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Short Communication

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

Philipp O. Tsvetkov[a,b], Ivan B. Cheglakov[c], Armen A. Ovsepyan[c], Oleg Y. Mediannikov[d], Alexander O. Morozov[d], Georgy B. Telegin[c] and Sergey A. Kozin[d,*]
[a]*INSERM UMR 911, Aix-Marseille Université, CRO2, Marseille, France*
[b]*The Institute of General Pathology and Pathophysiology, Moscow, Russia*
[c]*Branch of Shemyakin and Ovchinnikov Institute of bioorganic chemistry of Russian academy of sciences, Pushchino, Russia*
[d]*Kimonella Ventures Ltd., Limassol, Cyprus*

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Abstract. Two tetrapeptides, HAEE and RADD, which are ionic-complementary to the primary zinc recognition site of amyloid-β (Aβ), have been reported to inhibit zinc-induced dimerization of the Aβ metal-binding domain and slow Aβ 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 (Aβ) 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 Aβ 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 Aβ [3, 4]. Several therapeutics that were purported to reduce Aβ 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 Aβ plaques and are critical for pathological Aβ aggregation [6]. Experimental structural data for Aβ oligomers complexed with zinc ions are unavailable; however, the role of the N-terminal residues 1–16 as the Aβ metal-binding domain has been established [7, 8], and the region 11–14 (EVHH) was determined as the primary zinc recognition site of Aβ [9]. This site also constitutes intermolecular interface within

*Correspondence to: Sergey A. Kozin, PhD, Kimonella Ventures Ltd., 205, Makarios III Avenue, Victory House, CY3030, Limassol, Cyprus. Tel.: +7 498 309 06 81; E-mail: kozinsa@gmail.com.

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the zinc-induced dimers and oligomers of Aβ isoforms [10–14]. Recently, two tetrapeptides, HAEE and RADD, which are ionic-complementary to ^{11}EVHH^{14} region of Aβ, have been reported to block zinc-induced dimerization of the Aβ metal-binding domain and slow Aβ 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-NH_2) and RADD (Ac-RADD-NH_2) 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× 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 μL 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 μg/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 ± 3.1	–
RADD	5	2	7	Synthetic Ac-RADD-NH_2 (22.5 μg in 125 μL of PBS)/9	50	5.2 ± 1.6	$p < 0.001$
HAEE	7	2	7	Synthetic Ac-HAEE-NH_2 (22.5 μg in 125 μL of PBS)/9	70	5.8 ± 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 μm) 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), H_2O 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 (±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 AβPP/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 μg in 125 μL 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 sagital brain sections (8 μm-thick) were analyzed histochemically using

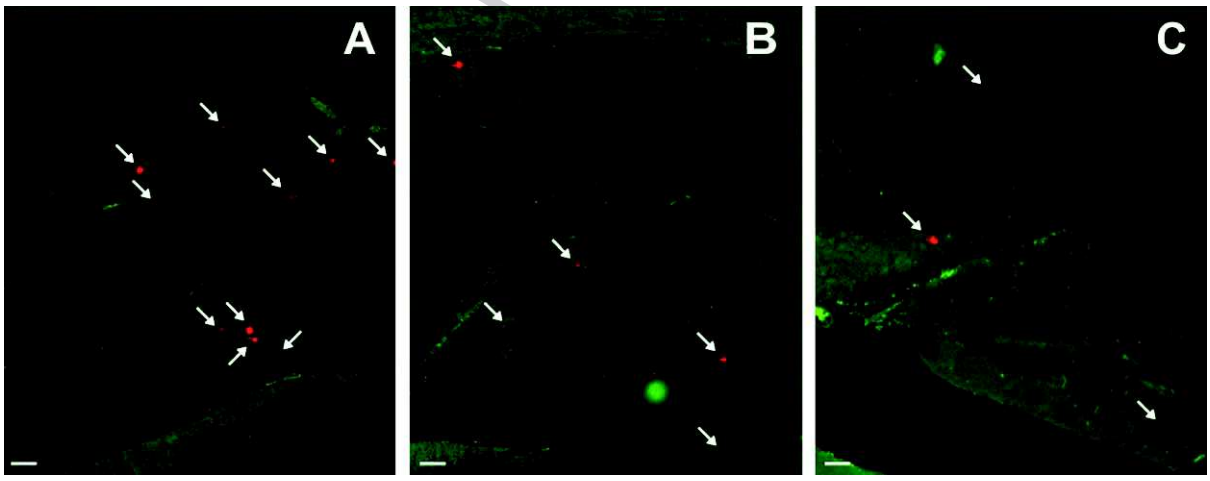

Fig. 1. Representative fluorescent micrographs of brain sections through the hippocampus for 7-month-old B6C3-Tg(APPswe, PSEN1dE9)85Dbo/j intact transgenic mice (A), the transgenic mice intravenously injected by synthetic HAEE (B) or RADD (C) tetrapeptides. Scale bars: A, B, and C, 50 μm.

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 HAEE- 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 Aβ regions are widely probed in peptide-based approach as drug candidate for disease-modifying therapy of AD [22–24]. Soluble dimers and oligomers of Aβ are considered as potential molecular agents triggering the amyloid cascade in AD progression [25, 26]. Zinc-induced dimers of Aβ appear to participate in this process [27]. Some chemical modifications within the metal-binding domain of Aβ (e.g., isomerization of Asp7 or phosphorylation of Ser8) facilitate zinc-dependent dimerization of the domain [13, 28]. The Aβ 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 HAEE and RADD used in our study have been designed to bind to the EVHH site [15] which mediates zinc-induced dimerization of Aβ [11–13]. Our data demonstrate that HAEE and RADD suppress pathological cerebral deposition of endogenous Aβ *in vivo*. Such short charged peptides seem to have satisfactory blood-brain barrier permeability and low immunogenicity. So HAEE 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 Aβ. Thus, the present work supports the use of ^{11}EVHH^{14} region of Aβ as promising drug target for preventing cerebral β-amyloidosis.

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