Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters 15 (2005) 3588–3591
Bioorganic & Medicinal Chemistry Letters

Neuroprotective effects of naturally occurring biflavonoids

Sam Sik Kang,[a,b] Ji Yeon Lee,[a] Yoo Keum Choi,[a] Sun Sook Song,[a] Ju Sun Kim,[b] Su Jin Jeon,[c] Yong Nam Han,[a,b] Kun Ho Son[c] and Byung Hee Han[a,b,*]

[a]*Department of Manufacturing Pharmacy, Seoul National University College of Pharmacy, Seoul 110-460, Republic of Korea*
[b]*Natural Products Research Institute, Seoul National University College of Pharmacy, Seoul 110-460, Republic of Korea*
[c]*Department of Food Science and Nutrition, Andong National University, Gyeongbuk 760-749, Republic of Korea*

Received 1 April 2005; revised 9 May 2005; accepted 12 May 2005

Abstract—We examined neuroprotective effects of naturally occurring biflavonoids on oxidative stress-induced and amyloid β peptide-induced cell death in neuronal cells. Among the nine biflavonoids tested, amentoflavone, ginkgetin, and isoginkgetin exhibited strong neuroprotection against cytotoxic insults induced by oxidative stress and amyloid β, suggesting their therapeutic potential against neurodegenerative diseases, including ischemic stroke and Alzheimer's disease.

Naturally occurring flavonoids are polyphenolic compounds present in a variety of fruits, vegetables, and seeds. They can be classified into flavanones, flavones, flavonols, and biflavones.[1] Flavonoids show many biological and pharmacological activities, including antioxidative, anti-inflammatory, antitumor, and antiviral effects.[2] Recently, growing lines of evidence have suggested that flavonoids have neuroprotective effects in many models of neurodegenerative diseases in vitro and in vivo.[3–6] For example, wogonin protected the rat brain against ischemia–reperfusion brain injury via inhibition of inflammatory activation of microglia.[3] Apigenin and kaempferol protected rat cortical neurons against amyloid β (Aβ)-induced neurotoxicity by inhibition of the caspase pathway, regardless of their anti-oxidative capacity.[7] Recently, we have reported that flavones, apigenin and luteolin, protect SH-SY5Y neuroblastoma cells from oxidative stress-induced neurotoxicity by inhibition of the caspase pathway, independent of their free-radical scavenging activity.[6] Therefore, flavonoids' neuroprotective action can be attributed to a variety of their biological activities.

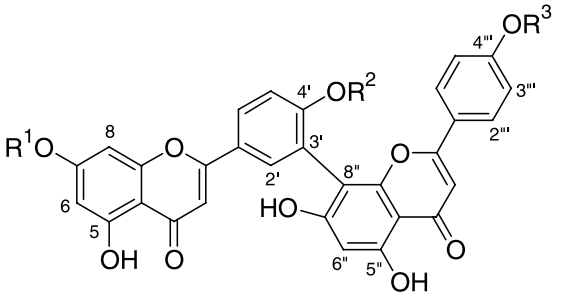
Naturally occurring biflavonoids literally consist of a dimer of flavonoids linked to each other by either a C–C or a C–*O*–C covalent bond. Although biflavonoids are known to display a variety of biological activities, such as anti-inflammatory activity,[8] inhibition of cytochrome P450 enzymes,[9] and antiviral activity,[10] their neuroprotective roles have not been known. In the present study, we explored whether natural biflavonoid protected neuronal cells against oxidative stress- and Aβ-induced neurotoxicity in cell-based assay systems.

Hinokiflavone was purchased from Extrasynthese (Genay Cedex, France). The rest of the biflavonoids were prepared as previously reported.[11,12] Briefly, bilobetin, ginkgetin, isoginkgetin, and sciadopitysin were purified from *Gingko biloba*. Ochnaflavone and 4′-*O*-methylochnaflavone were isolated from *Lonicera japonica*. Amentoflavone and isocryptomerin were purified from *Selaginella tamariscina*. As shown in Figure 1, these biflavonoids can be classified as three types: the amentoflavone-type (apigenin-C3′-C8″-apigenin), the ochnaflavone-type (apigenin-C3′-*O*-C4‴-apigenin), and the hinokiflavone-type (apigenin-C4′-*O*-C6″-apigenin).

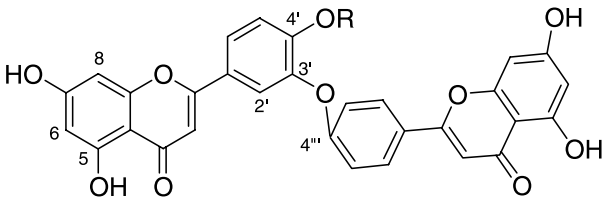
We first tested whether biflavonoids had cytoprotective effects on oxidative stress-induced cell death in SH-SY5Y neuroblastoma cells.[13] Oxidative stresses caused by the abnormal regulation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in cells and tissues have been implicated in many neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and ischemic and hemorrhagic stroke.[14–17] Moreover, in experimental models, oxidative stress plays a

Keywords: Flavonoids; Biflavonoids; Amentoflavone; Neuroprotection; Ischemic stroke; Alzheimer's disease.
* Corresponding author. Tel.: +8217408917; fax: +8217628322; e-mail: hanbh@snu.ac.kr

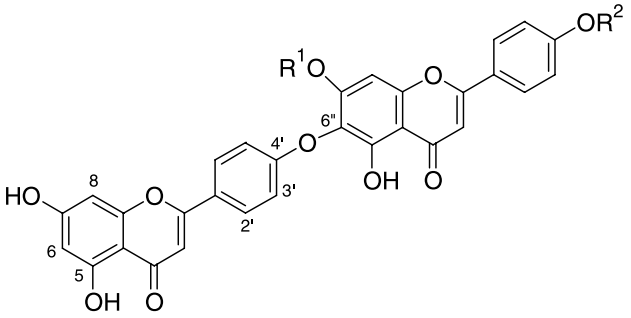
0960-894X/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2005.05.078



1: amentoflavone $R^1=R^2=R^3=H$, **2:** bilobetin $R^1=R^3=H$, $R^2=CH_3$
3: ginkgetin $R^1=R^2=CH_3$, $R^3=H$, **4:** isoginkgetin $R^1=H$, $R^2=R^3=CH_3$
5: sciadopitysin $R^1=R^2=R^3=CH_3$



6: ochnaflavone R=H, **7** 4'-*O*-methylochnaflavone R=CH_3



8: hinokiflavone $R^1=R^2=H$, **9:** isocryptomerin $R^1=CH_3$, $R^2=H$

Figure 1. Structure of biflavonoids.

critical role in neurodegeneration induced by a variety of insults, including excitotoxicity, Aβ, ischemia, and nerve growth factor withdrawal.

Similar to a previous study,[6] oxidative stress by treatment with 0.3 mM hydrogen peroxide resulted in ~50% cell death 24 h after the treatment, as determined by the MTT reduction assay in SH-SY5Y cells (data not shown). To test the neuroprotective effects, we treated cells with 0.4–10 μM biflavonoids dissolved in dimethylsulfoxide (DMSO) 1 h prior to the addition of 0.3 mM H_2O_2, followed by determination of the cell viability at 24 h later. We found that DMSO at a final concentration of 0.5% in media did not affect the solubility of flavonoids or the cell viability. As shown in Table 1, all tested biflavonoids at a concentration of 10 μM significantly reduced the H_2O_2-induced cell death. Overall, the order of potency for cytoprotection was amentoflavone-type (Compd **1–5**) ⩾ hinokiflavone-type (Compd **8–9**) > ochnaflavone-type (Compd **6–7**) biflavones. Within the amentoflavone-type biflavones, either –H or $–CH_3$ groups at the R^1, R^2, and R^3 position did not affect the neuroprotective effects because they showed similar inhibitory effects on ROS-induced cell death.

Table 1. Protective effects of biflavonoids on H_2O_2-induced cell death

Compound	% Inhibition (μM)[a]		
	0.4	2	10
1	27.0 ± 1.5*	39.9 ± 2.7*	62.9 ± 1.6*
2	25.3 ± 5.3	30.4 ± 5.5	32.9 ± 1.4*
3	11.3 ± 2.9	12.2 ± 2.2	35.7 ± 2.3*
4	40.1 ± 1.2*	28.7 ± 2.0*	55.1 ± 2.6*
5	39.5 ± 3.2*	52.1 ± 5.3*	69.5 ± 4.5*
6	−3.6 ± 0.9	−12.1 ± 0.5	32.7 ± 0.6*
7	1.8 ± 1.3	−1.2 ± 0.3	12.1 ± 2.5*
8	7.8 ± 2.0	19.8 ± 0.9	56.0 ± 3.7*
9	−3.5 ± 2.2	23.9 ± 1.9	68.1 ± 2.2*

[a] Protective effects of test compounds on hydrogen peroxide-induced cell death in SH-SY5Y cells. Data are presented as means ± SEM from triplicate samples.
* $p < 0.05$ as compared to the group treated only with H_2O_2.

Since biflavonoids showed such robust neuroprotection against ROS-induced insult, we asked further whether the anti-oxidative activity was attributed to their neuroprotective action. We observed that none of the nine biflavones we tested exhibited a free-radical scavenging activity up to 100 μM when assessed by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay as described (data not shown).[6,18] Thus, it did not appear that biflavones per se acted as antioxidants or free-radical scavengers, at least in a cell free system. These results suggested that biflavonoids' neuroprotection might be mediated by a direct blockade of the cell death cascades, but not by their anti-oxidative activity. To test this idea further, we examined the neuroprotective effects of biflavonoids against cytotoxic insult induced by staurosporine, which has been known to mediate apoptosis via the caspase-dependent mitochondrial pathway. Similar to neuroprotection against H_2O_2 insult, all biflavones, except for ochnaflavone, significantly reduced the staurosporin-induced cell death at a concentration of 10 μM (data not shown). Thus, these data further supported that biflavonoids' neuroprotection appeared to be mediated, in part if not all, by direct blockade of the signaling events leading to apoptosis upon cellular stresses.

Next, we examined the neuroprotective effects of biflavones against reactive nitrogen species (RNS)-induced cytotoxicity in SH-SY5Y cells. Nitric oxide (NO), produced by NO synthases in many types of cells, acts as a neurotransmitter in the central nervous system in the normal physiologic condition. In the pathologic condition, however, excessive generation of NO has been implicated in neuronal cell death in neurodegenerative diseases, such as following ischemic stroke.[19,20] NO is thought to generate highly cytotoxic free radicals, such as peroxynitrite, which causes oxidative damage to intracellular functional, such as proteins, lipid, and DNA. These oxidative damages, in turn, trigger intracellular signaling events, leading to neuronal cell death. Treatment of SH-SY5Y cells with a NO donor, sodium nitroprusside (SNP) at a concentration of 1.2 mM for

Table 2. Protective effects of biflavonoids on SNP-induced cell death

Compound	% Inhibition (μM)[a]		
	0.4	2	10
1	5.2 ± 0.9	16.4 ± 2.2*	40.5 ± 2.1*
2	2.5 ± 0.8	4.8 ± 0.8	11.0 ± 1.9*
3	−0.1 ± 1.2	7.8 ± 2.5	32.4 ± 3.2*
4	6.9 ± 2.4	10.5 ± 2.7	23.5 ± 3.3
5	2.8 ± 1.5	8.1 ± 2.6	4.6 ± 1.8
6	10.0 ± 1.2*	7.7 ± 0.6	9.3 ± 2.2*
7	2.9 ± 1.5	0.1 ± 2.6	1.6 ± 0.9
8	2.5 ± 0.6	−9.4 ± 1.2	15.0 ± 1.2*
9	−16.0 ± 0.6*	−14.8 ± 0.8*	−25.6 ± 0.1*

[a] Protective effects of test compounds on SNP-induced cell death in SH-SY5Y cells. Data are presented as means ± SEM from triplicate samples.

* $p < 0.05$ as compared to the group treated only with SNP.

Table 3. Protective effects of biflavonoids on $A\beta_{25-35}$-induced cell death

Compound	% Inhibition (μM)[a]		
	0.4	2	10
1	3.6 ± 1.1	25.4 ± 1.7*	31.8 ± 2.3*
2	9.8 ± 1.9	1.9 ± 1.2	−7.5 ± 0.3
3	29.5 ± 1.1*	43.6 ± 1.3*	5.6 ± 0.5
4	19.7 ± 0.5*	59.1 ± 1.2*	43.8 ± 0.7*
5	27.1 ± 0.9*	38.1 ± 0.5*	35.1 ± 1.3*
6	−12.7 ± 0.7*	−32.1 ± 0.5*	−1.9 ± 0.5
7	13.0 ± 0.2*	0.9 ± 0.9	−14.8 ± 0.1*
8	8.7 ± 1.4	−19.9 ± 2.2*	−10.0 ± 2.2
9	18.1 ± 1.3*	11.5 ± 0.9*	−0.2 ± 0.4

[a] Protective effects of test compounds on $A\beta_{25-35}$-induced cell death in SH-SY5Y cells. Data are presented as means ± SEM from triplicate samples.

* $p < 0.05$ as compared to the group treated only with $A\beta_{25-35}$.

24 h, resulted in a reduction by ~50% in cell viability, as determined by the MTT assay (data not shown). Under these conditions, inhibitory effects of biflavones on SNP-induced cytotoxicity were determined as described (Table 2). We found that amentoflavone, bilobetin, ginkgetin, ochnaflavone, and hinokiflavone at a concentration of 10 μM significantly reduced the SNP-induced cell death by 40.5 ± 2.1, 32.4 ± 3.2, 9.3 ± 2.2, and 15.0 ± 1.2, respectively, whereas the rest of the biflavones failed to demonstrate any significant neuroprotection. Although bilobetin and sciadopitysin showed strong neuroprotection against H_2O_2 insult, they failed to demonstrate neuroprotection against SNP insult.

We wanted to examine further whether biflavones showed protective effects against Aβ-induced cytotoxicity. Alzheimer's disease (AD) is a chronic neurodegenerative disease and is characterized by dystrophic neurites, neurofibrillary tangles, and senile plaques composed primarily of aggregated Aβ.[21–23] The Aβ peptide, a 40–42 a.a. peptide, is generated from the amyloid precursor protein (APP) by proteolytic cleavage. Recent studies have demonstrated that Aβ fragments ($A\beta_{1-42}$, $A\beta_{1-40}$, and $A\beta_{25-35}$) cause neuronal cell death via both apoptosis and necrosis in vitro.[24,25] Aβ-induced neurotoxicity appears to be mediated by multiple mechanisms including oxidative stress, intracellular calcium, and activation of the stress-activated protein kinase cascades.[26,27] Under our experimental conditions, treatment of rat PC12 cells with 1 μM $A\beta_{25-35}$ peptide resulted in a reduction by ~50% in the cell viability, as assessed by the MTT assay (data not shown). We found that the amentoflavone-type biflavones, except for bilobetin, significantly reduced $A\beta_{25-35}$-induced cytotoxicity with higher protective effects at 2 μM (Table 3). In contrast, the ochnaflavone- and hinokiflavone-type biflavones seemed to rather exacerbate the $A\beta_{25-35}$-induced neurotoxicity in PC12 cells.

We then tested neuroprotective effects of biflavonoids against neuronal cell death induced by a DNA-damaging agent, etoposide, of which neurotoxicity involved the multiple intracellular signaling events including p53 and caspase-dependent and -independent cell death pathways.[28,29] We found that in contrast to neuroprotection against oxidative stress, only the amentoflavone reduced the etoposide-induced cell death by 15.9 ± 1.0%, 21.7 ± 2.0%, and 12.3 ± 1.0% at concentrations of 0.4, 2, and 10 μM, respectively (data not shown). Besides amentoflavone, the other biflavonoids (0.4–10 μM) rather potentiated the etoposide-induced SH-SY5Y cell death. These data have suggested that the –H groups at the R^1, R^2, and R^3 position appeared to be essential for keeping neuroprotective action against cytotoxic insult by the DNA-damaging agent.

To our knowledge, this is the first report to suggest that naturally occurring biflavonoids protect neurons against a variety of cytotoxic insults, including oxidative stress, Aβ, and DNA-damaging agents. The amentoflavone-type biflavonoids consisting of an apigenin dimer linked by a C3′-C8″ covalent bond are more potent than the ochnaflavone and hinokiflavone derivatives in neuroprotective effects on oxidative stress-induced cell death. Furthermore, amentoflavone in the amentoflavone derivatives reveals the widest spectrum of neuroprotection against the diverse cytotoxic insults. Thus, it will be of interest to test further whether amentoflavone protects neurons in animal models of neurodegenerative diseases in vivo to explore its therapeutic potential as a neuroprotective agent. Our data suggest that the molecular mechanisms underlying these biflavones' neuroprotection appear to involve multiple molecular and cellular events which lead to apoptosis and necrosis. Further study is necessary to elucidate the molecular target(s) at which biflavones exert their neuroprotective action. This will provide further insight into the design of new therapeutic agents for neuroprotection.

Acknowledgments

The authors thank Hyung Geun Park and Sang Hee Kim for helpful comments. This work was supported by grants of Korea Health 21 R&D Project (01-PJ2-PG6-01NA01-0002 and 03-PJ1-PG1-CH06-0001) from the Ministry of Health and Welfare, Republic of Korea.

References and notes

1. Beecher, G. R. *J. Nutr.* **2003**, *133*, 3248S.
2. Ross, J. A.; Kasum, C. M. *Annu. Rev. Nutr.* **2002**, *22*, 19.
3. Lee, H.; Kim, Y. O.; Kim, H.; Kim, S. Y.; Noh, H. S.; Kang, S. S.; Cho, G. J.; Choi, W. S.; Suk, K. *FASEB J.* **2003**, *17*, 1943.
4. Youdim, K. A.; Spencer, J. P.; Schroeter, H.; Rice-Evans, C. *Biol. Chem.* **2002**, *383*, 503.
5. Dajas, F.; Rivera-Megret, F.; Blastina, F.; Arredono, F.; Abin-Carriquiry, J. A.; Costa, G.; Echeverry, C.; Lafon, L.; Heizen, H.; Ferreira, M.; Morquio, A. *Braz. J. Med. Biol. Res.* **2003**, *36*, 1613.
6. Kang, S. S.; Lee, J. Y.; Choi, Y. K.; Kim, G. S.; Han, B. H. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2261.
7. Wang, C. N.; Chi, C. W.; Lin, Y. L.; Chen, C. F.; Shiao, Y. J. *J. Biol. Chem.* **2001**, *276*, 5287.
8. Banerjee, T.; Valacchi, G.; Ziboh, V. A.; van der Vliet, A. *Mol. Cell. Biochem.* **2002**, *238*, 105.
9. von Moltke, L. L.; Weemhoff, J. L.; Bedir, E.; Khan, I. A.; Harmatz, J. S.; Goldman, P.; Greenblatt, D. J. *J. Pharm. Pharmacol.* **2004**, *56*, 1039.
10. Lin, Y. M.; Flavin, M. T.; Schure, R.; Chen, F. C.; Sidwell, R.; Barnard, D. L.; Huffman, J. H.; Kern, E. R. *Planta Med.* **1999**, *65*, 120.
11. Lee, S. J.; Choi, J. H.; Son, K. H.; Chang, H. W.; Kang, S. S.; Kim, H. P. *Life Sci.* **1995**, *57*, 551.
12. Son, K. H.; Park, J. O.; Chung, K. C.; Chang, H. W.; Kim, H. P.; Kim, J. S.; Kang, S. S. *Arch. Pharmacol. Res.* **1992**, *15*, 365.
13. Neuroprotective activity of biflavonoids on oxidative stress was assessed in human neuroblastoma cells SH-SY5Y cells. Cells were plated at 1×10^5 cells per well in a 48-well plate in DMEM containing 10% fetal bovine serum and antibiotics at a humidified CO_2 incubator. A day after plating, cells were pre-treated with a test compound for 1 h and then treated with 0.3 mM hydrogen peroxide, 1.2 mM SNP, 200 nM staurosporine, or 100 μg/ml etoposide for 24 h. For neuroprotective effects on $A\beta_{25-35}$-induced cytotoxicity, PC12 cells were pre-treated with biflavonoids 1 h before the addition of 1 μM $A\beta_{25-35}$ to induce cell death for 24 h. To assess the cell viability, cells were incubated with MTT for 3 h at 37 °C, and OD was read at 595 nm using a spectrophotometer. Data were calculated as % inhibition of cell death as compared to the group treated with vehicle. Data indicate means ± SEM from triplicate samples. $^*p < 0.05$ as compared to the vehicle-treated group. Statistics were analyzed by analysis of variance followed by Dunn's multiple comparison method.
14. Klein, J. A.; Ackerman, S. L. *J. Clin. Invest.* **2003**, *111*, 785.
15. Jenner, P. *Ann. Neurol.* **2003**, *53*, S26.
16. Eckert, A.; Keil, U.; Marques, C. A.; Bonert, A.; Frey, C.; Schussel, K.; Muller, W. E. *Biochem. Pharmacol.* **2003**, *66*, 1627.
17. Lipton, S. A.; Choi, Y.-B.; Pan, Z.-H.; Lei, S. Z.; Chen, H.-S. V.; Sucher, N. J.; Loscalzo, J.; Singel, D. J.; Stamler, J. S. *Science* **1993**, *364*, 626.
18. Free-radical scavenging activity was determined using DPPH as described. In a 96-well plate, 50 μl of 0.2 mM DPPH dissolved in ethanol was mixed with an equal volume of 0.1 M Tris–HCl buffer containing various concentrations of flavonoids. After incubation for 20 min, OD was read at 517 nm.
19. Heales, S. J.; Bolanos, J. P.; Stewart, V. C.; Brookes, P. S.; Land, J. M.; Clark, J. B. *Biochim. Biophys. Acta* **1999**, *1410*, 215.
20. Baranano, D. E.; Snyder, S. H. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 10996.
21. Dominguez, D. I.; De Strooper, B. *Trends Pharmacol. Sci.* **2002**, *23*, 324.
22. Jellinger, K. A.; Bancher, C. *J. Neural Transm. Suppl.* **1998**, *54*, 77.
23. Hamdane, M.; Delobel, P.; Sambo, A. V.; Smet, C.; Begard, S.; Violleau, A.; Landrieu, I.; Delacourte, A.; Lippens, G.; Flament, S.; Buee, L. *Biochem. Pharmacol.* **2003**, *66*, 1619.
24. Ramalho, R. M.; Ribeiro, P. S.; Sola, S.; Castro, R. E.; Steer, C. J.; Rodrigues, C. M. *J. Neurochem.* **2004**, *90*, 567.
25. Behl, C.; Davis, J. B.; Klier, F. G.; Schubert, D. *Brain Res.* **1994**, *645*, 253.
26. Miranda, S.; Opazo, C.; Larrondo, L. F.; Munoz, F. J.; Ruiz, F.; Leighton, F.; Inestrosa, N. C. *Prog. Neurobiol.* **2000**, *62*, 633.
27. Behl, C. *Prog. Neurobiol.* **1999**, *57*, 301.
28. Seluanov, A.; Gorbunova, V.; Falcovitz, A.; Sigal, A.; Milyavsky, M.; Zurer, I.; Shohat, G.; Goldfinger, N.; Rotter, V. *Mol. Cell. Biol.* **2001**, *21*, 1552.
29. Saura, J.; MacGibbon, G.; Dragunow, M. *Brain Res. Mol. Brain Res.* **1997**, *48*, 382.