

Protective effects of a chalcone derivative against A β -induced oxidative stress and neuronal damage

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Amyloid β -peptide (A β -peptide)-induced oxidative stress is thought to be a critical component of the pathophysiology of Alzheimer's disease (AD). New chalcone derivatives, the Chana series, were recently synthesized from the retrochalcones of licorice. In this study, we investigated the protective effects of the Chana series against neurodegenerative changes *in vitro* and *in vivo*. Among the Chana series, Chana 30 showed the highest free radical scavenging activity (90.7%) in the 1,1-diphenyl-2-picrylhydrazyl assay. Chana 30 also protected against A β -induced neural cell injury *in vitro*. Furthermore, Chana 30 reduced the learning and memory deficits of A β ₁₋₄₂-peptide injected mice. Taken together, these results suggest that Chana 30 may be a promising candidate as a potent therapeutic agent against neurodegenerative diseases. [BMB reports 2011; 44(11): 730-734]

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease that is strongly correlated with aging, and its prevalence globally has dramatically increased as the lifespan of the world's population has increased. AD is clinically characterized by severe memory loss, impairment of cognitive performance and personality changes (1). Oxidative stress is a key component of the AD pathological cascade (2). Oxidative stress, as measured by many parameters, including the modification of DNA, lipid peroxidation, protein oxidation and ROS formation, has been observed in the neurodegenerative brain tissue of AD patients (3, 4). Several studies have suggested that amyloid beta (A β) peptide induced the oxidative stress observed in the neurodegenerative AD brain (5-7). The presence of A β plaques is

one of the major pathological hallmarks of AD (8). This disease is thought to be caused by the accumulation of A β to the extent that the A β level reach a toxic level in the brain (9). It has been known that A β peptide-induced toxicity is mediated by free radicals, which cause lipid peroxidation of neural cell membranes via inhibition of antioxidants (10, 11). Proper intracellular balance of oxidants and antioxidants is vital for cell function and an imbalance of the oxidative state is involved in much of the pathology mediated by free radicals (12). Therefore, antioxidant therapies have been investigated as a potential means to reduce reactive oxygen species (ROS)-based damage in AD. Natural antioxidant products are thought to be promising potential candidate neuroprotective agents because natural antioxidants such as phytochemicals have been shown to prevent neuronal cell death via scavenging of free radicals and enhancing the cellular antioxidant system.

Licorice (the roots of *Glycyrrhiza inflata*) has been used in folk medicine to relieve rheumatic and other types of pain and is also known to have a healing effect on ulcers (13). Retrochalcones, including echinatin and the licochalcone series (licochalcone A-E), have been isolated from licorice, and have been shown to produce antioxidative effects such as the scavenging of superoxide (13), as well as anti-bacterial (14), anti-inflammatory (15) and anti-diabetic (16) activities. In this study, we synthesized new chalcone derivatives based on retrochalcone from licorice and investigated the protective effects of these synthesized chalcones on A β peptide-induced oxidative damage via free radical scavenging *in vitro* and determine if these chalcones could reduce learning and memory deficits in A β -treated mice.

RESULTS AND DISCUSSION

Free radical scavenging activity and the effects on cell viability of the Chana series

The free radical scavenging activity of the synthetic chalcone series, Chana 1 to 40, was screened using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. In this screen, Chana 30 was shown to have the highest scavenging activity among the Chana compounds (Fig.1B). In fact, the scavenging activity of Chana 30 was also significantly higher than that of epigallocatechin

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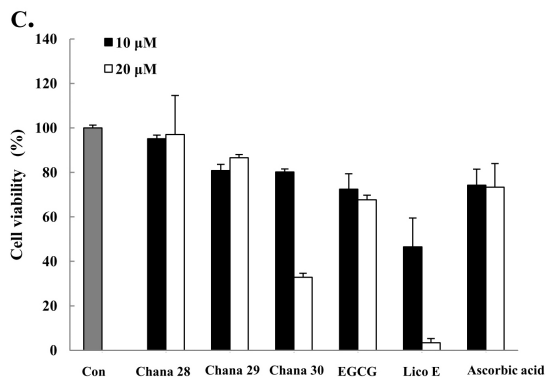
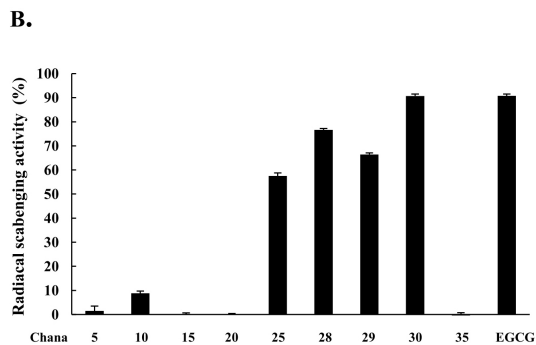
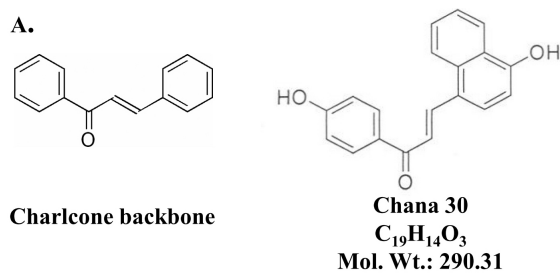


Fig. 1. Effects of Chana chalcones on PC12 cells. (A) The chemical structures of the chalcone backbone and the synthesized Chana 30 compound. (B) DPPH free radical scavenging activity of the Chana series was measured. (C) PC12 cells were treated with various concentrations (10, 20, and 50 μM) of Chana 28, 29, and 30 in serum-free media for 24 h. Cells were treated with either Lico A, Lico E, or ascorbic acid (same concentrations as above) for comparison. EGCG: epigallocatechin gallate, Lico E: Licochalcone E. The data are the mean ± SE (n = 5). Data are represented as mean ± standard error (n = 5).

gallate (EGCG), which was used as a positive control. Chana 28 and 29 also exhibited scavenging activities that were higher than of the other Chana compounds. To ensure that the compounds with increased scavenging ability were not toxic to cells, the viability of PC12 cells was monitored by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after the cells were treated with Chana 28, 29, and 30 (Fig. 1 C). Cells were treated with Chana 28, 29, or 30 at 10 and 20 μM for 24 h, and ascorbic acid was used as a positive control. 10

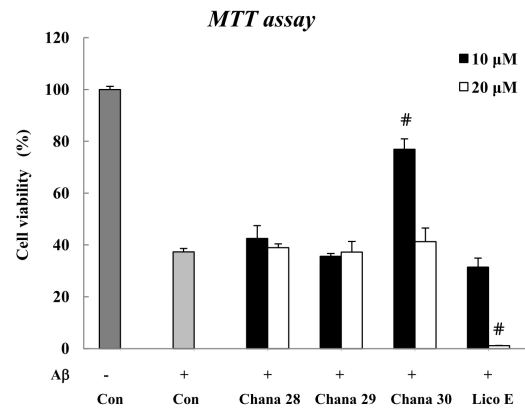


Fig. 2. Protective effect of the Chana series against Aβ₁₋₄₂-induced cell damage. Cell viability of the Chana series was evaluated using the MTT assay and the control group was not treated with the test samples. PC12 cells were treated with 10 or 20 μM of the Chana samples in serum-free media for 24 h. All groups were treated with 25 μM Aβ₁₋₄₂ except the control group. Cells were also treated with Lico E at the same concentrations for comparison. Sample treated groups were pre-incubated at 10 or 20 μM, in serum-free media for 24 hr before the addition of the Aβ peptide. The data are the mean ± SE (n = 5). #P < 0.05 vs. Aβ-injected group.

μM of all three of the Chana series compounds (28, 29 and 30) reduced cell viability by less than 20%. Interestingly, the viability of cells treated with the natural retrochalcone, Licochalcone E (Lico E), was lower than that of those treated with Chana 28, 29 or 30.

Protective effects of Chana chalcones against Aβ-induced cytotoxicity *in vitro*

To examine the protective effects of the selected Chana compounds (28-30) against Aβ₁₋₄₂-induced neuronal cell death, cell viability was measured in PC12 cells treated with these compounds and Aβ₁₋₄₂. Aβ₁₋₄₂ treatment of PC12 cells decreased cell viability by 37.33 ± 1.28% compared to control cells (Fig. 2). Among the selected Chana compounds, only Chana 30 at 10 μM significantly protected the cells against the effects of Aβ₁₋₄₂.

Aβ peptides have been shown to induce the peroxidation of lipids and proteins (10), and this activity may at least in part account for the neurodegeneration in AD brains (8). Recent evidence has indicated that natural compounds with antioxidant activity can reduce oxidative stress and exhibit neuroprotective effects. Caffeic acid derivatives from romaine lettuce (17), acubin from *Eucommia ulmoides japonica* (18) and anthocyanins from strawberries (19) have all been shown to reduce oxidative-induced neurodegenerative damage and retrochalcones have been previously reported to have free radical scavenging activity (13). In this study, the scavenging activity of Chana 30 was found to be similar to that of EGCG and higher than that of licochalcone E, and Chana 30 at 10 μM protected cells from Aβ peptide-induced neuronal cell death (Fig.

2). These results suggest that Chana 30 can potently protect against $A\beta_{1-42}$ induced oxidative damage *in vitro* and may be an efficacious therapeutic agent against $A\beta$ -peptide induced cell death via free radical scavenging.

The memory and learning deficits in $A\beta_{1-42}$ -treated mice are inhibited by Chana 30

Based on the *in vitro* neuroprotective effects of Chana 30, we performed an *in vivo* study to investigate the effects of Chana 30 on the learning and memory deficits in $A\beta$ -treated mice. To determine if Chana 30 improved learning and memory, the Y-maze and the passive avoidance test were performed. In this experiment, Chana 30 was administered orally at a dose of 20 or 50 mg/kg for 4 weeks (Fig. 3A). We used an AD animal model that is based on the intracerebroventricular (ICV) injection of $A\beta$, which has been previously reported to induce memory deficits (20, 21). There were no significant differences in body weight between the experimental groups during the experiment (Table 1), and all experimental animals survived. Pretreatment with Chana 30 was found to dramatically inhibit the $A\beta$ -induced decrease in spontaneous alternation behavior and treatment with 20 and 50 mg/kg Chana 30 reduced the spontaneous alternation behavior to 24.7% and 19.3%, respectively, relative to the untreated control mice, while mice injected with only $A\beta_{1-42}$ displayed a 41.2% decrease in such behavior (Fig. 3B). Moreover, the effects of Chana 30 were greater than those of the positive controls, Selegiline (12.5%) and Aricept (16.8%).

In the passive avoidance paradigm, the $A\beta_{1-42}$ -treated mice displayed a significantly reduced STL (64% reduction) when compared to the control group in the passive avoidance test (Fig. 3C). On the other hand, the Chana 30-treated groups had STL times of 194 s (20 mg/kg group) and 99.5 s (50 mg/kg), both of which were significantly higher than the STL of the group treated with only $A\beta_{1-42}$. In fact, these STL times were also higher than those of the controls and the positive control (Selegiline and Aricept) groups, indicating that Chana 30 could reduce $A\beta$ -induced learning and memory deficits to a greater extent than the positive controls, Selegiline and Aricept, which are currently being used to treat Alzheimer's.

In conclusion, Chana 30 protected cells against $A\beta$ peptide-induced oxidative stress and mice against $A\beta$ peptide injection-induced learning and memory deficits. These effects likely were the result of the scavenging activity of the newly synthesized retrochalcone Chana 30. Taken together, these results suggest that Chana 30 is a potential potent preventive therapeutic agent against AD.

MATERIALS AND METHODS

Cell cultures

PC12 cells (a rat pheochromocytoma cell line) were obtained from American Type Culture Collection (ATCC, Manassas, VA) and were maintained in RPMI-1640 (Gibco BRL, Gaithersburg,

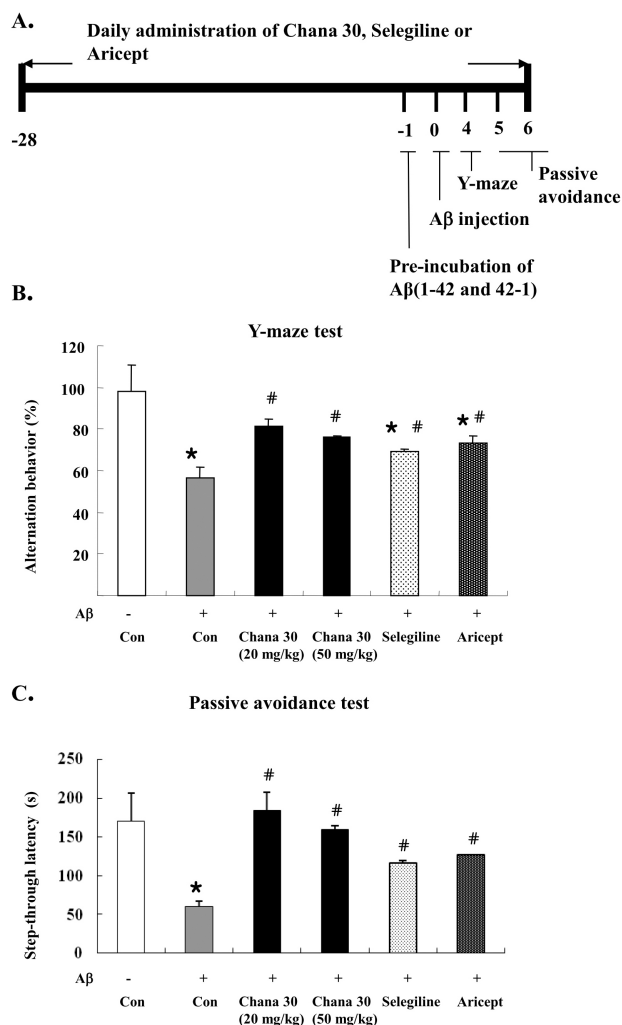


Fig. 3. Effects of Chana 30 in $A\beta$ -injected mice. (A) Schedule for pretreatment with Chana 30 *in vivo*. The Y-maze test and the passive avoidance test were performed to evaluate the *in vivo* neuroprotective effects of Chana 30 at concentrations of 20 and 50 mg/kg. ICR mice were treated with Chana 30, Selegiline, or Aricept by oral administration for 34 days. After 28 days of oral administration, mice were administered $A\beta_{1-42}$ (mice who did not receive Chana 30, Selegiline, or Aricept were administered either $A\beta_{1-42}$ or $A\beta_{42-1}$ (control mice) by ICV injection). $A\beta$ peptides were dissolved in PBS and incubated at 37°C for 24 h in order to aggregate prior to injection. $A\beta$ peptides were injected into each mouse (410 pmol /each) at the bregma by ICV injection. Four days after the injection of the $A\beta$ peptides, the Y-maze test was performed, and the passive avoidance test was performed in the following 2 days. (B) Spontaneous alternation behavior, which is regarded as a measurement of spatial memory, was measured by the Y-maze test in $A\beta$ -injected mice. (C) Step-through latency (%) was evaluated in a passive avoidance task in $A\beta$ -injected mice. Chana 30 (20 or 50 mg/kg), Selegiline (3 mg/kg), or Aricept (1 mg/kg) were administered orally for 28 days, and mice were ICV injected with $A\beta_{1-42}$. Control mice were injected with $A\beta_{42-1}$. Data are presented as mean \pm standard error (n = 8). *P < 0.05 vs. the $A\beta_{42-1}$ -injected control group, *P < 0.05 vs. $A\beta_{42-1}$ -injected control group, *P < 0.05 vs. control group, #P < 0.05 vs. $A\beta_{1-42}$ -injected group.

Table 1. Changes in body weight of mice co-treated with A β and Chana 30

	Control	A β (42-1)	A β (1-42)			
			Chana 30		Selegiline	Aricept
			20 (mg/kg)	50 (mg/kg)	3 (mg/kg)	1 (mg/kg)
Initial	20.3 \pm 1.3	20.3 \pm 1.4	19.3 \pm 1.6	19 \pm 1.5	19.8 \pm 1.4	20.0 \pm 1.4
Final	35.8 \pm 0.6	36.7 \pm 0.6	37.2 \pm 0.3	36.6 \pm 0.9	37.0 \pm 0.7	36.0 \pm 0.7

Control mice were injected with A β ₄₂₋₁. Samples were administrated by oral for 28 days, followed by A β ₄₂₋₁ was injected. Data are the mean \pm SE (n = 8).

MD, USA) medium that contained 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin (Gibco BRL). Cells were maintained at 37°C in a humidified atmosphere that contained 5% CO₂.

DPPH free radical scavenging activity

The DPPH scavenging activity of thirty-four synthetic Chana compounds were screened to evaluate their anti-oxidant properties. The procedure used for this assay was similar to the method described by Blois, with slight modifications (22). 1,000 μ g/ml of sample was dissolved in methanol and serially diluted to 5, 10, 20, 50, and 100 μ g/ml in ethanol. In each reaction, the solutions were mixed with 0.25 mM DPPH and incubated for 30 min at room temperature. The optical density of the solutions was then measured at 517 nm. The DPPH radical scavenging activity was calculated according to the following equation: scavenging activity (%) = $[(A_0 - A_1) / A_0] \times 100$, where A₀ is the absorbance of the blank and A₁ is the absorbance of the sample.

Cell viability measurement

Cell viability was measured by MTT reduction to determine the cytotoxicity and/or the neuroprotective effects of the Chana chalcones (Chana 28, 29 and 30) at various concentrations on PC12 and U87MG cells. MTT reduction was initiated by the addition of 10 μ l per well of a 2 mg/ml MTT solution. The cells were then incubated for 2 h at 37°C. The reaction was stopped by the addition of 100 μ l dimethyl sulfoxide (DMSO). The absorbance was then measured (excitation at 570 nm, emission at 630 nm) using a microplate reader (Model 550, BIO-RAD, CA). Cell viability was expressed as the relative percentage compared to the cell viability of an untreated control culture.

Animals

Male ICR mice (5 weeks old) were obtained from Samtako Co. (Osan, Korea). Animals were allowed to adapt to the environment for 1 week before experimental use and were maintained at constant temperature (23 \pm 1°C) and humidity (60 \pm 10%) and on a 12 h light/dark cycle (light on 07 : 00-19 : 00 h).

Mice were supplied with a standard pellet diet (Purina Korea) and tap water *ad libitum*. Chana 30 was dissolved in tap water at a concentrations of 20 and 50 mg/kg and administered orally daily for 34 days. Aricept (1 mg/kg) and Selegiline (3 mg/kg) were administered orally for 28 days and used as positive controls. After 21 days of treatment with Chana 30 or the positive control (Aricept and Selegiline), mice were administered A β ₁₋₄₂ by ICV injection (410 pmol/mouse). Mice not treated with Chana 30 or a positive control were ICV injected with either A β ₁₋₄₂ or the non-toxic reverse fragment A β ₄₂₋₁ (control group). A β peptides were dissolved in phosphate buffered saline (PBS) and pre-incubated at 37°C for 24 h to allow for fibril formation. The peptides were injected into mice (10 μ l per mouse) at the bregma by ICV injection using a Hamilton microsyringe (25-G needle, 2.5 mm depth) (26). All experiments were conducted according to the guidelines of the Committee on Care and Use of Laboratory Animals of Yonsei University.

Y-maze test

Spontaneous alternation behavior in a Y-maze was used as a measure of the short-term spatial recognition memory of Chana 30-treated and untreated mice (23). The Y-maze test was performed 4 days after A β injection. The maze was made of black-painted plastic, and each arm of the maze was 33 cm long, 15 cm high, 10 cm wide, and positioned at an equal angle. Each mouse, all of which had never been exposed to the maze previously, was placed at the end of one arm and allowed to freely explore the maze during an 8-min period.

Passive avoidance test

A step-through passive avoidance test was used to evaluate the effects of Chana 30 on A β -induced learning and memory deficits (24). The passive avoidance test was carried out 6 days after A β injection. The apparatus (Model PACS-30, Columbus Instruments Int.,) was divided into two chambers of equal size (23.5 \times 15.5 \times 15.5 cm). One chamber was illuminated, while the other was darkened. During the training trial, each mouse was gently placed in the illuminated compartment, and when the mouse entered the darkened compartment, the door

was closed, and the mouse received an inescapable electrical shock (0.5 mA, 1 s). In the test trials, which were given 1 day after the training trial, mice were again placed in the lighted compartment, and the latency to enter the darkened compartment was measured. If the mouse did not enter the darkened compartment within 300 s, the trial was ended, and the mouse was assigned a latency of 300 s.

Statistical analysis

The results were expressed as mean \pm standard deviation. Differences among means were examined by Duncan's multiple range tests with SPSS 12.0 (SPSS Inc., Chicago, IL), and a P value of <0.05 was considered significant.

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