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ORIGINAL ARTICLE

Effect of low-concentration amyloid-β 1–42 (Aβ42) on human neuroblastoma SH-SY5Y cell viability: neuroprotective potential of combination use with carnosic acid, rebamipide, edaravone, and resveratrol

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Abstract Toxic amyloid-beta (Aβ) is known to generate symptoms of Alzheimer's disease (AD); however, less is known regarding the neurotoxicity of Aβ at lower concentrations. Moreover, the neuroprotective potential of combination treatment with plant biophenols and existing drugs is not well understood. In this study, we estimated the no-observed adverse effect level (NOAEL) of Aβ 1–42 (Aβ42) against cultured human neuroblastoma SH-SY5Y cells, and examined the neuroprotective effect of combination pretreatment with 10 μM carnosic acid, 30 nM rebamipide, 10 μM edaravone, and 10 μM of resveratrol (the "CRER" blend) on weak but toxic Aβ42-treated SH-SY5Y cells. We evaluated the NOAEL of Aβ42 at 500 nM in these cells. Aβ42 at 1–8 μM reduced cell viability; however, the "CRER" blend ameliorated this Aβ42-induced decrease in viability. The "CRER" blend induced the expression of Aβ-degrading enzymes including matrix metalloproteinase-14 (MMP-14) and neprilysin, while also enhancing the expression of the inducible α-secretase TACE (tumor necrosis factor-α-converting enzyme), sirtuin 1 (SIRT1), and brain-derived neurotrophic factor (BDNF). Collectively, our results indicate that the "CRER" may aid in the prevention of Aβ toxicity by enhancing MMP-14, neprilysin, TACE, SIRT1, and BDNF. Thus, the "CRER" blend may prove to be a promising strategy for the prevention of Aβ-mediated disorders, particularly AD.

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Key words: low-concentration Aβ42; cell viability; combination treatment; SH-SY5Y cells; Alzheimer's disease.

Introduction

Alzheimer's disease (AD) is the most common cause of dementia[1)], with amyloid-beta (Aβ) oligomers generating symptoms of AD pathology[2)] even in the absence of senile plaques[3)]. Aβ 1–40 (Aβ40) and Aβ 1–42 (Aβ42) are two major forms of Aβ peptides that are produced enzymatically from the amyloid precursor protein (APP)[4)]. Compared with Aβ40, Aβ42 exhibits higher neurotoxicity as a result of its higher hydrophobicity, which ultimately leads to faster oligomerization and aggregation[5)]. These Aβ monomers are physiologically produced by β-site APP-cleaving enzyme-1 (β-secretase) together with γ-secretase[6)]. Aβ production is decreased through the enhancement of α-secretases, such as ADAM10 (a disintegrin and metalloproteinase-10) and TACE (tumor necrosis factor-α-converting enzyme), which cleave APP at the α-site within the Aβ domain. In addition, Aβ-degrading enzymes (ADEs) such as matrix metalloproteinase-14 (MMP-14, alias membrane type-1 [MT-1]-MMP)[7)], neprilysin (NEP)[8, 9)], insulin-degrading enzyme (IDE), and endothelin-converting enzyme 1 (ECE1) have important roles in detoxi-

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fying Aβ[9].

A hypothetical model of dynamic biomarkers for AD has suggested that a substantial Aβ load occurs in the brain before the appearance of clinical symptoms[10]. Thus, this preclinical phase of AD provides a critical opportunity for preventive intervention. In this context, we previously found that carnosic acid, a phenolic diterpene compound in the labiate herb rosemary[11], reduces Aβ production by inducing TACE expression in both human neuron and astrocyte models[12, 13]. In addition, we also demonstrated that carnosic acid, rebamipide (a gastrointestinal protective drug[14]), and gnetin C (a resveratrol dimer[15]) can attenuate the Aβ-induced apoptosis by reducing the intracellular accumulation of Aβ oligomers[16-18], and that rebamipide and gnetin C enhance MMP-14 expression[17, 18] in a human neuron model. It has been reported that edaravone (a brain-penetrant drug and free radical scavenger[11]) down-regulates Aβ production by increasing α-site APP-cleavage[19]. Resveratrol is a naturally polyphenolic phytoalexin and is well known to have cytoprotective functions[20]. In particular, resveratrol is protective against neurotoxic factors, including Aβ peptides[21], and is reported to suppress the motor neuron degeneration in a transgenic mouse model of amyotrophic lateral sclerosis[22]. Some resveratrol oligomers such as gnetin C appear to have biological activities that are superior to monomeric resveratrol[18, 23]; however, gnetin C may be somewhat hard-to-use due to its light-susceptibility and current high-cost.

The SH-SY5Y cell line is a thrice clone from the neuroblastoma cell line SK-N-SH. The cells were originally derived from a bone marrow biopsy of a four-year-old female neuroblastoma patient. Although the AD literature has primarily focused on the voluntary nerve system, autonomic nerve system dysfunction in AD has also been reported[24]. The SH-SY5Y cell line is the most cited *in vitro* model system for detecting the impact of toxicants on mature and developing neurons. In addition, SH-SY5Y cells have the advantages of low cost, feasibility (ease of culture), reproducibility, and large data resources (through the literature), and may allow for the investigation of genes relevant in early cortical development[25].

The Aβ42 peptide at 10 μM or higher has clearly demonstrated neurotoxicity *in vitro*[16-18]. However, less is known regarding the neurotoxicity of Aβ42 at lower concentrations. Moreover, the neuroprotective potential of combination treatment with plant biophenols (such as carnosic acid and resveratrol[10, 26, 27]) and existing drugs (such as rebamipide[14] and edaravone[11]) is not well understood. Therefore, in the present study, we estimated the no-observed adverse effect level (NOAEL) of Aβ42 against cultured human neuroblastoma SH-SY5Y cells as a model of human neurons, and examined the neuroprotective effect of combination pretreatment with carnosic acid (10 μM), rebamipide (30 nM), edaravone (10 μM), and resveratrol (10 μM) (hereafter referred to as the "CRER" blend) on weak but toxic Aβ42 (up to 8 μM)-treated SH-SY5Y cells. We herein demonstrated "CRER" -induced TACE, MMP-14, NEP, sirtuin 1 (SIRT1), and brain-derived neurotrophic factor (BDNF). SIRT1 has been reported to activate multiple neuroprotective responses against cellular stresses[28]. BDNF has also been shown to protect neurons against cellular damage, including Aβ-induced toxicity[29].

Materials and Methods

1. Reagents

Dulbecco's Modified Eagle Medium (DMEM)/Nutrient F-12 Ham (F12) (1:1) containing GlutaMAX™-I, Advanced DMEM/F12, fetal bovine serum (FBS), and Zymax™ horseradish peroxidase (HRP)-conjugated anti-goat IgG were purchased from Invitrogen™/Thermo

Fisher Scientific Inc. (Frederick, MD, USA). Bovine serum albumin (BSA) and 3-[(3-cholamidopropyl) dimethylammonio]propanesulfonate (CHAPS) were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA). Carnosic acid, rebamipide, and edaravone were generously donated by Nagase Co., Ltd. (Osaka, Japan), Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan), and Mitsubishi Tanabe Pharma Corporation (Osaka, Japan), respectively. Resveratrol (natural, #185-0721), dimethyl sulfoxide (DMSO), sodium dodecyl sulphate (SDS) and the enzyme-linked immunosorbent assay (ELISA) kit for Aβ42 (Human/Rat βAmyloid (1-42) ELISA Kit Wako, High-Sensitive) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The ELISA kit for BDNF (BDNF Emax® ImmunoAssay System, #G7610) was purchased from Promega Corporation (Madison, WI, USA). The human Aβ42 monomer was obtained from Peptide Institute, Inc. (Ibaraki, Japan). The CellQuanti-MTT™ assay kit was obtained from BioAssay Systems (Hayward, CA, USA). The NucleoSpin® RNA II total RNA isolation kit was obtained from Macherey-Nagel GmbH & Co. KG (Düren, Germany). The iScript™ Advanced cDNA synthesis kit and SsoAdvanced™ Universal SYBR® Green Supermix solution for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) were both obtained from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Oligonucleotide primers for qPCR were custom synthesized by Fasmac/Greiner Japan (Atsugi, Japan). The bicinchoninic acid (BCA™) protein assay kit was obtained from Pierce®/Thermo Scientific Inc. (Waltham, MA, USA). Polyvinylidene fluoride (PVDF) membranes and the Luminata™ Crescendo Western HRP substrate were obtained from Millipore Corporation (Billerica, MA, USA). The anti-Aβ 1–16 monoclonal antibody (6E10, mouse ascites, #SIG-39300) for Aβ oligomers was obtained from BioLegend, Inc. (San Diego, CA, USA), formerly known as Covance. Antibodies against poly-(ADP-ribose) polymerase (PARP) (rabbit, #9542), MMP-14/MT1-MMP (D1E4, rabbit monoclonal, #13130), and TACE/a disintegrin and metalloprotease-17 (ADAM17) (raised against Val200, rabbit, #3976) were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). The anti-NEP antibody (N2C1, internal, rabbit, #GTX111680) was obtained from GeneTex Inc. (Irvine, CA, USA). Antibodies to SIRT1 (E104, C-terminal, rabbit, #ab32441) and β-actin (ACTN (C4), mouse monoclonal, #ab3280) were purchased from Abcam plc (Cambridge, UK). Anti-rabbit and anti-mouse IgG-HRP were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

2. Cell Culture

Human neuroblastoma SH-SY5Y cells (ECACC No. 94030304) were obtained from the European Collection of Authenticated Cell Cultures (Porton Down, UK), and cultured in DMEM/F12 containing GlutaMAX™-I supplemented with 10% FBS[18, 30]. Once the cells were 80% confluent, they were used for experimental purposes and the medium was immediately replaced with fresh Advanced DMEM/F12 containing GlutaMAX™-I supplemented with 3% FBS. In experiments with plant biophenols (carnosic acid and resveratrol) and existing drugs (rebamipide and edaravone), cells were pretreated with the biophenols for 1 h prior to the addition of the Aβ42 monomer. Aβ42, carnosic acid, and resveratrol were dissolved in DMSO. The maximum final concentration of DMSO in the culture medium was 1.2%. Control cells were treated with vehicle alone.

3. Cell viability

Cell viability was measured with a CellQuanti-MTT™ assay kit according to the manufacturer's protocol with slight modifications[16-18]. Briefly, SH-SY5Y cells were cultured in 96-well culture plates at 4×10^4 cells per well in 100 μL of

Table 1. Oligonucleotide primers used for real-time quantitative PCR

cDNA	Forward sequence (5′ to 3′)	Reverse sequence (5′ to 3′)
BDNF	AGCCTCCTCTTCTCTTTCTGCTGGA	CTTTTGTTGTCTATGCCCCTGCCTT
18S rRNA	ACTCAACACGGGAAACCTCA	AACCAGACAAATCGCTCCAC

Abbreviations used: BDNF, brain-derived neurotrophic factor.

medium. The MTT reagent (15 μL) was then added to each well, and the cells were incubated for an additional 4 h. The formazan crystals that are only produced by living cells were solubilized by adding 100 μL/well of solubilizer (10% SDS, 3.2 mM HCl) and the plates were incubated for ~2 h at 37°C. The absorbance (A570) of the resulting colored solution was measured with a microplate reader (iMark, Bio-Rad). Each experiment was performed in triplicate.

4. ELISA

Cell culture supernatants were mixed with 0.2% BSA and 0.075% CHAPS to minimize the loss of Aβ42, which can occur by adhesion to the storage tubes. Aliquots of the samples were stored at −80°C until further use. Levels of Aβ42 in the cell-conditioned medium (extracellular Aβ42) and the diluted whole cell lysates (intracellular Aβ42) were determined using an ELISA kit and the iMark/Bio-Rad microplate reader[13, 14]. BDNF levels in the conditioned medium were also determined.

5. RT-qPCR

Total RNA was extracted from cells, and single-strand cDNA was synthesized using the iScript™ Advanced cDNA synthesis kit for RT-qPCR[17, 30]. The CFX96™ Real-Time PCR System (Bio-Rad) was used for quantitative analyses of 18S rRNA and the mRNA of the gene listed in Table 1. The concentration of the primers used was at 500 nM. Amplification was performed using the SsoAdvanced™ Universal SYBR® Green Supermix solution. The following reaction conditions were used: 95°C for 30 s; 40 cycles of 95°C for 5 s, 55°C for 10 s, and 60°C for 15 s; and an increase from 65–95°C, 0.5°C/5 s, for the melting step. Data were analyzed using CFX Manager™ Version 2.1 software.

6. Western blot analysis

Cellular proteins were detected by Western blotting, as described previously[16-18]. Briefly, cells cultured in 35 mm-diameter dishes were washed twice with ice-cold 20 mM phosphate-buffered saline (PBS, pH 7.4). The cells were then lysed in 100 μL of Laemmli's reducing sample buffer with passing through a 26-gage needle 10 times and then incubated on ice for 30 min. The lysates were then centrifuged at 10,000 × *g* for 20 min at 4°C, and the supernatants were transferred to fresh tubes. After the BCA protein assay, aliquots of the solutions containing 5, 10, or 20 μg protein were mixed with 6× loading buffer and boiled for 6 min. The samples were then subjected to electrophoresis on 7.5% or 10–20% SDS-polyacrylamide gels, and the proteins were transferred onto PVDF membranes. The membranes were blocked by incubation in Tris-buffered saline with Tween 20 (TBS-T, pH 7.4, 50 mM Tris-HCl, 250 mM NaCl, and 0.1% Tween-20) containing 1% nonfat dry milk for 1 h at room temperature. The membranes were then incubated with the anti-Aβ oligomer, anti-PARP, anti-MMP-14, anti-NEP, anti-TACE, or anti-SIRT1 antibodies (1:1000) overnight at 4°C, followed by a third incubation with the appropriate secondary antibodies (HRP-conjugated anti-rabbit, or anti-mouse IgG, 1:5000) for 1 h at room temperature. Immunoreactive bands were detected with an enhanced chemilu-

minescence (ECL) detection system. Blots were then re-probed with the anti-β-actin antibody (1:5000) as a loading control.

7. Densitometric quantification

The integrated optical density of the immunoreactive bands was quantified using ImageJ 1.49i software (National Institute of Health, Bethesda, MD, USA), and normalized to the β-actin band density.

8. Statistics

Values are expressed as the mean ± SD, and statistical significance was analyzed by the unpaired two-tailed *t*-test or one-way analysis of variance. Significance was assigned to a probability (*P*) value less than 0.05.

Results

1. Cytotoxic concentration of Aβ42 against cultured human neuroblastoma SH-SY5Y cells

We first evaluated the NOAEL of Aβ42 against cell viability in cultured human neuroblastoma SH-SY5Y cells using the MTT assay (Fig. 1). After treatment for 24 h, Aβ42 did not affect the cell viability up to 500 nM (Figs. 1A and 1B); however, Aβ42 decreased the cell viability at 600 nM and higher (Figs. 1B). Even after treatment for 48 h (Fig. 1C), Aβ42 at 500 nM did not significantly show toxicity or elicit morphological changes in the cells. Therefore, we estimated the NOAL of Aβ42 against SH-SY5Y cells at 500 nM.

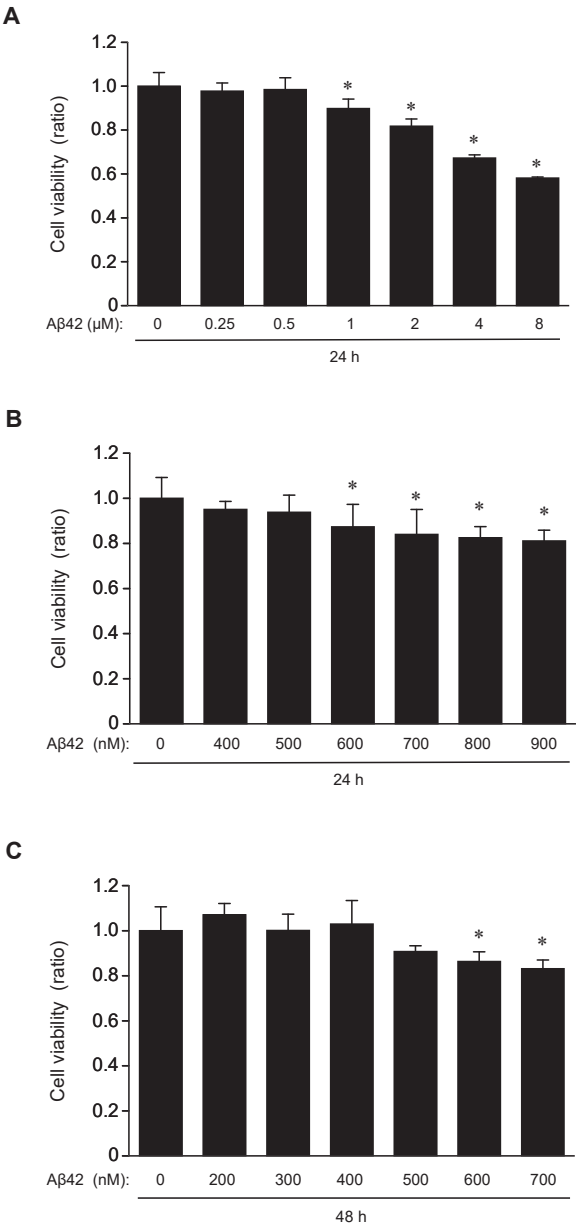

Fig. 1 Cell viability of cultured SH-SY5Y cells. Cells were treated with different concentrations of amyloid-β 1–42 (Aβ42) up to 8 μM for 24 h (A, B) or 48 h (C). Dimethyl sulfoxide (DMSO) was used as a solvent for Aβ42. Cell viability was quantified by the MTT assay and the mean ± SD of three experiments is shown; $^{*}P < 0.05$ vs. Aβ42-free control (0.8% DMSO).

2. A combination with carnosic acid, rebamipide, edaravone, and resveratrol efficiently ameliorates the decreased cell viability induced by Aβ42

We next examined cell viability in Aβ42-treated SH-SY5Y cells that were pretreated with 10 μM of carnosic acid, 30 nM of rebamipide, 10 μM of edaravone, and/or 10 μM of resveratrol (Fig. 2). We decided on these four agents (Fig. 2A) and their concentrations based on our previous studies[11-13, 16-18]. Cells were pretreated with the agent(s) or their combinations for 1 h, followed by 10 μM of Aβ42 for 24 h (Fig. 2B). Control cells were treated with DMSO during the experiment. Consistent with

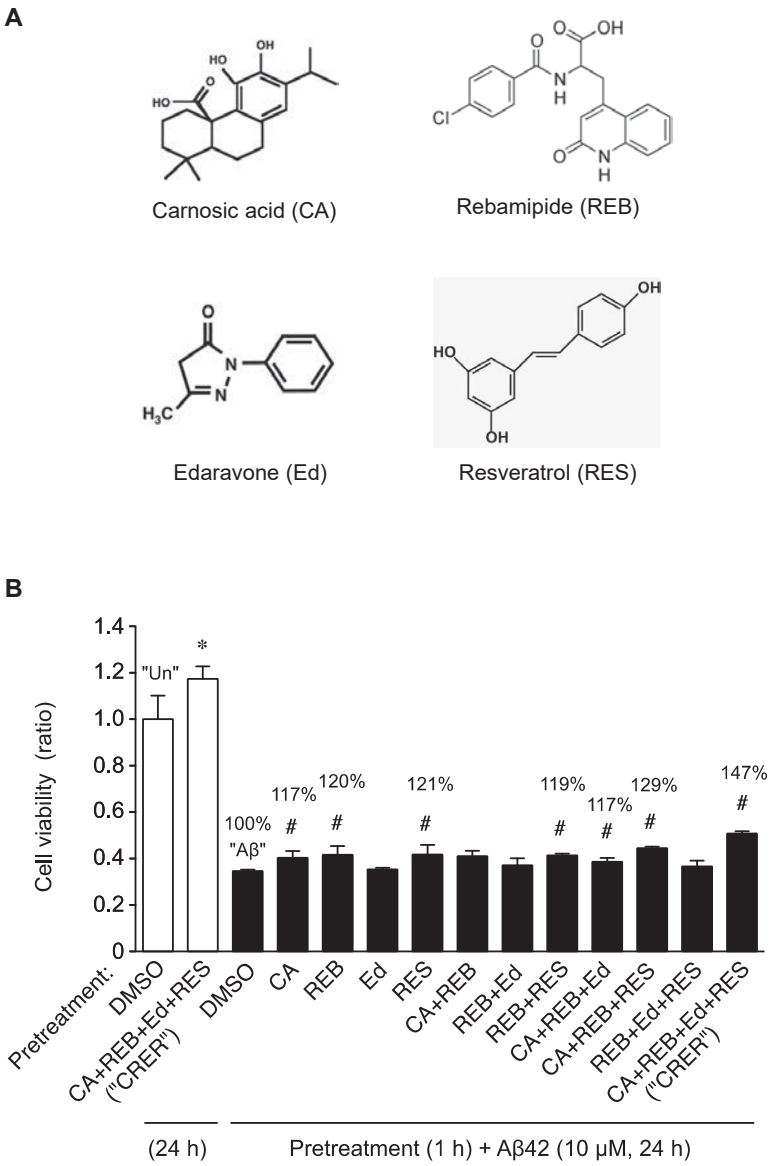

Fig. 2 (A) Chemical structures of carnosic acid (CA, 332.4 g/mol), rebamipide (REB, 370.8 g/mol), edaravone (Ed, 174.2 g/mol), and resveratrol (RES, 228.3 g/mol). (B) Ameliorative effects of CA (10 μM), REB (30 nM), Ed (10 μM), or RES (10 μM) and their combinations on the high-dose amyloid-β 1–42 (Aβ42)-lowered cell viability in cultured SH-SY5Y cells. After pretreatment with the neuroprotective agents for 1 h, the cells were incubated with 10 μM of Aβ42 for 24 h. Dimethyl sulfoxide (DMSO) was used as a vehicle control. Cell viability was quantified by the MTT assay and the mean ± SD of three experiments is shown; $^{*}P < 0.05$ vs. "Un" control (1.2% DMSO) and $^{\#}P < 0.05$ vs. "Aβ" control (10 μM Aβ42).

our previous studies[16-18], Aβ42 reduced cell viability to 35%; however, among the agent(s) or their combinations, the combination with 10 μM carnosic acid, 30 nM rebamipide, 10 μM edaravone, and 10 μM resveratrol (the "CRER" blend) ameliorated the toxicity most efficiently (47%). Therefore, we used the "CRER" blend for the following experiments.

3. The "CRER" blend ameliorates reduced cell viability and an apoptotic marker aggravated by low-dose Aβ42

We next examined cell viability and cleaved PARP, an apoptotic marker[31], in Aβ42 (0.5–8

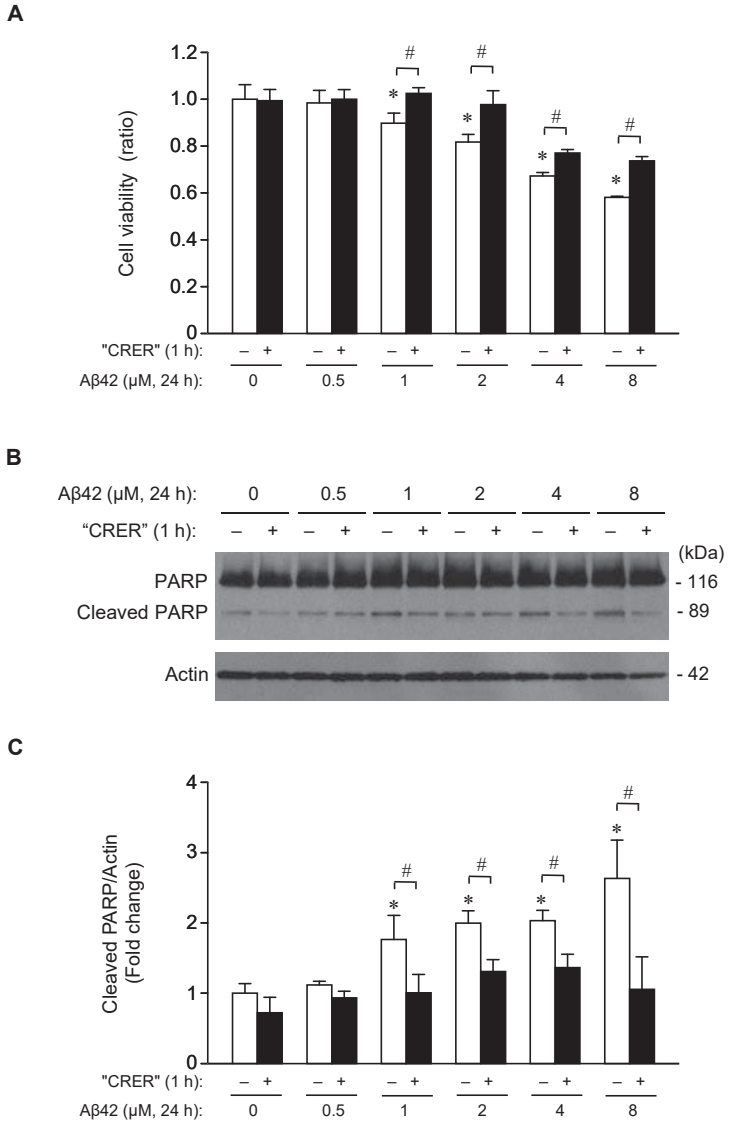

Fig. 3 Ameliorating effects of combination treatment with 10 μM of carnosic acid, 30 nM of rebamipide, 10 μM of edaravone, and 10 μM of resveratrol (the "CRER" blend) on the low-dose amyloid-β 1–42 (Aβ42)-reduced cell viability in cultured SH-SY5Y cells. After pretreatment with the "CRER" blend for 1 h, the cells were incubated with 0.5–8 μM of Aβ42 for 24 h. Dimethyl sulfoxide (DMSO) was used as a vehicle control. The mean ± SD of three experiments is shown; $^{*}P < 0.05$ vs. control (1.2% DMSO) and $^{\#}$ denotes $P < 0.05$. (A) Cell viability was quantified by the MTT assay. (B) The cellular protein level of cleaved poly-(ADP-ribose) polymerase (PARP) was detected by Western blotting. (C) The integrated optical density of the protein band in (B) was quantified using ImageJ 1.49i and was normalized to that of actin.

μM)-treated SH-SY5Y cells that were pretreated with the "CRER" blend (Fig. 3). As shown in Fig. 3A, the "CRER" blend most fully recovered the reduced viability in cells treated with 1 and 2 μM Aβ42, and moderately improved the viability in cells treated with 4 and 8 μM Aβ42. At the same time, the "CRER" blend suppressed the increased levels of cleaved PARP in cells treated with 1–8 μM Aβ42 (Figs. 3B and 3C).

4. The "CRER" blend reduces intracellular Aβ oligomers in cells treated with high-dose Aβ42, and suppresses both intra- and extracellular Aβ monomers in those with low-dose Aβ42

To clarify the effects of "CRER" pretreatment on the levels of intracellular Aβ oligomers, we

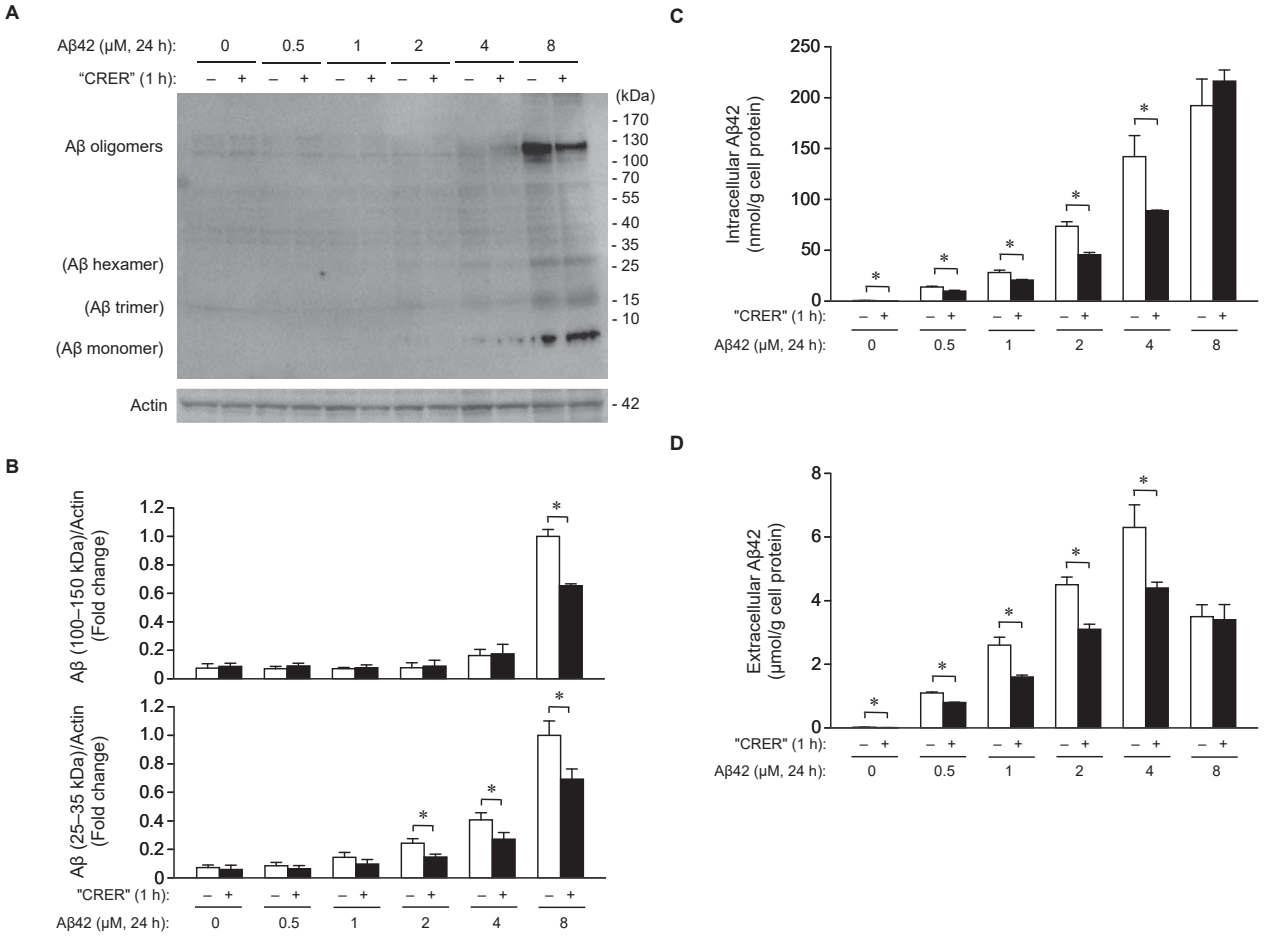

Fig. 4 Effects of combination treatment with 10 μM of carnosic acid, 30 nM of rebamipide, 10 μM of edaravone, and 10 μM of resveratrol (the "CRER" blend) on the presence of amyloid-β (Aβ) oligomers and monomers in the low-dose Aβ42-challenged SH-SY5Y cells. After pretreatment with the "CRER" blend for 1 h, the cells were incubated with 0.5–8 μM of Aβ 1–42 (Aβ42) for 24 h. Dimethyl sulfoxide (DMSO) was used as a vehicle control. The mean ± SD of three experiments is shown. * denotes $P < 0.05$. (A) Aβ oligomers in whole cell lysates were detected by Western blotting (samples were separated in a 10–20% sodium dodecyl sulfate-polyacrylamide gradient gel; probed with a mouse anti-Aβ monoclonal antibody, 1–16 (6E10), ascites), and actin was used as a loading control. The data shown are representative of three experiments. (B) The integrated optical density of the blot for high molecular weight oligomers (100–150 kDa) in (A) was quantified using ImageJ 1.49i and normalized to that of actin. (C and D) Under the same condition as shown in (A), intracellular (C) and extracellular (D) Aβ42 monomers in whole cell lysates and cell-conditioned medium, respectively, were analyzed by enzyme-linked immunosorbent assay (ELISA), and normalized to total cell protein determined by the BCA assay.

analyzed the generation of Aβ oligomers in whole lysates of cells preincubated with the "CRER" blend for 1 h and then treated with the Aβ42 monomer (0.5–8 μM) for 24 h (Figs. 4A and 4B). In cells treated with Aβ42 at 8 μM, "CRER" pretreatment reduced the level of intracellular Aβ oligomers (high molecular weight oligomers[32, 33], 100–150 kDa) by 35%. Yet in cells treated with Aβ42 at 4 μM or lower, the levels of the 100–150 kDa Aβ oligomers were not significant and the "CRER" blend did not appear to affect the levels. However, the "CRER" blend suppressed the levels of an Aβ oligomer (*ca.* 30 kDa) in cells treated with Aβ42 at 2–8 μM. Moreover, "CRER" pretreatment decreased the levels of both intracellular and extracellular Aβ42 monomers in cells treated with Aβ42 at 4 μM or lower (Figs. 4C and 4D).

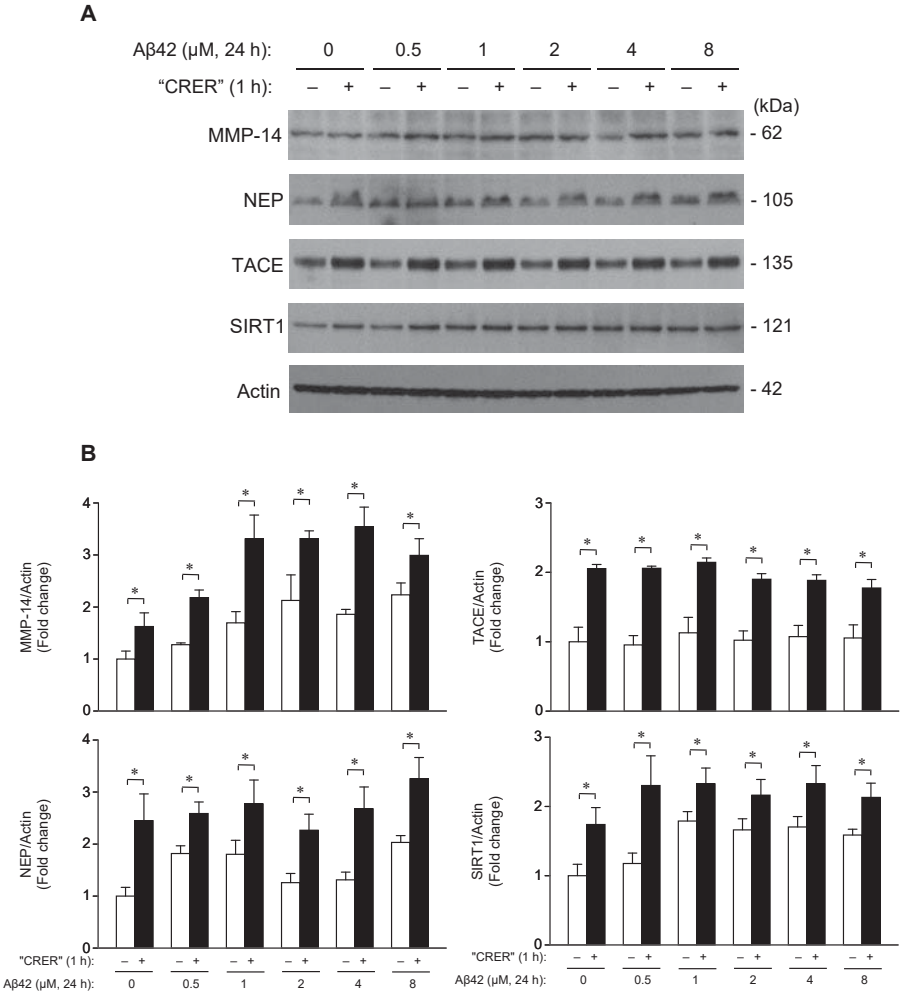

Fig. 5 Effects of combination treatment with 10 μM of carnosic acid, 30 nM of rebamipide, 10 μM of edaravone, and 10 μM of resveratrol (the "CRER" blend) on the protein expression of amyloid-β-degrading enzymes (ADEs) and sirtuin 1 (SIRT1) in the low-dose Aβ42-challenged SH-SY5Y cells. Matrix metalloproteinase-14 (MMP-14, also termed as membrane type-1 MMP [MT1-MMP]), neprilysin (NEP), and tumor necrosis factor-α-converting enzyme (TACE) were examined as ADEs. After pretreatment with the "CRER" blend for 1 h, the cells were incubated with 0.5–8 μM of Aβ 1–42 (Aβ42) for 24 h. Dimethyl sulfoxide (DMSO) was used as a vehicle control. The mean ± SD of three experiments is shown. * denotes $P < 0.05$. (A) Proteins in whole cell lysates were detected by Western blotting (samples were separated in a 7.5% sodium dodecyl sulfate-polyacrylamide gel), and actin was used as a loading control). The data shown are representative of three experiments. (B) The integrated optical density of the protein band in (A) was quantified using ImageJ 1.49i and was normalized to that of actin.

5. The "CRER" blend enhances the expression of ADEs and SIRT1

Based on the aforementioned results, we next investigated "CRER"-mediated changes in the expression of genes that can potentially affect Aβ levels (Fig. 5A). We focused on enzymes known to be related to Aβ degradation, including MMP-14 and NEP, and the inducible α-secretase TACE. We found that "CRER" pretreatment significantly enhanced the expressions of these enzymes at the protein level even in the presence of Aβ42 at 0.5–8 μM (Fig. 5B). Simultaneously, the "CRER" blend was found to induce SIRT1 protein expression (Fig. 5B).

6. The "CRER" blend enhances BDNF expression even in the presence of low-dose Aβ42

We next observed that the "CRER" blend enhanced the mRNA expression (Fig. 6A) and protein secretion (Fig. 6B) of BDNF in SH-

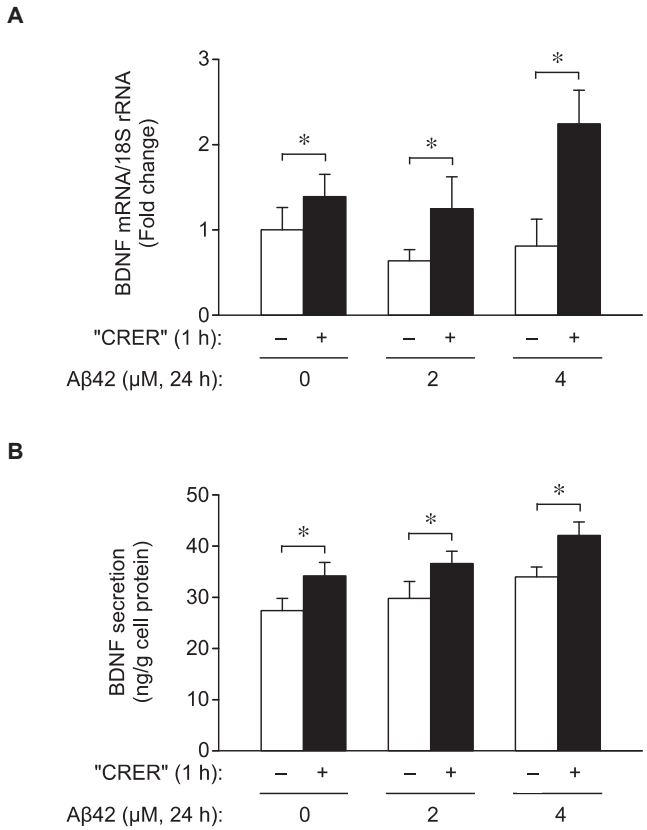

Fig. 6 Effects of combination treatment with 10 μM of carnosic acid, 30 nM of rebamipide, 10 μM of edaravone, and 10 μM of resveratrol (the "CRER" blend) on the expression and secretion of brain-derived neurotrophic factor (BDNF) in the low-dose Aβ42-stimulated SH-SY5Y cells. After pretreatment with the "CRER" blend for 1 h, the cells were incubated with 2 or 4 μM of Aβ 1–42 (Aβ42) for 24 h. Dimethyl sulfoxide (DMSO) was used as a vehicle. The mean ± SD of three experiments is shown. * denotes $P < 0.05$. (A) The mRNA expression of BDNF was analyzed by real-time quantitative PCR and was normalized to 18S rRNA levels (relative value). (B) The secreted level of BDNF in the cell-conditioned medium was analyzed by enzyme-linked immunosorbent assay (ELISA) and normalized to total cell protein determined by the BCA assay.

SY5Y cells. This increase was also observed in the presence of 2 and 4 μM Aβ42 after 24 h.

Discussion

In the present study, we first examined the neurotoxicity of Aβ42 at lower concentrations in cultured human neuroblastoma SH-SY5Y cells, and estimated the NOAEL of Aβ42 at 500 nM in SH-SY5Y cells. The normal levels of Aβ42 *in vivo* are not considered to exceed 500 nM. Accumulated evidence suggests that Aβ plays beneficial roles in human physiology, such as in antimicrobial activity, tumor suppression, sealing leaks in the blood–brain barrier (BBB), promoting recovery from brain injury, and regulating synaptic function[34]. A soluble form of Aβ42 is normally secreted into the extracellular space within the brain and then cleared by the cerebrospinal fluid (CSF) and vascular system. Because CSF is in direct contact with the brain, its composition is affected by biochemical changes in the brain. CSF Aβ42 has been shown to reflect the deposition of amyloid plaques. The mean level of CSF Aβ42 in AD is reportedly 382.2 ng/L (85 pM), whereas the level in the healthy controls is 755.6 ng/L (170 pM)[35]. It is hypothesized that the abnormally low CSF Aβ42 levels in AD patients reflected the increased deposition of Aβ42 into senile plaques, resulting in reduced CSF Aβ42

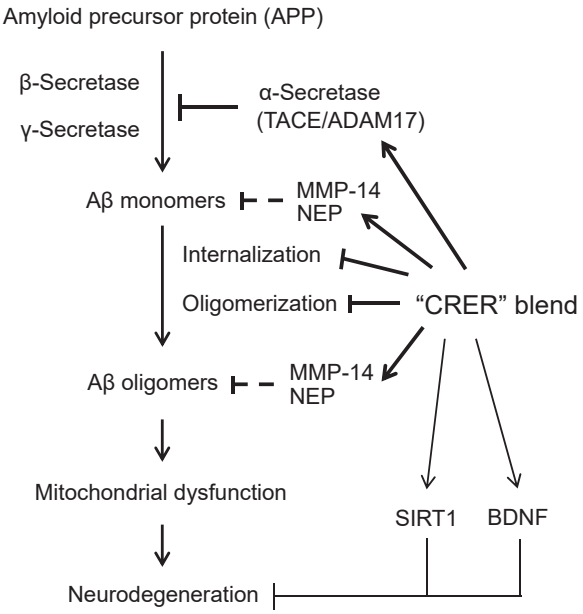

Fig. 7 Neuroprotective potential of combination treatment with 10 μM of carnosic acid, 30 nM of rebamipide, 10 μM of edaravone, and 10 μM of resveratrol (the "CRER" blend) in cultured SH-SY5Y cells. We focused on the processing of amyloid precursor protein (APP) and the reduction in cell viability induced by amyloid-β (Aβ). The "CRER" blend may play a role in neuroprotection by enhancing α-secretase TACE/ADAM17 and Aβ-degrading enzymes (ADEs) such as MMP-14 and NEP, in cooperation with inhibiting both the internalization and oligomerization of Aβ monomers. In addition, "CRER"-induced SIRT1 and BDNF may also support the survival of the cells. Abbreviations used: ADAM17, a disintegrin and metalloproteinase-17; BDNF, brain-derived neurotrophic factor; MMP-14, matrix metalloproteinase-14 (also termed as MT1-MMP, membrane type-1 MMP); NEP, neprilysin; SIRT1, sirtuin 1; and TACE, tumor necrosis factor-α-converting enzyme.

concentrations. However, how well CSF Aβ42 can identify AD pathology and predict future AD symptoms remains unclear[36]. It is challenging to measure Aβ42 concentration within the brain parenchyma in healthy subjects. Nevertheless, the evaluation of the cell toxicity of Aβ42 in a neuronal model may help clarify the role of Aβ physiology in AD pathology in future studies.

Our next results demonstrated that the full "CRER" blend has an ameliorating effect on the Aβ42 (10 μM)-reduced cell viability compared with other incomplete blends. The "CRER" blend consisted of 10 μM carnosic acid, 30 nM rebamipide, 10 μM edaravone, and 10 μM resveratrol. These plant biophenols (carnosic acid and resveratrol) and existing drugs (rebamipide and edaravone) serve primarily to reduce cellular oxidative stress. Indeed, carnosic acid induces the transcription factor Nrf2, which is a master regulator of the antioxidant response[11]. Rebamipide can cross the BBB after oral administration where it inhibits the generation of reactive oxygen species[14]. Edaravone has been shown to ameliorate postischemic neural dysfunction in patients with acute stroke[11]. While edaravone alone did not significantly ameliorate the Aβ42-induced decrease in cell viability observed in this study, it did have an ameliorative effect to the highest level of the full "CRER" blend. Resveratrol also shows free radical scavenging activity, together with a remarkable range of cytoprotective properties[20, 21]. These findings support the hypothesis that the "CRER" blend is helpful for attenuating the Aβ-induced oxidative stress, as Aβ is known to generate reactive oxygen species in the AD brain[37]. The "CRER" blend may also be helpful for patients with gas-

troenteritis and/or stroke to prevent associated dementia. This is partly because 40% of stroke patients have AD lesions[38].

Since edaravone and rebamipide are prescribed medicines, it is not realistic to expect that healthy subjects use them in everyday life. Rather, in clinical situations, it can be supposed that patients with acute stroke (including those with AD complication) prescribed edaravone ingest rebamipide, carnosic acid, and resveratrol simultaneously to improve the AD prevention effect, even in the chronic phase, and that patients prescribed rebamipide also ingest other "CRER" blend components to enhance the AD prevention effect.

This study also demonstrated that "CRER" pretreatment effectively improves the deteriorated viability of the cells challenged with Aβ42 at 1–8 μM. Because pretreatment decreased the levels of cleaved PARP, the "CRER" blend may suppress apoptosis induced by Aβ42 at 1–8 μM. Importantly, in cells treated with Aβ42 up to 2 μM, the "CRER" blend was able to fully recover the Aβ42-reduced cell viability. This means the "CRER" blend may increase the NOAEL of Aβ42 from 500 nM to 2 μM in the cells.

Consistent with our previous studies[16-18], Aβ oligomers are primarily between 100–150 kDa and are apparently generated in cells treated with Aβ42 at 8 μM. The "CRER" blend effectively reduced the Aβ oligomers that are similar to amylospheroids (ASPD, 128 kDa), which have been shown to cause mitochondrial calcium dyshomeostasis resulting in neurodegeneration[39]. Under such a high-dose Aβ42 (8 μM) load, the "CRER" blend may play a role in inhibiting intracellular Aβ oligomerization. On the other hand, under low-dose Aβ42 loads (up to 4 μM), Aβ oligomers only faintly appeared; however, the "CRER" blend reduced the levels of the Aβ oligomer between 25–35 kDa (also at 8 μM Aβ42) and both intracellular and extracellular Aβ monomers. The reduction of such potentially toxic Aβ peptides may contribute to the increased cell viability in Aβ-challenged cells.

In addition, we confirmed that the "CRER" blend promotes the syntheses of MMP-14, NEP, TACE, and SIRT1 even in cells treated with Aβ42. MMP-14 is reported to degrade both soluble and fibrillar Aβ peptides[7], and it may play a role in reducing both Aβ monomers and oligomers in cells challenged with Aβ. Thus, both the enhanced α-secretase TACE[40] and the induced Aβ-decomposing enzymes, including MMP-14 and NEP[8, 9], may be responsible for the suppression of intracellular Aβ accumulation. Moreover, "CRER"-induced SIRT1 may also suppress neurodegeneration by promoting unique cytoprotective systems such as heat shock proteins[22].

We also report here that "CRER" consistently up-regulates BDNF expression and secretion. The neurotrophin BDNF has been shown to support the survival and outgrowth of many types of neurons such as sensory, ganglion, dopaminergic, cholinergic, GABAergic, and motor[41]. In addition, a substantial body of evidence has shown that BDNF protects neurons against cellular damage, including Aβ-induced toxicity[29]. Therefore, these factors may comprehensively contribute to the ameliorated cell viability in cells treated with the "CRER" blend. Additional research is warranted in order to clarify the underlying mechanisms of the protective and/or restorative activities of the "CRER" blend on Aβ-induced damage and apoptosis in neurons.

Collectively, our findings indicate that the "CRER" blend reduces Aβ production and ameliorates Aβ-induced neurotoxicity. Possible mechanisms by which the "CRER" blend may execute these effects include an increase in α-site APP-cleavage and Aβ decomposition through the enhancement of TACE and the induction of MMP-14 and NEP, respectively (Fig. 7). Moreover, the "CRER" blend may also play a role in reducing both the internalization and

oligomerization of exogenous Aβ monomers. Reduction of intracellular Aβ peptides by the "CRER" blend may contribute to the preservation or recovery of neuronal cell viability, ultimately protecting cells from Aβ accumulation. Thus, the "CRER" blend may be a promising strategy to prevent the progression of Aβ-mediated disorders, such as AD.

Conflict of Interest Statement

All authors have no conflicts of interest directly relevant to the content of this article.

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