Gnetin C, a resveratrol dimer, reduces amyloid-β 1–42 (Aβ42) production and ameliorates Aβ42-lowered cell viability in cultured SH-SY5Y human neuroblastoma cells

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ABSTRACT
Accumulation and oligomerization of amyloid-beta (Aβ) peptides have been known to be a potent cause of neurodegenerative diseases such as Alzheimer’s disease (AD). To expand the possibilities of preventing AD, we investigated the effects of resveratrol dimers, gnetin C and ε-viniferin, on Aβ 1–42 (Aβ42) production and the reduced cell viability observed after Aβ42 treatment (monomers, 10 μM) in cultured SH-SY5Y human neuroblastoma cells. Among them, addition of gnetin C (20 μM) into the media reduced Aβ42 production most efficiently. Gnetin C suppressed the expression of β-site amyloid precursor protein-cleaving enzyme-1 (BACE1, β-secretase). Furthermore, gnetin C ameliorated the Aβ42-reduced cell viability most significantly. Concomitantly, gnetin C reduced intracellular Aβ oligomers (ca. 15 and 130 kDa) and elevated both levels of intracellular and extracellular Aβ monomers. Under the treatment with or without Aβ42, gnetin C upregulated the expression of matrix metalloproteinase-14 (MMP-14) which is assumed to be an Aβ-decomposing enzyme. Gnetin C may thereby prevent Aβ toxicity by suppressing BACE1 and enhancing MMP-14, together with reducing both internalization and oligomerization of exogenous Aβ monomers. The use of gnetin C may lead to the prevention of Aβ-mediated diseases, particularly AD.

Alzheimer’s disease (AD) is a degenerative brain disease and the most common cause of dementia; accounts for an estimated 60–80% of cases (1). Importantly, amyloid-beta (Aβ) oligomers generate symptoms of AD pathology, even in the absence of senile plaques, as seen in transgenic mice that lack the ability to generate Aβ fibrils (37). This finding has strongly supported the Aβ oligomer hypothesis as a molecular basis for AD (2, 7, 15). Aβ 1–40 (Aβ40) and Aβ 1–42 (Aβ42) are two major forms of Aβ peptides produced enzymatically from the amyloid precursor protein (APP) (33). These Aβ monomers are physiologically produced by β-site APP-cleaving enzyme-1 (BACE1, β-secretase) together with presenilin-1 (the main part of γ-secretase) (24). Whereas, the Aβ production is prevented by enhancing α-secretases, such as a disintegrin and metalloproteinase-10 (ADAM10) and tumor necrosis factor-α-converting enzyme (TACE), which cleave APP at the α-site within Aβ domain. In addition, there are Aβ-decomposing enzymes, e.g., neprilysin (NEP) (12, 38), insulin-degrading enzyme (IDE), endothelin-converting enzyme 1 (ECE1) (38) and matrix metalloproteinase-14 (MMP-14, alias membrane type-1 [MT1]-MMP) (18). Compared to Aβ40, Aβ42 exhibits higher neuro-
toxicity as a result of its higher hydrophobicity, thus, leading to faster oligomerization and aggregation (3). Aβ 1–43 (Aβ43) is also frequently detected in AD brains (31, 39), and it is reported to be more neurotoxic than Aβ42 and can promote Aβ42 polymerization (22, 30); however, physiological Aβ43 production in vitro is nearly undetectable (40) and its deteriorating effect on cell viability is comparable to Aβ42 (8).

A hypothetical model of dynamic biomarker for AD suggests that a substantial Aβ load in the brain occurs before the appearance of clinical symptoms (34). 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, reduces Aβ production by inducing TACE expression in both human neuron and astrocyte models (21, 40). We also demonstrated that in a human neuron model, carnosic acid and rebamipide, a gastrointestinal protective drug, attenuate the Aβ-induced apoptosis by reducing the intracellular accumulation of Aβ oligomers (8, 22), and that rebamipide enhances the expression of MMP-14 (8).

Resveratrol is a red grape-derived bi phenol and is well known to have cytoprotective functions (9, 23), in particular, against neurotoxic factors such as Aβ peptides (13, 20, 28). Some resveratrol oligomers appear to have biological activities that are superior to monomeric resveratrol (6). However, the neuroprotective functions of resveratrol dimers have not been well known (26, 27). In the present study, we focused on two resveratrol dimers (19), gnetin C isolated from melinjo (Gnetum gnemon L.) and ε-viniferin from grapevines; and examined the effects of these dimers on Aβ42 production and Aβ42-induced neurotoxicity using cultured human neuroblastoma cells as a model of human neurons.

MATERIALS AND METHODS

Reagents. Dulbecco’s Modified Eagle Medium (DMEM)/Nutrient F-12 Ham (F12) (1 : 1) containing GlutaMAX™-I, Invitrogen™ horseradish peroxidase (HRP)-conjugated anti-goat IgG, and Lipofectamine™ RNAiMAX were purchased from Invitrogen™/Thermo Fisher Scientific Inc. (Frederick, MD, USA). Bovine serum albumin (BSA), 3-[3-cholamidopropyl]dimethylammonio]propanesulfonate (CHAPS) and an anti-actin antibody (rabbit polyclonal, #5060) were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA). Resveratrol (natural, #185-0721), gnetin C (standard ≥ 97.0% [HPLC], #070-06141), ε-viniferin (standard ≥ 98.0% [HPLC], #226-02021), dimethyl sulfoxide (DMSO), sodium dodecyl sulfate (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). Human Aβ42 monomer was from Peptide Institute, Inc. (Ibaraki, Japan). The Cell-Quanti-MTT™ assay kit was from BioAssay Systems (Hayward, CA, USA). The NucleoSpin® RNA II total RNA isolation kit was from Macherey-Nagel GmbH & Co. KG (Düren, Germany). An iScript™ Advanced cDNA synthesis kit and SsoAdvanced™ Universal SYBR® Green Supermix solution for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) were 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 from Pierce™/Thermo Scientific Inc. (Waltham, MA, USA). Polyvinylidene fluoride (PVDF) membranes and the Lumina™ Crescendo Western HRP substrate were from Millipore Corporation (Billerica, MA, USA). An antibody against MMP-14/membrane type-1 MMP (MT1-MMP) (D1E4, rabbit monoclonal, #13130) was purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-APP antibody (C2C3, C-term, rabbit, #GTX101336) was from GeneTex Inc. (Irvine, CA, USA). Antibodies to β-actin (ACTN, C4) and mouse monoclonal, #ab3280) was from Abcam plc (Cambridge, UK). An anti-Aβ 1–16 monoclonal antibody (6E10, mouse ascites, #SIG39300) was from BioLegend, Inc. (San Diego, CA, USA), formerly known as Covance. Anti-rabbit and anti-mouse IgG-HRP were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Select Validated siRNA for target gene MMP-14 (#s8877) was from Ambion/Life Technologies/Applied Biosystems (Carlsbad, CA, USA). Nonsilencing negative control siRNA (102728) was from Qiagen (Hilden, Germany).

Cell Culture. SH-SY5Y human neuroblastoma 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 (21). Once the cells were 80% confluent, they were used for experimental purpose; immediately, the medium was replaced with fresh Advanced DMEM/F12 containing GlutaMAX™-I supplemented with 3% FBS. In experiments with biophenols
used for quantitative analyses of 18S rRNA and mRNAs of the genes listed in Table 1. The primers were used at 500 nM. The amplification was performed in an SsoAdvanced™ Universal SYBR® Green Supermix solution and 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 with CFX Manager™ Version 2.1 software.

**Western blot analysis.** Cellular proteins were detected by Western blotting, as described previously (8, 22). Briefly, cells cultured in a 35 mm-diameter dish were washed twice with ice-cold 20 mM phosphate-buffered saline (PBS, pH 7.4). The cells were then lysed in 50 μL of Laemmli reducing sample buffer with shorter pulses of ultrasonication for 30 s and then incubated on ice for 30 min. Lysates were 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 subjected to electrophoresis on a 10–20% SDS-polyacrylamide gel, and proteins were transferred onto PVDF membranes. The membranes were blocked by incubating 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 an anti-Aβ 1–16, anti-MMP-14, or anti-APP (1 : 1000) antibody overnight at 4°C, followed by a third incubation with the appropriate secondary antibodies (HRP-conjugat-

(resveratrol or its dimers), cells were pretreated with the biophenol for 1 h prior to the addition of Aβ42. Aβ42 and the biophenols were dissolved in DMSO; the maximum final concentration of DMSO in culture medium was 1.1%, and control cells were treated with vehicle alone.

**Cell viability.** Cell viability was measured by using a CellQuanti-MTT™ assay kit according to the manufacturer’s protocol with slight modifications (22). SH-SY5Y cells were cultured in 96-well culture plates at 4 × 10^4 cells per well in 100 μL of medium. The MTT reagent (15 μL) was then added per well, and the cells were incubated for an additional 4 h. Formazan crystals only produced by living cells were solubilized by adding 100 μL/well of solubilizer (10% SDS, 3.2 mM HCl) and the plates were placed overnight 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.

**ELISA.** Cell culture supernatants were mixed with 0.2% BSA and 0.075% CHAPS to minimize the loss of Aβ42 by adhesion to storage tubes. Aliquots of the samples were stored at −80°C until further use. The levels of Aβ42 in the cell-conditioned medium and whole cell lysates were determined using an ELISA kit and an iMark/Bio-Rad microplate reader (22, 40).

**RT-qPCR.** Total RNA was extracted from cells, and single-strand cDNA was synthesized using an iScript™ Advanced cDNA synthesis kit for RT-qPCR (8). A CFX96™ Real-Time PCR System (Bio-Rad) was used for quantitative analyses of 18S rRNA and mRNAs of the genes listed in Table 1. The primers were used at 500 nM. The amplification was performed in an SsoAdvanced™ Universal SYBR® Green Supermix solution and 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 with CFX Manager™ Version 2.1 software.
ed anti-rabbit, or anti-mouse IgG, 1:5000) for 1 h at room temperature. The immunoreactive bands were detected by an enhanced chemiluminescence (ECL) detection system. The same blot was then reprobed with an anti-β-actin antibody (1:5000).

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

**RNA interference (RNAi).** Cells were transfected with the target gene siRNA or non-silencing control siRNA (8), using the Lipofectamine™ RNAiMAX transfection reagent, according to the manufacturer’s protocol. After 48 h, the cells were washed, and fresh Advanced DMEM/F12 containing GlutaMAX™-I media supplemented with 3% FBS was added. The cells were then treated with gnetin C alone or with both gnetin C and Aβ42 for 24 h.

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

**RESULTS**

**Safety tests of gnetin C and ε-viniferin**

We first evaluated the no adverse effect level of two resveratrol dimers (gnetin C and ε-viniferin) on cell viability in SH-SY5Y human neuroblastoma cells, using the MTT assay (Fig. 1). After treatment for 48 h, ε-viniferin did not affect the cell viability up to 100 μM (also resveratrol, up to 50 μM), but gnetin C decreased the cell viability at 30 μM or higher. Therefore, we mainly used the 20 μM dose of gnetin C or ε-viniferin as a condition to test our hypothesis in the following experiments.

**Gnetin C and ε-viniferin lower Aβ42 production**

We next examined the effects of gnetin C and ε-viniferin on the endogenous production of Aβ monomers in cultured SH-SY5Y cells (Fig. 2). The specificity of the ELISA kit allowed us to accurately determine the level of secreted Aβ42. Untreated control cells constitutively released Aβ42 into the media for 24 h. In contrast, gnetin C treatment (10 or 20 μM) effectively lowered Aβ42 secretion by 49 or 63%, respectively. ε-viniferin (10 or 20 μM) moderately decreased Aβ42 production by 21 or 34%, respectively; and was comparable to the cells treated with 10 or 20 μM of resveratrol, which reduced Aβ42 secretion by 21 or 33%, respectively.

**Gnetin C suppresses BACE1 expression and enhances MMP-14 expression**

Based on the aforementioned results, we decided to investigate the gnetin C-mediated changes in expression of genes that potentially affect Aβ levels (Fig. 3). As for secretases, we found that gnetin C treatment significantly suppressed, at the transcriptional level, the expression of the β-secretase, BACE1, for both
Fig. 3 Effects of resveratrol (RES) dimers on mRNA expressions for secretases and amyloid-β (Aβ)-decomposing enzymes in cultured SH-SY5Y cells. Cells were treated with 20 μM of gnetin C (GNC), ε-viniferin (EVN) or RES for 6 or 24 h. Dimethyl sulfoxide (DMSO) was used as a vehicle control. mRNA expression was analyzed by real-time quantitative PCR and normalized to 18S rRNA expression. The mean ± SD of three experiments is shown. * denotes P < 0.05. (A) Four secretases, i.e., a disintegrin and metalloproteinase-10 (ADAM10), tumor necrosis factor-α-converting enzyme (TACE), β-site APP-cleaving enzyme-1 (BACE1) and presenilin-1 (PS1) were examined. (B) Four Aβ-decomposing enzymes, i.e., nephrilysin (NEP), insulin-degrading enzyme (IDE), endothelin-converting enzyme 1 (ECE1) and matrix metalloproteinase-14 (MMP-14) were examined.
Fig. 4 Effects of knockdown of gnetin C (GNC)-induced matrix metalloproteinase-14 (MMP-14) in cultured SH-SY5Y cells. Cells were transfected with siRNA against MMP-14 or control siRNA for 48 h and then treated with GNC for 24 h. Dimethyl sulfoxide (DMSO) was used as a solvent for GNC. The level of amyloid precursor protein (APP) was also checked. The mean ± SD of three experiments is shown. *denotes \( P < 0.05 \). (A) mRNA expression was analyzed by real-time quantitative PCR and normalized to 18S rRNA expression. (B and C) Protein expression was detected by Western blotting. The data shown are representative of three experiments. (D and E) The integrated optical density of the protein band in (B and C) was quantified using ImageJ 1.49i and was normalized to that of actin.

and 24 h (Fig. 3A). Gnetin C did not have much remarkable effects on mRNA expressions of ADMA10 (a constitutive α-secretase), TACE (an inducible α-secretase) and presenilin-1 (a core part in γ-secretase complex).

Next, we focused on the other enzymes related to Aβ decomposition, i.e., NEP, IDE, ECE1 and MMP-14 (Fig. 3B). It was eye-catching that MMP-14
mRNA was eminently induced by gnetin C for both 6 and 24 h, although the others were decreased for 24 h (IDE, for 6 h also).

Incidentally, another resveratrol dimer, ε-viniferin, moderately increased the expressions of TACE, IDE and ECE1 for 24 h, showing the same or analogous expression patterns to those of resveratrol.

**Knockdown of MMP-14 suppresses the gnetin C-induced protein expression**

To confirm that the gnetin C-induced upregulation of MMP-14 expression levels are dependent on gnetin C treatment, we performed gene knockdown of MMP-14 by siRNA and examined the protein level (Fig. 4). Both gnetin C-induced and constitutive expressions of MMP-14 were clearly inhibited, at both the mRNA (Fig. 4A) and protein (Fig. 4B and 4D) levels, by the addition of respective MMP-14 siRNA. In contrast, gnetin C and/or the siRNA did not affect APP protein expression (Fig. 4C and 4E). These results confirmed that gnetin C moderately but significantly enhanced MMP-14 protein expression without affecting the protein level of APP.

**Gnetin C efficiently ameliorates the cell viability lowered by Aβ42**

We next examined cell viability in Aβ42-treated SH-SY5Y cells that were pretreated with 10 or 20 μM of gnetin C, ε-viniferin or resveratrol (Fig. 5). The cells were pretreated with the biophenol for 1 h and then with 10 μM of Aβ42 for 24 h. Control cells were treated with DMSO during the experiment. Consistent with our previous studies (8, 22), Aβ42 reduced the cell viability to 36%; however, 10 and 20 μM of gnetin C efficiently (46 and 81%, respectively) ameliorated the toxicity. Gnetin C alone did not affect cell viability up to a concentration of 20 μM (Fig. 1). Therefore, we used 20 μM gnetin C for the following experiments.

Incidentally, 10 and 20 μM of resveratrol partially (33 and 46%, respectively) improved the toxicity, although ε-viniferin did not.

**Gnetin C reduces intracellular Aβ oligomers**

To clarify the effects of gnetin C pretreatment on the levels of intracellular Aβ oligomers, we analyzed the generation of Aβ oligomers in whole lysates of cells preincubated with gnetin C (20 μM) for 1 h and then treated with Aβ42 monomer (10 μM) for 24 h. As shown in Fig. 6A and 6B, the gnetin C pretreatment reduced the level of intracellular Aβ oligomers (high molecular weight oligomers, 100–150 kDa) (29, 41) by 65%. An intracellular 15-kDa Aβ oligomer (Aβ trimer) was lowered by gnetin C and resveratrol by 51 and 52%, respectively.

**DISCUSSION**

In the present study, we have examined resveratrol dimers, gnetin C and ε-viniferin, as possible preventive measures against neurotoxicity induced by Aβ accumulation. This is the first study to reveal that gnetin C suppressed the level of Aβ42 production and improved the cell viability of human neuroblastoma cells treated with Aβ42.

Our results demonstrate that gnetin C has an efficiently suppressive effect on Aβ42 production compared to resveratrol and ε-viniferin. Uniquely, gnetin C consistently down-regulates β-secretase (BACE1)
Fig. 6 Effects of gnetin C (GNC) and resveratrol (RES) on the presence of amyloid-β (Aβ) monomers and oligomers in cultured SH-SY5Y cells. After pretreatment with 20 μM of GNC or RES for 1 h, the cells were incubated with 10 μM of Aβ1-42 (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 Aβ trimers (ca. 15 kDa) or high molecular weight oligomers (100–150 kDa) in (A) was quantified using ImageJ 1.49i and normalized to that of actin. (C) Under the same condition as shown in (A), extracellular and intracellular Aβ42 monomers in cell-conditioned medium and whole cell lysates, respectively, were analyzed by enzyme-linked immunosorbent assay (ELISA), and normalized to total cell protein determined by the BCA assay.

expression without inducing the other secretases (α- and γ-secretases) or their substrate, APP. Since BACE1 together with presenilin-1 (the main part of γ-secretase) produces Aβ monomers from APP (24, 40), the decline of BACE1 leads to reduce Aβ42 production. Furthermore, gnetin C promotes the synthesis of an Aβ-decomposing enzyme, MMP-14 (18), although the other Aβ-decomposing enzymes such as NEP (12, 38), IDE and ECE1 (38) are not increased by gnetin C. Thus, the suppressed BACE1 and the induced MMP-14 expression may be responsible for the decrease of Aβ production and release from the cells.

On the other hand, resveratrol and ε-viniferin unlike gnetin C enhanced α-, β-, or γ-secretase, or Aβ-decomposing enzymes such as NEP, IDE, or ECE1. In particular, resveratrol and ε-viniferin enhanced the expression of α-secretases, ADAM10 or TACE (16). These mechanisms may comprehensively contribute to the reduced Aβ42 production in the
Gnetin C as an Aβ suppressor

Since gnetin C elevates the levels of both intracellular and extracellular Aβ monomers in cells treated with Aβ42, gnetin C may play roles in inhibition against internalization and oligomerization of exogenous Aβ monomers. Although the detailed mechanisms are not known, these functions can reduce intracellular Aβ accumulation. Indeed, the Aβ oligomers generated in the cells treated with Aβ42 were effectively reduced by gnetin C. Noteworthily, the main size of the Aβ oligomers that were reduced by gnetin C in this study was between 100–150 kDa, similar to that of Aβ assemblies, termed amylospheroids (ASPD, 128 kDa) (25). The amylospheroids have been shown to cause presynaptic calcium overload by interacting with sodium pumps (specifically, the neuron-specific Na+/K+-ATPase α3 subunit), leading to neurodegeneration (25). The reduction of such toxic Aβ oligomers may in turn, contribute to the increased cell viability in Aβ-treated cells.

Moreover, in addition to its antioxidant activity, gnetin C itself has radical scavenging activity similar to that of ascorbic acid and dl-α-tocopherol (13). This supports the hypothesis that gnetin C is a helpful agent for attenuating the Aβ-induced oxidative stress, since Aβ generates reactive oxygen species (ROS) in the AD brain (4, 5, 10). More works will be required to clarify the mechanisms of the protective and/or restorative activities of gnetin C on Aβ-induced damage and apoptosis in neuronal cells. Finally, the clinical and prophylactic efficacy of gnetin C in AD pathologies remains to be evaluated, although melinjo, a plant which is rich in resveratrol dimers including gnetin C (19), is commonly cultivated and traditionally used as a vegetable in Southeast Asia, such as Indonesia. Interestingly, melinjo extracts are reported to exhibit biological activities, such as anticancer (6), vasculoprotective (17) and antioxidant (32) properties, which may lead to longevity (11). Gnetin C as well as other melinjo-derived biophenols is free from harmful side effects (35, 36), leading to a high level of expectation for its potential use in the prevention and/or treatment of AD.

Collectively, gnetin C reduces Aβ production and
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