researchers (SK, ICh). To determine reproducibility of the plaques counts, an intra-class correlation (ICC) was calculated yielding

good inter-rater reliability between the two researchers (ICC >0.85). For each group of mice, the average values ($\pm$ s.d.) of the plaques number per section were calculated.

Statistical methods used for data analysis

The Shapiro-Wilk test was used to check the normality of the distribution. The Mann-Whitney test was used for pairwise comparison between examined groups. The significance level applied was 99.9% ($p < 0.001$). Statistical analysis was performed using STATISTICA 8.0 (StatSoft Inc., Tulsa, OK, USA).

RESULTS

We have investigated the ability of synthetic tetrapeptides HAEE and RADD to reduce cerebral β -amyloidosis in A β PP/PS1 doubly transgenic mouse model of AD. These mice demonstrate cognitive features of AD-like pathology, and possess significant amounts of dense-core congophilic amyloid plaques starting from 4 to 6 month age regardless of sex [20, 21]. The experimental groups included male and female animals were subjected to intravenous injections of tetrapeptides HAEE or RADD (22.5 μ g in 125 μ L of PBS) starting from 2 months of age. After serial (at 12–16 days intervals) inoculations with the respective peptide, the host mice were sacrificed at the age of 7 months.

The brains were extracted, and sagittal brain sections (8 μ m-thick) were analyzed histochemically using

Fig. 1. Representative fluorescent micrographs of brain sections through the hippocampus for 7-month-old B6C3-Tg(APPswe, PSEN1dE9)85Dbo/j intact transgenic mice (A), the transgenic mice intravenously injected by synthetic HAEE (B) or RADD (C) tetrapeptides. Scale bars: A, B, and C, 50 μ m.

Congo Red staining (Fig. 1). Hippocampus was chosen as the target region for manual counting of stained congophilic amyloid plaques by using fluorescence microscopy in the sections representing the brain layer located from 0.48 to 1.92 mm relative to the midline in lateral stereotaxic coordinates. The congophilic plaques found in the brains of all experimental animals were similar in terms of their localization and size distribution in the brain parenchyma (Fig. 1). However, quantitative analysis has revealed significantly lower number of congophilic amyloid plaques per section in both HAAE- and RADD-inoculated 7-month-old transgenic mice (−59.2% and −63.4%, respectively, $p < 0.001$) compared to untreated littermates (Table 1; Fig. 1).

DISCUSSION

Synthetic peptides and peptidomimetics that bind to specific A β regions are widely probed in peptide-based approach as drug candidate for disease-modifying therapy of AD [22–24]. Soluble dimers and oligomers of A β are considered as potential molecular agents triggering the amyloid cascade in AD progression [25, 26]. Zinc-induced dimers of A β appear to participate in this process [27]. Some chemical modifications within the metal-binding domain of A β (e.g., isomerization of Asp7 or phosphorylation of Ser8) facilitate zinc-dependent dimerization of the domain [13, 28]. The A β species bearing isomerized Asp7 and by this reason susceptible to zinc-driven dimerization has been shown to trigger cerebral amyloidosis *in vivo* [29].

The tetrapeptides HAAE and RADD used in our study have been designed to bind to the EVHH site [15] which mediates zinc-induced dimerization of A β [11–13]. Our data demonstrate that HAAE and RADD suppress pathological cerebral deposition of endogenous A β *in vivo*. Such short charged peptides seem to have satisfactory blood-brain barrier permeability and low immunogenicity. So HAAE and RADD may ultimately be clinically useful for disease-modifying treatment of AD. The observed anti-aggregation action of the tetrapeptides presumably is based on blocking the formation of zinc-bound dimers of A β . Thus, the present work supports the use of ¹¹EVHH¹⁴ region of A β as promising drug target for preventing cerebral β -amyloidosis.

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REFERENCES

- [1] Gandy S, Simon AJ, Steele JW, Lublin AL, Lah JJ, Walker LC, Levey AI, Krafft GA, Levy E, Checler F, Glabe C, Bilker WB, Abel T, Schmeidler J, Ehrlich ME (2010) Days to criterion as an indicator of toxicity associated with human Alzheimer amyloid-beta oligomers. *Ann Neurol* **68**, 220-230.
- [2] Hardy J, Selkoe DJ (2002) The amyloid hypothesis of Alzheimer's disease: Progress and problems on the road to therapeutics. *Science* **297**, 353-356.
- [3] Bharadwaj PR, Verdile G, Barr RK, Gupta V, Steele JW, Lachenmayer ML, Yue Z, Ehrlich ME, Petsko G, Ju S, Ringe D, Sankovich SE, Caine JM, Macreadie IG, Gandy S, Martins RN (2012) Latrepirdine (Dimebon™) enhances autophagy and reduces intracellular GFP-A β ₄₂ levels in yeast. *J Alzheimers Dis* **32**, 949-967.
- [4] Mangialasche F, Solomon A, Winblad B, Mecocci P, Kivipelto M (2010) Alzheimer's disease: Clinical trials and drug development. *Lancet Neurol* **9**, 702-716.
- [5] Karran E, Mercken M, De Strooper B (2011) The amyloid cascade hypothesis for Alzheimer's disease: An appraisal for the development of therapeutics. *Nat Rev Drug Discov* **10**, 698-712.
- [6] Bush AI (2013) The metal theory of Alzheimer's disease. *J Alzheimers Dis* **33**, S277-S281.
- [7] Faller P, Hureau C (2009) Bioinorganic chemistry of copper and zinc ions coordinated to amyloid- β peptide. *Dalton Trans*, 1080-1094.
- [8] Zirah S, Kozin SA, Mazur AK, Blond A, Cheminant M, Segalas-Milazzo I, Debey P, Rebuffat S (2006) Structural changes of region 1-16 of the Alzheimer disease amyloid β -peptide upon zinc binding and *in vitro* aging. *J Biol Chem* **281**, 2151-2161.
- [9] Tsvetkov PO, Kulikova AA, Golovin AV, Tkachev YV, Archakov AI, Kozin SA, Makarov AA (2010) Minimal Zn²⁺ binding site of amyloid- β . *Biophys J* **99**, L84-L86.
- [10] Alies B, Lapenna G, Sayen S, Guillon E, Hureau C, Faller P (2012) Insights into the mechanisms of amyloid formation of Zn^{II}-Ab11-28: pH-dependent zinc coordination and overall charge as key parameters for kinetics and the structure of Zn^{II}-Ab11-28 aggregates. *Inorg Chem* **51**, 7897-7902.
- [11] Kozin SA, Kulikova AA, Istrate AN, Tsvetkov PO, Zhokhov SS, Mezentssev YV, Kechko OI, Ivanov AS, Polshakov VI, Makarov AA (2015) The English (H6R) familial Alzheimer's disease mutation facilitates zinc-induced dimerization of the amyloid- β metal-binding domain. *Metallomics* **7**, 422-425.
- [12] Kozin SA, Mezentssev YV, Kulikova AA, Indeykina MI, Golovin AV, Ivanov AS, Tsvetkov PO, Makarov AA (2011) Zinc-induced dimerization of the amyloid- β metal-binding domain 1-16 is mediated by residues 11-14. *Mol Biosyst* **7**, 1053-1055.
- [13] Kulikova AA, Tsvetkov PO, Indeykina MI, Popov IA, Zhokhov SS, Golovin AV, Polshakov VI, Kozin SA, Nudler E, Makarov AA (2014) Phosphorylation of Ser8 promotes zinc-induced dimerization of the amyloid- β metal-binding domain. *Mol Biosyst* **10**, 2590-2596.
- [14] Miller Y, Ma B, Nussinov R (2010) Zinc ions promote Alzheimer A β aggregation via population shift of polymorphic states. *Proc Natl Acad Sci U S A* **107**, 9490-9495.
- [15] Mediannikov O, Morozov A. Peptide compound useful for inhibiting amyloid plaque formation. *France Patent*

- 2,966,827, filed October 10, 2010, and issued August 22, 2014.
- [16] Yardeni T, Eckhaus M, Morris HD, Huizing M, Hoogstraten-Miller S (2011) Retro-orbital injections in mice. *Lab Anim (NY)* **40**, 155-160.
- [17] Peters OM, Connor-Robson N, Sokolov VB, Aksinenko AY, Kukharsky MS, Bachurin SO, Ninkina N, Buchman VL (2013) Chronic administration of dimebon ameliorates pathology in TauP301S transgenic mice. *J Alzheimers Dis* **33**, 1041-1049.
- [18] Peters OM, Shelkownikova T, Tarasova T, Springe S, Kukharsky MS, Smith GA, Brooks S, Kozin SA, Kotelevtsev Y, Bachurin SO, Ninkina N, Buchman VL (2013) Chronic administration of dimebon does not ameliorate amyloid- β pathology in 5xFAD transgenic mice. *J Alzheimers Dis* **36**, 589-596.
- [19] Surgucheva I, Ninkina N, Buchman V, Grasing K, Surguchov A (2005) Protein aggregation in retinal cells and approaches to cell protection. *Cell Mol Neurobiol* **25**, 1051-1066.
- [20] Borchelt DR, Ratovitski T, van Lare J, Lee MK, Gonzales V, Jenkins NA, Copeland NG, Price DL, Sisodia SS (1997) Accelerated amyloid deposition in the brains of transgenic mice coexpressing mutant presenilin 1 and amyloid precursor proteins. *Neuron* **19**, 939-945.
- [21] Garcia-Alloza M, Robbins EM, Zhang-Nunes SX, Purcell SM, Betensky RA, Raju S, Prada C, Greenberg SM, Bacskai BJ, Frosch MP (2006) Characterization of amyloid deposition in the APPswe/PS1dE9 mouse model of Alzheimer disease. *Neurobiol Dis* **24**, 516-524.
- [22] Cho PY, Joshi G, Johnson JA, Murphy RM (2014) Transthyretin-derived peptides as β -amyloid inhibitors. *ACS Chem Neurosci* **5**, 542-551.
- [23] Parthasarathy V, McClean PL, Hölscher C, Taylor M, Tinker C, Jones G, Kolosov O, Salvati E, Gregori M, Masserini M, Allsop D (2013) A novel retro-inverso peptide inhibitor reduces amyloid deposition, oxidation and inflammation and stimulates neurogenesis in the APPswe/PS1dE9 mouse model of Alzheimer's disease. *PLoS One* **8**, e54769.
- [24] Wang Q, Liang G, Zhang M, Zhao J, Patel K, Yu X, Zhao C, Ding B, Zhang G, Zhou F, Zheng J (2014) De novo design of self-assembled hexapeptides as β -amyloid ($A\beta$) peptide inhibitors. *ACS Chem Neurosci* **5**, 972-981.
- [25] Jucker M, Walker LC (2013) Self-propagation of pathogenic protein aggregates in neurodegenerative diseases. *Nature* **501**, 45-51.
- [26] Kaye R, Lasagna-Reeves CA (2013) Molecular mechanisms of amyloid oligomers toxicity. *J Alzheimers Dis* **33**, S67-S78.
- [27] Istrate AN, Tsvetkov PO, Mantsyzov AB, Kulikova AA, Kozin SA, Makarov AA, Polshakov VI (2012) NMR solution structure of rat $A\beta$ (1-16): Toward understanding the mechanism of rats' resistance to Alzheimer's disease. *Biophys J* **102**, 136-143.
- [28] Tsvetkov PO, Popov IA, Nikolaev EN, Archakov AI, Makarov AA, Kozin SA (2008) Isomerization of the Asp7 residue results in zinc-induced oligomerization of Alzheimer's disease amyloid β (1-16) peptide. *Chembiochem* **9**, 1564-1567.
- [29] Kozin SA, Cheglakov IB, Ovsepyan AA, Telegin GB, Tsvetkov PO, Lisitsa AV, Makarov AA (2013) Peripherally applied synthetic peptide isoAsp7- $A\beta$ (1-42) triggers cerebral β -amyloidosis. *Neurotox Res* **24**, 370-376.