Regular Article

Enhancement of Neprilysin Activity by Natural Polyphenolic Compounds and Their Derivatives in Cultured Neuroglioma Cells

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The onset of Alzheimer's disease (AD) is characterized by accumulation of amyloid *β* peptide (A*β*) in the brain. Neprilysin (NEP) is one of the major A*β*-degrading enzymes. Given findings that NEP expression in the brain declines from the early stage of AD before apparent neuronal losses are observed, enhancement of NEP activity and expression may be a preventive and therapeutic strategy relevant to disease onset. We screened for compounds that could enhance the activity and expression of NEP using a polyphenol library previously constructed by our research group and investigated the structure–activity relationships of the identified polyphenols. We found that amentoflavone, apigenin, kaempferol, and chrysin enhanced the activity and expression of NEP, suggesting that chemical structures involving a double bond between positions 2 and 3 in the C ring of flavones are important for NEP enhancement, while catechol or pyrogallol structures, except for the galloyl group of catechins, abolished these effects. Moreover, natural compounds, such as quercetin, were not effective *per se*, but were changed to effective compounds by adding a lipophilic moiety. Using our study findings, we propose improvements for dietary habits with experimental evidence, and provide a basis for the development of novel small molecules as disease-modifying drugs for AD.

Key words neprilysin, flavone, polyphenol, aliphatic derivative, Alzheimer's disease

INTRODUCTION

For almost three decades, there has been an intensely growing but still unmet need to develop clinically effective therapeutic agents for the treatment of Alzheimer's disease (AD) and dementia.[1)] The amyloid cascade hypothesis has been driving the vast majority of therapeutic drug developments in AD, since it was initially proposed in 1992.[2,3)]

The pathological cascade in AD starts with accumulation of amyloid *β* peptide (A*β*) in the brain,[4)] and thus removal of A*β* from the brain is considered a fundamental disease-modifying treatment. Given that A*β* accumulation in the brain occurs slowly for two decades before apparent presentation of clinical symptoms after 60 years of age, people may be able to prevent onset of disease by improving certain lifestyle factors, such as dietary habits and exercise habits, from 30–40 years of age, as long as they are not carriers of mutations in the causal genes for AD (*APP*, *PSEN1*, and *PSEN2*).

Neprilysin (NEP) serves as the major A*β*-degrading enzyme relevant to the onset of AD,[5,6)] and contributes to effective clearance of A*β* secreted from the pre-synapses of neurons in a wide range of limbic regions, including the cerebral cortex and hippocampal formation. The expression levels of NEP and its endogenous positive regulator somatostatin in brain regions vulnerable to amyloid pathology were shown to decline with aging in both normal elderly people and sporadic AD patients.[7–10)] However, the rate of decline was much more rapid in AD patients. Therefore, targeting NEP could be a curative therapy for AD.

Nearly 60% of medicines on the market have been developed by imitating the structures of naturally-occurring compounds.[11)] Frequently, natural products contained in foods are utilized for medications as they stand or as derivatives. This fact led us to consider whether there were natural products that could upregulate NEP in the brain. In previous studies, certain polyphenols, such as (−)-epigallocatechin-3-gallate (EGCg) and resveratrol, were shown to enhance NEP expression in the brain and improve cognitive function in senescence-accelerated mice.[12–14)] Furthermore, resveratrol decreased brain A*β* deposition and improved cognitive function through enhanced expression of NEP and reduced expression of *β*-site amyloid precursor protein-cleaving enzyme 1 in AD model mice.[13,14)]

In the present study, we screened for NEP modulators in neuroglioma H4 cells using a polyphenol library and identified some compounds that could enhance the activity and expression of NEP. Subsequently, as a trial to increase the potential of the identified polyphenols for upregulation of NEP, we synthesized some derivatives by adding a hydrophobic moiety to the polyphenols and tested their activity.

MATERIALS AND METHODS

Chemistry

General

The ^{1}H- and ^{13}C-NMR spectra were measured using a Var-

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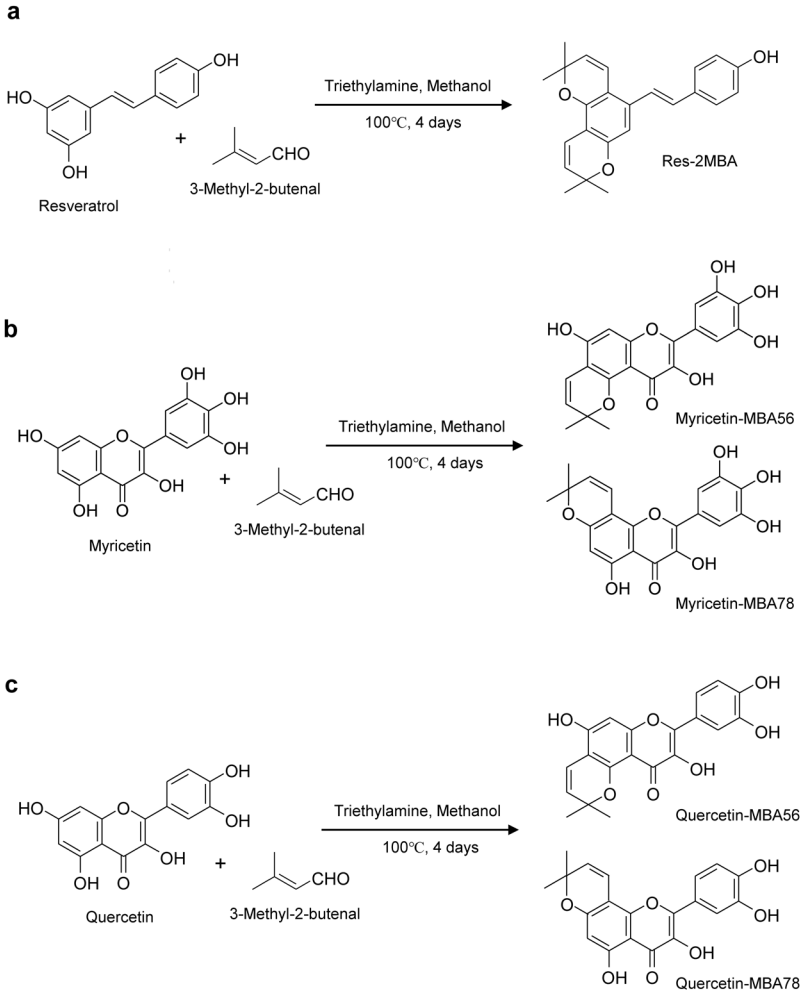


Fig. 1. Scheme for the Synthesis of Aliphatic Polyphenol Derivatives

(a–c) The aliphatic polyphenol derivatives Res-2MBA (a), myricetin-MBA56 and myricetin-MBA78 (b), and quercetin-MBA56 and quercetin-MBA78 (c) were synthesized by coupling with 3-methyl-2-butenal. The details were described in Materials and Methods.

ian Unity plus 500 spectrometer (^{1}H: 500 MHz; ^{13}C: 125 MHz) or JEOL JNM-AL 400 spectrometer (^{1}H: 400 MHz; ^{13}C: 100 MHz). The mass spectra were obtained with a JEOL JMS-700N mass spectrometer. Silica gel 60N (100–210 μm) (Kanto Chemical Co., Inc., Tokyo, Japan) was used for column chromatography. TLC was conducted with silica gel 60 F254 plates (Merck KGaA, Darmstadt, Germany). Reagents and solvents were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and Nacalai Tesque Inc. (Kyoto, Japan).

General Synthetic Procedure for Aliphatic Polyphenol Derivatives

A methanolic solution (10 mL) containing the desired polyphenol (1.3 mmol), 3-methyl-2-butenal (3.3 mmol), and triethylamine (200 mg) in a screw vial was heated at 100 °C for 2–4 d.[15] The reaction mixture was concentrated, and the products were separated by silica gel column chromatography (hexane-ethyl acetate) and preparative TLC (hexane–ethyl acetate, 7 : 3, v/v) to give the desired products.

Experimental Characterization Data

5-(4-Hydroxystyryl)-2,2,8,8-tetramethyl-2*H*, 8*H*-pyrano[2,3-h]chromene (Res-2MBA)

Resveratrol (300 mg), 3-methyl-2-butenal (280 mg), and triethylamine (200 mg) were dissolved in methanol (10 mL) and heated at 100 °C for 4 d (Fig. 1a).

^{1}H-NMR (400 MHz, CD_3OD) δ: 1.39 (3H, s), 1.41 (3H, s), 5.61 (1H, d, J = 7 Hz), 5.63 (1H, d, J = 7 Hz), 6.57 (1H, s), 6.61 (1H, d, J = 10 Hz), 6.72 (1H, d, J = 10 Hz), 6.77 (2H, br d, J = 7 Hz), 6.91 (1H, d, J = 16 Hz), 7.15 (1H, d, J = 16 Hz), 7.38 (2H, br d, J = 7 Hz). ^{13}C-NMR (100 MHz, CD_3OD) δ: 27.8 (2C), 28.1 (2C), 76.7, 77.1, 106.0, 110.8, 114.1, 116.6 (2C), 117.8, 120.3, 122.5, 129.16 (2C), 129.20, 130.2, 130.6, 132.2, 135.9, 150.3, 154.7, 158.8. FABMS m/z: 362 ($[M + H]^+$).

Myricetin-MBA56 and Myricetin-MBA78

Myricetin (1.0 g, 3.1 mmol), 3-methyl-2-butenal (10 g), and trimethylamine (4.4 g) were dissolved in methanol (100 mL) and heated at 100 °C for 2 d. The reaction mixture was concentrated and separated by silica gel column chromatography (hexane-ethyl acetate, 2 : 1, v/v) to give a mixture (97.1 mg) of myricetin-MBA56 and myricetin-MBA78. Further separation by repeated silica gel column chromatography was conducted to give myricetin-MBA56 (13.3 mg) and myricetin-MBA78 (13.1 mg) (Fig. 1b).

5,9-Dihydroxy-8-(3,4,5-trihydroxyphenyl)-2,2-dimethylpyrano-[2,3-f]chromen-10(2*H*)-one (Myricetin-MBA56)

^{1}H-NMR (500 MHz, CD_3OD) δ: 1.45 (6H, s), 5.69 (1H, d, J = 7 Hz), 6.37 (1H, s), 6.67 (1H, d, J = 7 Hz), 7.34 (2H, s). ^{13}C-NMR (125 MHz, CD_3OD) δ: 28.5, 79.0, 95.5, 105.2, 105.7,

108.5, 116.1, 122.9, 129.5, 137.0, 137.5, 146.7, 148.3, 156.4, 157.3, 160.6, 177.4. FABMS *m/z*: 385 ($[M+H]^+$).

2-(3,4,5-Trihydroxyphenyl)-3,5-dihydroxy-8,8-dimethyl-4*H*,8*H*-pyrano[2,3-f]chromen-4-one (Myricetin-MBA78)

^{1}H-NMR (400 MHz, CD_3OD) δ: 1.45 (6H, s), 5.72 (1H, d, $J=7$ Hz), 6.15 (1H, s), 6.88 (1H, d, $J=7$ Hz), 7.40 (2H, s).

Quercetin-MBA56 and Quercetin-MBA78

Quercetin-MBA56 and quercetin-MBA78 were prepared in a similar manner to the method described for myricetin (Fig. 1c).

5,9-Dihydroxy-8-(4,5-dihydroxyphenyl)-2,2-dimethylpyrano-[2,3-f]chromen-10(2*H*)-one (Quercetin-MBA56)

^{1}H-NMR (400 MHz, CD_3OD) δ: 1.45 (6H, s), 5.70 (1H, d, $J=7$ Hz), 6.40 (1H, s), 6.67 (1H, d, $J=7$ Hz), 6.87 (1H, d, $J=7$ Hz), 7.63 (1H, brd, $J=7$ Hz), 7.72 (1, brs). FABMS *m/z* 369 ($[M+H]^+$).

2-(4,5-Dihydroxyphenyl)-3,5-dihydroxy-8,8-dimethyl-4*H*,8*H*-pyrano[2,3-f]chromen-4-one (Quercetin-MBA78)

^{1}H-NMR (400 MHz, CD_3OD) δ: 1.45 (6H, s), 5.70 (1H, d, $J=7$ Hz), 6.15 (1H, s), 6.84 (1H, d, $J=7$ Hz), 6.89 (1H, d, $J=7$ Hz), 7.67 (1H, brd, $J=7$ Hz), 7.76 (1, brs). ^{13}C-NMR (126 MHz, CD_3OD) δ: 28.4, 79.2, 99.9, 102.4, 105.2, 11.7, 115.8, 116.3, 121.7, 124.1, 128.5, 137.5, 146.3, 147.8, 148.9, 152.1, 160.5, 161.9, 177.5. FABMS *m/z* 369 ($[M+H]^+$).

Biological Evaluation

Reagents

The natural compound library used in the present study was composed of polyphenols prepared in previous studies or purchased from FUJIFILM Wako Pure Chemical Corporation, Tokyo Chemical Industry Co., Ltd., Nacalai Tesque Inc., and Merck KGaA. The details are listed in Supplementary Table 1. The polyphenols were dissolved in dimethyl sulfoxide (DMSO) (Nacalai Tesque Inc.) and stored at −20 °C as 10 mM stock solutions until use. Other chemical reagents were commercially available grade.

Cell Culture and Drug Treatment

Human neuroglioma cells (H4 cells) overexpressing human wild-type amyloid-β precursor protein (APP) (H4-BH1)[16] and human APP with the Swedish mutation (H4-NL cells)[17] were cultured in Dulbecco's modified Eagle's medium (Nacalai Tesque Inc.) supplemented with 10% fetal bovine serum (Merck KGaA) and 100 U/mL penicillin/100 μg/mL streptomycin solution (Nacalai Tesque Inc.) at 37 °C under 5% CO_2. H4-NL cells were plated on 6-well culture plates at a density of approximately 1.2×10^5 cells per well. After 24 h, the culture medium was replaced with Opti-MEM™ I reduced-serum medium (Thermo Fisher Scientific K.K., Tokyo, Japan) containing 10 μM polyphenol (final DMSO concentration: 0.1%).

Measurement of NEP Activity

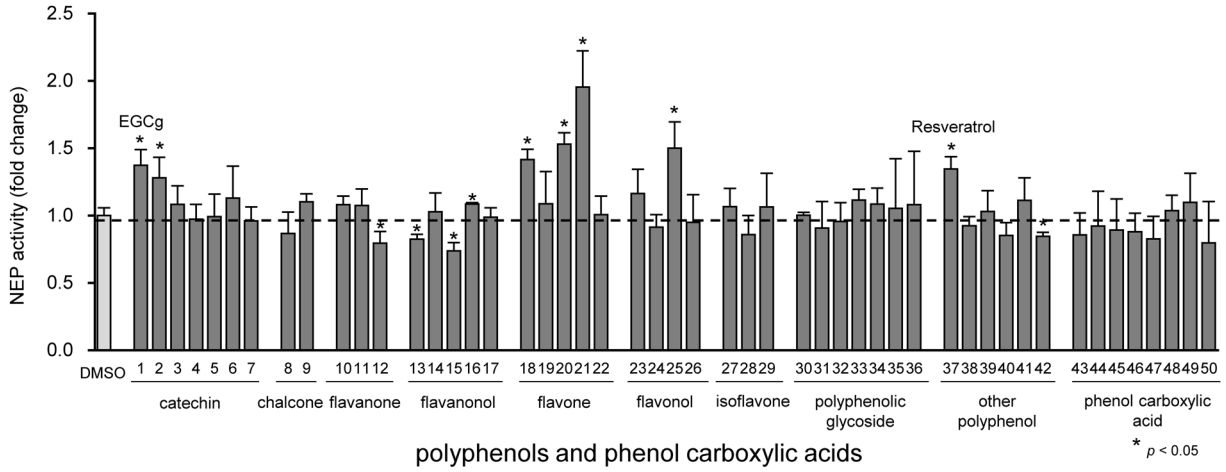
Cells treated with polyphenols for 48 h were harvested and lysed in 40 μL of lysis buffer (50 mM Tris–HCl pH 7.4, 0.15 M NaCl, 1% Triton X-100 (Nacalai Tesque Inc.), protease inhibitor cocktail Complete™ ethylenediaminetetraacetic acid (EDTA)-free (Roche Diagnostics GmbH, Mannheim, Germany), 10 μM ZLLLal (Peptide Institute, Osaka, Japan), and 0.7 μg/mL pepstatin A (Peptide Institute)) with a pellet mixer and then stood on ice for 1 h. The obtained cell lysates were centrifuged at $21000\times\boldsymbol{g}$ for 30 min at 4 °C, and the supernatants were collected for measurement of NEP activity and Western blot analysis. Protein concentrations were determined with a BCA Protein Assay Kit (TaKaRa Bio Inc., Shiga, Japan). NEP activity was determined as described previously.[18] Briefly, cell lysates were incubated in 100 mM MES buffer (pH 6.5) containing 0.1 mM succinyl-L-alanyl-L-alanyl-L-phenylalanine-4-methyl-coumaryl-7-amide (succinyl-Ala-Ala-Phe-MCA) (Bachem, Bubendorf, Switzerland), as a substrate for neutral endopeptidases, at 37 °C. After 1 h of incubation, 5 μg (0.02 units equivalent)/mL leucine aminopeptidase (Merck KGaA) and 10 μM phosphoramidon (Peptide Institute Inc.) were added to cleave the phenylalanine residue from Phe-MCA. The fluorescence intensity of free 7-amino-4-methylcoumarin was measured with excitation at 390 nm and emission at 460 nm using an Infinite M1000 microplate reader (Tecan Group Ltd., Männedorf, Switzerland). The NEP-dependent neutral endopeptidase activity was determined, based on the decrease in rate of digestion caused by 10 μM thiorphan (Merck KGaA), a specific inhibitor of NEP.

Western Blot Analysis

The cell lysates were dissolved in sodium dodecyl sulfate (SDS) sample buffer at a concentration of 0.3 μg/μL, and aliquots containing 3 μg of protein were separated by 7% gradient SDS-polyacrylamide gel electrophoresis and electrotransferred onto 0.45-μm polyvinylidene difluoride membranes (Immobilon-P; Merck KGaA). The membranes were probed with a goat polyclonal antibody against human NEP (1 : 500 dilution; R&D Systems, Minneapolis, MN, U.S.A.; Cat#AF1182; RRID:AB_354652), followed by HRP-conjugated anti-goat immunoglobulin G (IgG) (Cytiva, Tokyo, Japan). Immunoreactive bands on the membranes were visualized with an enhanced chemiluminescence kit (Immunostar LD; FUJIFILM Wako Pure Chemical Corporation), and the band intensities were determined with a densitometer (LAS4000; Fuji Photo Film, Tokyo, Japan) using Science Lab 97 Image Gauge software (ver. 3.0.1; FUJIFILM Wako Pure Chemical Corporation). The relative immunoreactive protein content in each sample was calculated by reference to a standard curve constructed with certain samples in the control group to reduce alterations in the immunoreactivity and intensity of ECL signals in different experiments. The membranes were reprobed with an anti-β-actin antibody (1 : 10000 dilution; Sigma-Aldrich, St. Louis, MO, U.S.A.; Cat#A1978; RRID:AB_476692) to confirm that equal amounts of total protein had been loaded.

RNA Isolation and Quantitative PCR (qPCR)

Total RNA was extracted from H4-NL cells treated with polyphenols or DMSO (vehicle) for 48 h using a Nucleospin RNA Kit (TaKaRa Bio Inc.). After first-strand cDNA was synthesized using a PrimeScript™ RT Master Mix Kit (Perfect Real Time; TaKaRa Bio Inc.), qPCR was conducted using a Probe qPCR Mix Kit (TaKaRa Bio Inc.) and a LightCycler 96 system (Roche Diagnostics GmbH) with the following two-step amplification profile: 40 cycles of 5 s at 95 °C and 40 s at 60 °C. Relative quantification of *MME* (gene symbol for NEP) RNA expression was carried out by normalization to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and expressed as (MME mRNA/GAPDH mRNA). The nucleotide sequences of the probes and forward/reverse primers were Hs_MME-Probe (CGGCATGGTCATAGGACACGAAATCACC) and Hs_MME-F (GCAGTCCAACTCATTGAACTATGG)/Hs_MME-R (TCTTTGTTAAAGTTTCTGCCATTGT) for human MME, and Hs_GAPDH-Probe (CCCACTCCTCCACCTTTGACGCTGG)



1_EGCg	11_Eriodictyol	21_Amentoflavone	31_Apiin	41_Cyanidin chloride
2_ECg	12_Pinocembrin	22_Luteolin	32_Rutin	42_Sulfuretin
3_EGC	13_Fustin	23_Fisetin	33_2''-Rhamnosyl vitexin	43_Caffeic acid
4_EC	14_S,S-Taxifolin	24_Quercetin	34_Kaempferol-3-rhamnoside	44_Protocatechuic acid
5_EC-M1	15_R,R-Taxifolin	25_Kaempferol	35_Kaempferol 3-O-α-(2-O-β-glucosyl)-rhamnoside	45_Kojic acid
6_EGC-M1	16_Dihydrokaempferol	26_Myricetin	36_Kaempferol 3-O-α-(2-O-6'''-p-coumaroyl-β-glucosyl)-rhamnoside	46_trans-p-Coumaric acid
7_EGC-M3	17_(+)-Dihydromyricetin	27_Genistein	37_Resveratrol	47_Ethyl 4-hydroxy-3-methoxycinnamate
8_2',4',6'-Trihydroxydihydrochalcone	18_Chrysin	28_Mallonyl genistin	38_Curcumin	48_Ferulic acid
9_Phloretin	19_Baicalein	29_Liquiritin	39_Chlorogenic acid	49_Syringic acid
10_Naringenin	20_Apigenin	30_Quercitrin hydrate	40_3-Hydroxyphloretin	50_Sinapinic acid

Fig. 2. Screening for Natural Compounds That Enhance NEP Activity in H4-NL Cells

NEP activity was measured in Triton X-100-soluble lysates of H4-NL cells that had been treated with 10 μM polyphenols for 48 h. The experiments were individually performed with a pair of controls and one or two compounds, and the data were normalized to the value of each control and shown together for the figure. Some compounds with flavone and flavonol structures strongly enhanced NEP activity. Data are shown as means ± S.D. ($n = 3$–4). Statistical analyses were performed using Student's *t*-test. *$p < 0.05$, significant difference *versus* the control group (DMSO treatment).

and Hs_GAPDH-F (CTCCTCTGACTTCAACAGCGA)/Hs_GAPDH-R (CCAAATTCGTTGTCATACCAGGA) for human GAPDH.

Statistical Analysis Data were expressed as mean ± standard deviation (S.D.). All statistical analyses were conducted using SigmaPlot software ver. 14.0 (Systat Software Inc., San Jose, CA, U.S.A.). Comparisons of mean values among more than three groups were conducted by one-way ANOVA followed by a *post-hoc* Student–Newman–Keuls test if the data passed the Shapiro–Wilk normality test and Brown–Forsythe equal variance test. If the normality test or equal variance test was not passed for ANOVA, a Kruskal–Wallis one-way ANOVA on the ranks was performed. Values of $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Design Several studies worldwide have reported that polyphenols and their metabolites are capable of preventing AD.[19,20] As polyphenols need to bind to receptors or binding proteins on the cell surface or pass through the membrane to enter the cytoplasm and upregulate NEP, a hydrophilic nature of polyphenols would hinder these behaviors. Previous studies tried to confer a hydrophobic nature on polyphenols through *O*-esterification of one or more of their hydroxyl groups using carboxylic acids. In the present study, we investigated another strategy that would avoid ester hydrolysis in the cells or *in vivo*. Our strategy involved the synthesis of aliphatic polyphenols through heterocyclic ring formation by covalent binding with an aliphatic chain using the reactions of different polyphenols with 3-methyl-2-butenal. The synthesis design was essentially determined by the availability of the polyphenols and aliphatic aldehydes and the empirical simplicity of the synthesis and purification.

Synthesis The synthesis of polyphenol derivatives followed the procedure reported by Zamora *et al.*[15] with some modifications. The synthesis involved cyclization of the selected polyphenols, namely resveratrol, myricetin, and quercetin, with 3-methyl-2-butenal. The reactivity of some positions was higher than that of other positions, and thus only limited number of adducts were produced. Resveratrol gave Res-2MBA, while myricetin gave two derivatives: myricetin-MBA56 and myricetin-MBA78. Quercetin also gave two derivatives: quercetin-MBA56 and quercetin-MBA78. The structures and purities of the prepared compounds were confirmed by ^{1}H- and ^{13}C-NMR analyses as well as by mass spectrometry.

Biological Evaluation

Screening for Natural Polyphenols Capable of Enhancing NEP Activity in APP-H4 Cells

To identify compounds capable of enhancing NEP activity, we used a polyphenol library consisting of compounds prepared by our research group and commercially available compounds. In the first screening, we treated H4-NL cells with 42 polyphenols and 8 phenol carboxylic acids (Supplementary Table 1) at a concentration of 10 μM for 48 h, and assayed NEP activity in the cell lysates (Fig. 2). Consistent with previous studies, EGCg and resveratrol each enhanced NEP activity up to approximately 1.4-fold. Amentoflavone enhanced NEP activity most potently among the compounds tested, followed by chrysin, apigenin, and kaempferol, which showed comparable effects to EGCg and resveratrol. Thus, some flavones, flavonols, and flavanols appeared to have positive effects on NEP

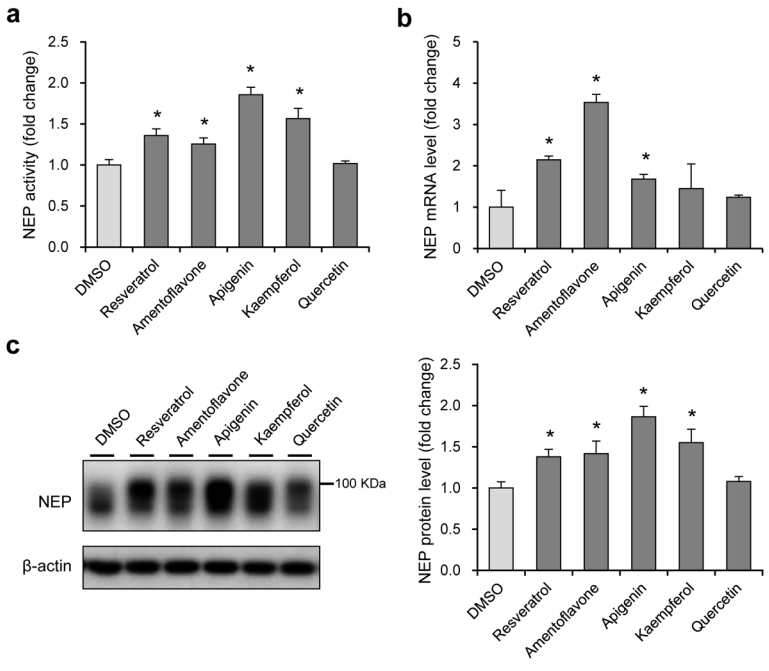


Fig. 3. Polyphenols Enhance Expression of NEP at the mRNA and Protein Levels in H4-BH1 Cells

(a–c) H4-BH1 cells treated with 10 μM polyphenols for 48 h were measured for their NEP activity (a), NEP mRNA (b), and NEP protein (c) levels using fluorescent substrate, qPCR, and quantitative Western blotting assays, respectively. In (b) and (c), the NEP mRNA and protein levels were normalized by the GAPDH or β-actin levels as internal standard molecules, respectively. Data are presented as means ± S.D. ($n = 4$). Statistical analyses were performed by one-way ANOVA, followed by a *post-hoc* test. *$p < 0.05$, significant difference *versus* the control group (DMSO treatment).

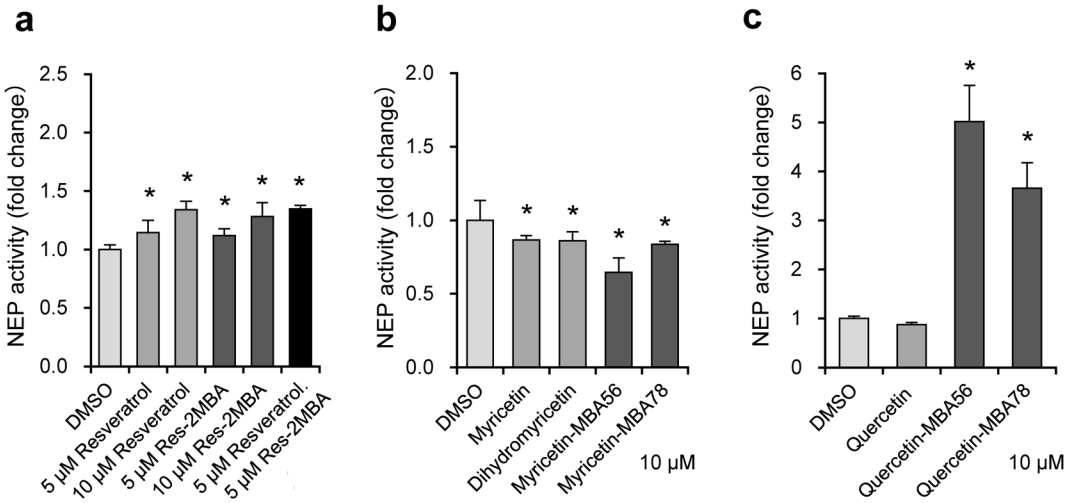


Fig. 4. Effects of Polyphenol Derivatives on NEP Activity in H4-BH1 Cells

(a–c) The derivatives of resveratrol (a), myricetin (b), and quercetin (c) with 3-methyl-2-butenal added as an aliphatic moiety to their original structures were synthesized and examined for their potential to enhance NEP activity in neuronal cells. Data are presented as means ± S.D. ($n = 4$). Statistical analyses were performed by one-way ANOVA, followed by a *post-hoc* test. *$p < 0.05$, significant difference *versus* the control group (DMSO treatment).

upregulation. Meanwhile, chalcones, flavanones, flavanonols, isoflavones, polyphenolic glycosides, phenol carboxylic acids, and other polyphenols were almost inactive for enhancement of NEP activity.

Analysis of NEP Expression Enhanced by Selected Polyphenols

To confirm the results in the first screening, we selected four flavones (amentoflavone, chrysin, apigenin, kaempferol), together with resveratrol as a positive control and quercetin as a negative control, and analyzed their effects on NEP activity and NEP expression at the mRNA and protein levels using another cell line, H4-BH1, overexpressing human wild-type APP (Fig. 3). Following treatment with the selected compounds (10 μM), NEP activity showed similar increases to those observed in the first screening, and the protein expression level of NEP increased significantly ($p < 0.05$) in parallel with the increased NEP activity. However, the changes in NEP protein or activity were not in part correlated with the change in the increased NEP mRNA levels, suggesting that these polyphenols may affect the translational process from mRNA or lysosome-mediated protein turnover/degradation of NEP. These findings suggested that the effective compounds enhanced NEP activity through protein expression, namely transcriptional and translational activation or protein stabilization

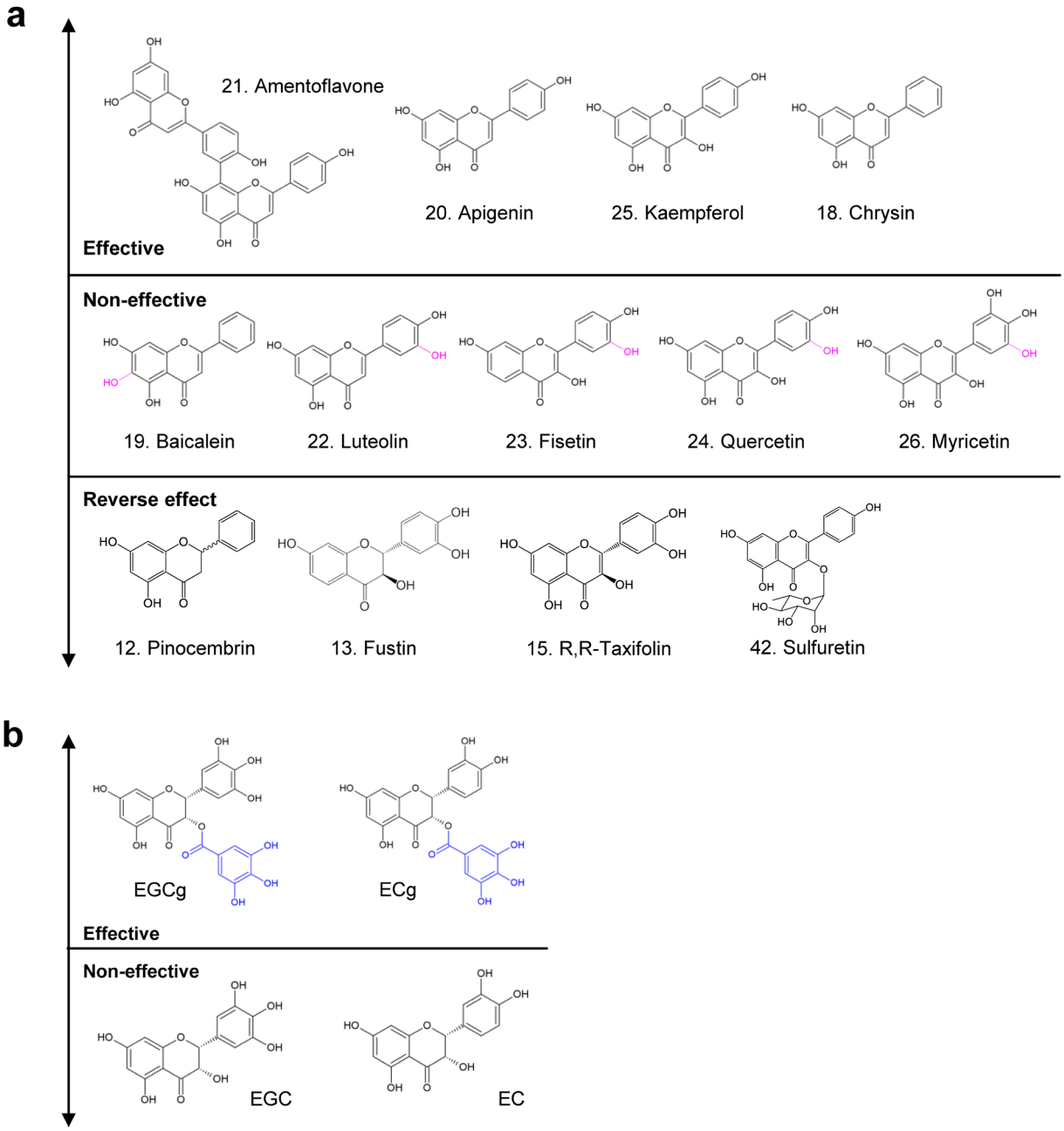


Fig. 5. Summary of Effective and Ineffective Compounds

(a) Structures of flavones and flavonols. The hydroxy group indicated in magenta disturbed the enhancement of NEP activity. (b) Structures of catechins. The galloyl group indicated in blue is essential for enhancement of NEP activity in catechins.

of NEP, rather than by activating NEP through direct binding leading to an allosteric change. As the negative control, quercetin had no effect on the activity and did not increase the NEP mRNA and protein expression levels.

Effects of Aliphatic Moiety-Adducted Polyphenol Derivatives on NEP Activity

We tried to decrease the hydrophilicity of the polyphenols and investigated the effects of the prepared derivatives on NEP upregulation. We selected three polyphenols, resveratrol, myricetin and quercetin, of which the first is effective and the other two are not. We added 3-methyl-2-butenal to the A-ring (dihydroxy benzene), a structure with almost no effect on NEP upregulation in this study, according to a previously reported method. The effect of the Res-2MBA derivative on NEP upregulation was similar to that of the original compound. However, the effects of myricetin-MBA56 and myricetin-MBA78 on NEP upregulation were slightly decreased. Meanwhile, quercetin-MBA56 and quercetin-MBA78 strongly increased NEP activity in H4-BH1 cells, although the original compound was ineffective (Fig. 4).

Structure–Activity Relationships

We successfully determined the basic polyphenolic structures in the 50 compounds that had the potential to upregulate NEP activity in cultured neuronal cells. The most potent structures were flavones (chrysin, apigenin, amentoflavone), followed by a flavonol (kaempferol) and flavanols (catechins: EGCg and epicatechin gallate (ECg)). The structures of these derivatives are shown in Supplementary Table 1.

As described above, several flavones and flavonols were able to enhance NEP activity (Figs. 2, 3). Focusing on the

basic structures of these flavones and flavonols, a hydroxy group at position 5′ in the B ring showed a tendency to hinder the effects, while either the presence or absence of a hydroxy group at position 3 in the C ring had no effect (Fig. 5a). Amentoflavone, a dimer-like structure of apigenin, showed the most potent effect among the compounds tested and its effect was about 2-fold stronger than that of apigenin.

Although flavanols (catechins) have no double bond and no carbonyl group in the C ring, a galloyl group appeared essential for their ability to enhance NEP activity, because epigallocatechin, epicatechin, and their metabolites without a galloyl group were almost ineffective (Fig. 5b).

Other flavonoids, such as chalcones without the C ring, flavanones, flavanonols, and anthocyanidins without a double bond between positions 2 and 3 in the C ring, isoflavones with a carbonyl group in the ring B at position 3 and pentacyclic flavonoids with the C ring did not increase NEP expression, suggesting that a structure with a double bond between positions 2 and 3 in the C ring is necessary for NEP enhancement and that the catechol structure and pyrogallol structure appear to weaken the effect of NEP enhancement.

A double bond between positions 2 and 3 and a carbonyl group at position 4 in the C ring of polyphenols are known to be required for various activities.[21,22] One of these is antioxidant activity, in which the catechol structure plays an important role. Rice-Evans *et al.*[23] investigated the structure-antioxidant activity-relationship of polyphenols using the trolox equivalence antioxidant capacity method, and revealed that a double bond between positions 2 and 3, a carbonyl group at position 4, and a hydroxy group at position 3 in the C ring were required to enhance antioxidant activity. The catechol structure and the pyrogallol structure have high responsiveness and are easily oxidized, because the two hydroxy groups have coupling potency and can be transformed into *o*-benzoquinone by oxidation.[24] Therefore, polyphenols with the catechol or pyrogallol structure can easily bind to amino acid residues in proteins. Furthermore, they showed that the catechol structure in the B ring is important for the antioxidant activity. Flavonols with the catechol structure in the B ring, such as quercetin, had about 3.5-fold higher antioxidant activity than kaempferol. However, our results showed that flavones and flavonols with the catechol or pyrogallol structure had no effect on NEP expression (Fig. 5a). As one possible reason, the compounds may be rapidly oxidized and become bound to other substances. Meanwhile, EGCg and ECg enhanced NEP activity despite having the catechol or pyrogallol structure (Fig. 5b), indicating that the galloyl group has a critical role for enhancement of NEP activity because it confers a more hydrophobic nature and easily binds to the cell membrane for internalization. This property and difference may be important for enhancement of NEP activity in catechins.

A Possible Mechanism for NEP Upregulation by Polyphenols

Several studies have demonstrated that EGCg and resveratrol possess various activities, including antioxidation,[25,26] anti-inflammation,[27,28] and neuroprotection,[29–33] that are beneficial for human health. In particular, their therapeutic effects on neurodegenerative diseases appear to be attributed to transcriptional or translational regulation of certain genes *via* activation of the extracellular cAMP-responsive element-binding protein (CREB) signaling pathway[34] and suppression of histone deacetylase 1 (HDAC1).[35] Previous studies revealed that NEP was downregulated by HDAC1-dependent epigenetic inhibition[36] and that its level decreased in parallel with the decreased level of phosphorylated CREB (active form) in the brain of AD model mice,[37] suggesting that EGCg may enhance NEP activity *via* the CREB signaling pathway.

Resveratrol was able to ameliorate AD-like pathologies and improve cognitive dysfunction in AD model mice.[38] In that report, resveratrol was described to act as a sirtuin 1 (Sirt1) activator. In another study, loss of Sirt1 in the brain increased miR-134 expression and miR-134 suppressed translation of CREB mRNA,[39] resulting in deactivation of the CREB signaling pathway. Resveratrol acts to decrease the miR-134 level and facilitate the CREB signaling pathway *via* activation of Sirt1.[40] Thus, it is possible that EGCg and resveratrol may upregulate NEP *via* the CREB signaling pathway.

Some flavones identified as NEP modulators in the present study were reported to act *via* a similar mechanism to resveratrol and EGCg. Apigenin had the potential to decrease Aβ plaques and improve impaired learning and memory function in AD model mice.[41] Interestingly, apigenin and kaempferol can activate the CREB signaling pathway,[41,42] and thus these flavones may upregulate NEP through activation of this pathway. Somatostatin, an endogenous positive regulator of NEP that is regulated by CREB,[43] enhanced NEP gene expression and promoted translocation of NEP to the cell membrane.[44] These observations suggest that flavones may enhance NEP expression and activity *via* the actions of somatostatin increased by CREB.

Aliphatic Derivatization of Natural Polyphenols

Based on the results of our trials, two compounds were ineffective, but the third changed from an ineffective compound to an effective compound. The latter findings indicate that our synthesis strategy can confer additional biological activity on an ineffective polyphenol as it stands, in some cases. In this respect, the actions of both intracellular signaling and uptake into cells may be important and necessary for activation of the CREB signaling pathway.

CONCLUSION

Because Aβ accumulation in the human brain occurs slowly and gradually for two decades before apparent presentation of clinical symptoms after the age of 60 years, people may be able to postpone the onset of AD by improving lifestyle factors, such as dietary habits, from the age of 30–40 years. For example, polyphenols can be consumed in many daily foods, including red wine, coffee, green tea, and chocolate. Notably, many studies on the relationship between green tea and cognitive function have been published. Higher consumption of green tea suppressed cognitive impairment and decreased the risk of AD onset. Although green tea contains many kinds of polyphenols, intake of only catechins isolated from green tea enhanced working memory.[45] The present results can provide an effective improvement method for daily dietary habits. Furthermore, we demonstrated that an ineffective compound could be changed to an effective compound by addition of a hydrophobic region to a certain polyphenol using a simple synthetic method. This is the first step toward developing novel small-molecule disease-modifying drugs for AD. Our data suggest that polyphenols or their derivatives could be

utilized for their advantage of enhancing NEP activity to decelerate AD development. Our research will be helpful in solving the difficulties associated with the development of drugs for AD.

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Conflict of Interest N.I., K.S., and T.T. have patents: US Patent 9980937 and JP Patent 6797408 related to the application of aliphatic derivatives of polyphenols as therapeutic agents for Alzheimer's disease. The other authors have no conflicts of interest to declare.

Supplementary Materials This article contains supplementary materials.

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