Caspase 저해제로서의 DMVD 및 DMVD-fmk 유도체들의 합성과 그들의 신경보호효과 측정

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(2008. 7. 28 접수)

Syntheses of DMVD and DMVD-fmk Derivatives and Evaluation of their Neuroprotective Effects as Caspase Inhibitors

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(Received July 28, 2008)

Keywords: Caspase-3 Inhibitor, Apoptosis, DMVD, Fluoromethylketone

INTRODUCTION

Apoptotic cell suicide, or programmed cell death, is a fundamentally important biological process that is required to maintain the integrity and homeostasis of multicellular organisms. Inappropriate apoptosis, however, underlies the etiology of many human diseases, including neurodegenerative disorders, autoimmunity, and cancer. 1-4 Caspases (cysteine-aspartate-specific proteases), a family of cysteine proteases, are among the essential components of the apoptotic machinery, and are presently divided into 10 families on the basis of their sequences, phylogenetic similarities, and preference for the sites. All caspases have a stringent requirement for cleavage after aspartic acid although the preferred tetrapeptide motifs recognized by various caspases are different significantly.5-8 As proteolysis of caspase substrate by caspase is one of the critical mechanisms of apoptosis, the cleavage products of some of these substrates are effects of apoptosis themselves. Based on the caspase cleavage site of substrate, various caspase inhibitors such as z-VAD-fmk, z-DEVD-fmk, z-DEVD-cmk, and Ac-DEVD-cho, which exhibited a very good apoptotic effect as caspase inhibitors, have been developed. 9-11 Recently, we found that the neuronal microtubule-associated protein Tau, which is the main component of the neurofibrillary lesions found in Alzheimer’s disease (AD) and in other neurodegenerative disorders, is a substrate for caspase-3 in a culture model of neuronal apoptosis, and that the caspase cleavage site at the C-terminus of Tau has 418-DMVD-421.12 Because the identification and characterization of critical caspase substrates are the key to find new caspase-3 inhibitors, we further investigate the role of lipophilicity in the structural requirement of the DMVD motif by attaching various lipophilic moieties either at the side chain of the aspartic acid or at the N-terminal amino group of the aspartic acid. Here, we report synthetic methods of various lipophilic DMVD and DMVD-fmk derivatives and their biological activities as a novel caspase-3 inhibitor.
RESULTS AND DISCUSSION

Chemistry

The peptides 1, 2, 3, and 4 were prepared by solid phase peptide synthesis (SPPS). Thus, tetrapeptide TFA-DMVD-OH salt (1) was prepared by stepwise elongation of the peptide chain from Fmoc-Asp (OtBu)-Wang resin with the corresponding ester of an amino acid using solid phase peptide synthesis (SPPS), followed by cleavage of the resin with TFA in the presence of radical scavengers (2.5% ethanedithiol, 2.5% triisopropylsilane) (Scheme 1). The purity of the crude peptide was measured by HPLC, and the molecular weight was identified with ESI-MS.

In order to investigate the role of the lipophilicity of the N-terminal amino group on caspase inhibitory activity, the Fmoc-DMVDOH (2), where the lipophilic Fmoc group was attached to the terminal amino group in the aspartic acid group, was prepared by using the same synthetic protocol of TFA-DMVD-OH salt (1) without deprotection of the N-terminal Fmoc group at the final step. The different ester groups were introduced into the side chain of aspartic acid residue to increase lipophilicity. The allyl ester and cyclohexyl ester as orthogonal protecting groups for the β-carboxyl function of aspartic acid were attached in the last step for the synthesis of TFA-D(OAll)MVD-OH salt (3) and TFA-D(OcHx)MVD-OH salt (4) (Scheme 1).

Since the pentapeptidimetric compound 16 cannot be prepared by stepwise elongation of the peptide chain, it was prepared by the tripeptide-single amino acid [3+1] segment-coupling approach. The main segment Fmoc-D(OtBu)MVD-OH (17) was prepared by the general SPPS protocol using 2-chlorotriyl chloride resin instead of Wang resin. The synthesis of the minor segment was first attempted with fluorous tagging of the corresponding methylethylketone 7 by modification of the reported method (Scheme 2). Thus, z-Asp(OtBu)-OH (5) was converted to the methylethylketone 7 via Weinreb amide 6. The methylethylketone compound 7 was then converted to the corresponding silyl enol ether 8, which was reacted with 1-(chloromethyl)-4-fluoro-1,4-diazabicyclo[2.2.2]octane bis(tetrafluoroborate) to give the fluorousethyl compound 9. However, various attempts for the deprotection of z-group by hydrogenation methods resulted in the generation of many side products.

Consequently, alternative synthesis was employed to synthesize the desired product via nitro-aldol condensation followed by oxidation (Scheme 3). The t-butyl ester 12 was prepared from the direct reaction of carboxylic acid and t-butanol in the presence of concentrated sulfuric acid on powdered...
Syntheses of DMVD and DMVD-fmk Derivatives

![Scheme 3](image)

Reagents: (a) HSO₃, benzene, t-BuOH, MgSO₄, 25 °C; (b) 2-fluoroethanol, (COCl)₂, DMF, EtN, DCM, -78~25 °C; (c) Raney-Ni, Mg(OH)_2, H₂, (45 psi), 25 °C; (d) HOBt, DIC, DCM, 25 °C; (e) COCl₂, DMF, EtN, DCM, -78~25 °C.

Anhydrous magnesium sulfate with 89% yield. Condensation of the t-butylation 12 with fluoroacetaldehyde, which was first prepared from Swern oxidation of 2-fluoroethanol in *in-situ* condition, gave the nitro alcohol derivative 13 with 97% yield. Hydrogenation of the nitro alcohol 13 over Raney-Nickel gave the amino alcohol 14 with 52% yield. Finally, the main segment 17 and the minor segment 14 were coupled with DIC and HOBt condition to give the compound 15, which was subjected to Swern oxidation to give the desired compound 16 after purification by reversed liquid column chromatography. It is interesting to note that Dess-Martin oxidation of the compound 15 did not work in this system.

**Biological Evaluation**

The biological activities of the synthesized DMVD and DMVD-fmk derivatives were evaluated by measuring LDH release in cortical cell culture, and compared to z-VAD-fmk which is known to be a good caspase inhibitor (Table 1). All of the derivatives were found to be inactive while the compound 2 only showed weaker activity compared to z-VAD-fmk. These results indicate that free carboxylic acid at the side chain of aspartic acid is indispensable for the activity while the terminal amino group can be modified for better activity.

In conclusion, the lipophilic DMVD and DMVD-fmk derivatives were successfully synthesized by using a SPPS method and [3+1] segment coupling approach. Biological results showed that free carboxylic acid at the side chain of aspartic acid in DMVD is important for the neuroprotective activity.

**Table 1. Neuroprotective effects of DMVD and DMVD-fmk Derivatives.**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>N</th>
<th>LDH release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staurosporin (100 nM)</td>
<td>4</td>
<td>44.86±4.94</td>
</tr>
<tr>
<td>z-VAD-fmk</td>
<td>4</td>
<td>37.94±9.69</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>50&gt;</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>42±3±1.76</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>50&gt;</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>50&gt;</td>
</tr>
<tr>
<td>17</td>
<td>4</td>
<td>50&gt;</td>
</tr>
</tbody>
</table>

*Cortical cell cultures (DIV 10-12) were exposed to 100 nM staurosporin for 24 hr, alone or with 50 µM of DMVD and DMVD-fmk Derivatives. Neuronal death was analyzed 24 hr later by measuring LDH efflux into the bathing media, mean±SEM (n=8-12 culture wells per condition). *Experimental number.

**EXPERIMENTAL.**

**Instrument and Materials.** Advanced ChemTech Model 90 tabletop synthesizer was used for solid phase peptide synthesis. Fmoc amino acids and resins were purchased from Advanced Chemtech, NOVA Biochem, and Senn chemical companies. Other chemicals were purchased from Aldrich Company. Solvents were purchased from Mallinckrodt and Fisher Scientific Inc. The melting points were determined on a Fisher-Johns apparatus and are uncorrected. 1H NMR and 13C NMR spectra were recorded on a Varian Gemini 200 spectrometer at 200 MHz and 50 MHz. Chemical shifts are reported in ppm (δ) from tetramethylsilane as internal standard. TLC was performed on precoated silica gel F254 plate (Merck). Flash column chromatography was done by using Merck silica gel 60 (15-40
µm). HPLC were recorded on a Jasco 1580 series (PU 1580, UV 1575, LG 1580-54, DG 1580-54) and Waters 600 series (600 pump, 600 controller, 486 detector). HPLC columns were purchased from Vydis Company (Peptide & Protein C-18) and BIO-TEK instruments (PEAKMAX C-18). Mass spectra were committed to the KIST. Following abbreviation are used: ESI, electrospray ionization; TFA, trifluoroacetic acid; DIC, 1,3-diisopropylcarbodiimide; HOBt, 1-hydroxybenzotriazole; HBTU, O-benzotriazol-1-yl-N,N',N-triaminophosphonium hexafluorophosphate; LDH, lactate dehydrogenase; SEM, standard error means; HMTA, hexamethylenetetramine; Asp, aspartic acid; Met, methionine; Val, valine; z, benzylxycarbonyl; Fmoc, 9-fluorenlymethylxycarbonyl; fmk, fluoromethylketone; cHx, cyclohexyl; All, allyl; t-Bu, t-buty1; EA, ethyl acetate; DMF, N,N-dimethylformamide; DMA, N,N-dimethylacetamide; IPA, isopropyl alcohol; DMSO, dimethyl sulfoxide; TFE, tetrahydrofuran; DCM, dichloromethane; DPEA, diisopropylethylamine; EtN, triethylamine; CH3MgBr, methyl/magnesium bromide; TBDMS-OTf, tert-butyldimethylsilylchloromethanesulfonate; F-TEDA- BF3, 1-(chloromethyl)-4-fluoro-1,4-diazabicyclo[2.2.2]octane bis(tetrafluoroborate).

**General procedure of solid phase peptide synthesis**

1) Fmoc deprotection: Fmoc-AA-Resin was sealed into a reaction vessel (25 mL), and was swollen in DMA (2 min × 2). It was then washed twice with DMF and the Fmoc group was deprotected by 25% piperidine/DMF solution (7 min × 3).

2) Coupling: Fmoc-AA-OBt (activated amino ester) was prepared by mixing Fmoc-AA-OH, HOBt and DIC in DMA in the same equivalent amount. The mixture was stirred for 30 min before being used for coupling reaction. The deprotected resin was washed successively with DMA (20 mL × 3), IPA (20 mL × 2), and DCM (20 mL × 3). After drying the reaction vessel, the solution of Fmoc-AA-OBt (3.0 eq.) was added for coupling reaction. The mixture was shaken for 100 min at room temperature. The resin was washed with DMA (20 mL × 3), IPA (20 mL × 2), and DCM (20 mL × 3). Monitor coupling efficiency by Kaiser test.

3) Resin cleavage: The resin was cleaved off with TFA (92.5%), and water (2.5%) in the presence of scavengers (2.5% ethanedithiol and 2.5% trisopropylsilane) for 60 min.

**Synthesis**

TFA-H-Asp-Met-Val-Asp-OH salt (1). Fmoc-Asp(OtBu)-Wang resin (0.7 mmole/g, 100–200 mesh) was placed in a 25 mL reaction vessel, and the following steps were carried out: (1) deprotection of the Fmoc group with piperidine/DMF (1:4, V/V, 7 min × 3), (2) washing, DMA (20 mL × 4), IPA (20 mL × 2), and DCM (20 mL × 3), (3) coupling with activated ester which was prepared from the reaction of the corresponding Fmoc-amino acid (sequence, Fmoc-Val-OH, Fmoc-Met-OH, Fmoc-Asp(OtBu)-OH), HOBt (3.0 eq.), and DIC (3.0 eq.) in DMA (20 mL), and (4) washed with DMA (20 mL × 3), IPA (20 mL × 2), and DCM (20 mL × 3). The coupling time was 90 min, and sometimes, double coupling was applied. The completion of reaction was monitored by the Kaiser test. After coupling each residue to the resin step by step, the last Fmoc group was removed by the protocol described above. The resin was washed with DMA (20 mL × 4), IPA (20 mL × 2), and DCM (20 mL × 3). The desired peptide was cleaved off by 92.5% TFA, 2.5% water and scavengers (2.5% ethanedithiol and 2.5% trisopropylsilane) for 60 min. The resin was filtered and the TFA solution was concentrated under the reduced pressure at room temperature. The residue was diluted with 8–10 fold volumes of cold ether to give a white precipitate, which was then filtered and washed with cold ether three times to afford the title compound as a white solid. HPLC (0.1% TFA-water: 0.1% TFA-acetonitrile = 91:9, Rf = 5.7 min) showed the 95% purity. ESI MS [M+H]+ m/z 479.

Fmoc-Asp-Met-Val-Asp-OH (2). The compound was prepared by the similar reaction described for the synthesis of TFA-H-Asp-Met-Val-Asp-OH salt (1). White solid. HPLC (0.1% TFA-water: 0.1% TFA-acetonitrile = 65:35, Rf = 6.2 min) showed the 96% purity. ESI MS [M+H]+ m/z 701.

TFA-H-Asp(Oall)-Met-Val-Asp-OH salt (3). The compound was prepared by the similar reaction
described for the synthesis of TFA-H-Asp-Met-Val-Asp-OH salt (1). White solid. HPLC (0.1% TFA-water : 0.1% TFA-acetonitrile = 80 : 20, Rt = 4.0 min) showed the 97% purity. ESI MS [M+H]⁺ m/z 519.

TFA-H-Asp(OcHal)-Met-Val-Asp-OH salt (4). The compound was prepared by the similar reaction described for the synthesis of TFA-H-Asp-Met-Val-Asp-OH salt (1). White solid. HPLC (0.1% TFA-water : 0.1% TFA-acetonitrile = 76 : 24, Rt = 4.7 min) showed the 97% purity. ESI MS [M+H]⁺ m/z 519.

1-Butyl 3-benzyloxycarbonylamino-5-(methoxymethyleneamino)-4-oxobutanoate (6). To a solution of N-benzyloxycarbonyl aspartic acid (1.00 g, 2.93 mmol) in DCM/DMF (1:1, 20 mL) at 0 °C were added HBTU (1.30 g, 3.52 mmol) and DIPEA (0.620 mL, 3.52 mmol). After N,O-Dimethylhydroxylamine hydrochloride (0.340 g, 3.52 mmol) and DIPEA (0.620 mL, 3.52 mmol) were added to the solution, the resulting reaction mixture was stirred for 40 min, and then diluted with EA (30 mL). The organic phase was washed with saturated NaHCO₃ solution (40 mL x 2), water (40 mL x 3) and brine, dried over anhydrous MgSO₄, and concentrated to give a residue, which was recrystallized from ether/hexane to afford the title compound as a white solid (1.17 g, 90.0% yield). "H NMR (CDCl₃) δ 7.40~7.30 (s, 5H), 5.15~5.05 (s, 2H), 3.80~3.70 (s, 3H), 2.80~2.50 (m, 2H), 1.50~1.40 (s, 9H).

1-Butyl 3-benzyloxycarbonylamino-4-oxopentanoate (7). To a solution of 3 M CH₂MgBr in ether (10.0 mL, 30.0 mmol) diluted with anhydrous THF (50 mL) was added 1-butyl 3-benzyloxycarbonylamino-5-(methoxymethyleneamino)-4-oxobutanoate (2.00 g, 5.46 mmol) slowly at 0 °C. After the reaction mixture was stirred for 1 hr, it was quenched with the solution of NH₄Cl and H₂O (1:1, 40 mL), and then was diluted with EA (20 mL). The organic phase was washed with water (20 mL x 3), dried over anhydrous MgSO₄, and concentrated to give a residue, which was purified by column chromatography (EA/hexane=1:3) to afford the title compound as a colorless oil (1.45 g, 82.5% yield). "H NMR (CDCl₃) δ 7.40~7.30 (s, 5H), 5.20~5.10 (s, 2H), 4.50~4.40 (q, 1H), 3.60~2.60 (m, 2H), 2.30~2.20 (s, 3H), 1.50~1.30 (s, 9H). "C NMR (CDCl₃) δ 26.8, 28.0, 36.9, 57.0, 81.8, 127.9, 128.0, 128.4, 135.9, 155.8, 170.1, 205.6.

t-Butyl 3-benzyloxycarbonylamino-4-(t-butyldimethylsilyloxy)pent-4-enoate (8). To a solution of t-butyl 3-benzyloxycarbonylamino-4-oxopentanoate (1.00 g, 0.11 mmol) in DCM (20 mL) was added Et₃N (2 mL) followed by TBDMS-OTf (3.22 mL, 14.0 mmol) at -15 °C. After the reaction mixture was stirred for 40 min, it was diluted with EA (50 mL). The organic phase was washed with saturated NaHCO₃ solution (40 mL x 2), water (40 mL x 3) and brine, dried over anhydrous MgSO₄, and concentrated to give a residue, which was purified by column chromatography (EA:hexane=1:3, with 5 drops of Et₃N per 100 mL of solution) to afford the title compound as a yellow oil (0.70 g, 52% yield). "H NMR (CDCl₃) δ 7.45~7.30 (s, 5H), 5.20~5.10 (s, 2H), 4.55~4.40 (m, 1H), 4.40~4.10 (d, 2H), 2.65~2.50 (m, 2H), 1.50~1.40 (s, 9H), 1.00~0.85 (s, 9H), 0.30~0.10 (m, 6H).

1-Butyl 3-benzyloxycarbonylamino-5-fluoro-4-oxopentanoate (9). After the reaction of t-butyl 3-benzyloxycarbonylamino-4-(t-butyldimethylsilyloxy)pent-4-enoate (0.27 g, 0.62 mmol) and F-TEDA-Br (0.260 g, 0.74 mmol) in DCM (4 mL) was stirred for 3.5 hr at 0 °C, it was diluted with EA (30 mL). The organic phase was washed with saturated NaHCO₃ solution (40 mL x 2), water (40 mL x 3) and brine, dried over anhydrous MgSO₄, and concentrated to give a residue, which was purified by column chromatography (EA/hexane=1:4) to afford the title compound as a white oil (0.09 g, 44% yield). "H NMR (CDCl₃) δ 7.50~7.35 (s, 5H), 5.20~5.10 (s, 2H), 5.20~4.95 (m, 1H), 4.75~4.55 (m, 1H), 3.10~2.80 (m, 2H), 1.50~1.35 (s, 9H).

t-Butyl 3-nitropropionate (12). To a vigorously stirred suspension of anhydrous MgSO₄ (20.1 g, 168 mmol) in benzene (1.50 mL) was added concentrated H₂SO₄ (2.34 mL, 42.2 mmol). After the suspension was stirred for 15 min, 3-nitropropionic acid (5.13 g, 42.3 mmol) and tertiary butanol (20.1 mL, 210 mmol) were added. After the reaction mixture was sealed with stopper and stirred for 20 hr at
25 °C, it was quenched with saturated sodium bicarbonate solution (250 mL) and then stirred until all MgSO₄ dissolved. The organic layer was separated, washed with water (150 mL × 3) and brine, dried over anhydrous MgSO₄, and concentrated to afford the title compound as a yellow oil (6.58 g, 88.6% yield). ¹H NMR (CDCl₃) δ 4.65-4.35 (m, 2H), 3.70-3.50 (m, 1H), 2.50-2.35 (m, 2H), 1.50-1.40 (s, 9H); IR (cm⁻¹) 1740.4, 1153.9. ¹³C NMR (CDCl₃) δ 28.2, 39.3, 49.3, 71.7, 84.1, 85.8, 171.0.

Fmoc-Asp(OtBu)-Met-Val-Asp(OtBu)-fmk

(15). To a solution of Fmoc-Asp(OtBu)-Met-Val-Asp(OtBu)-fmk (0.074 g, 0.12 mmol) in DCM (2 mL) were added HOBT (0.016 g, 0.12 mmol) and DIC (0.018 mL, 0.12 mmol). After the reaction mixture was stirred for 40 min, r-butyl 5-fluoro-4-hydroxy-3-aminopentanoate (0.02 g, 0.09 mmol) was added. After the resulting mixture was stirred for 2 hr, it was diluted with EA. The organic phase was washed with saturated NaHCO₃ solution (10 mL × 2), water (10 mL × 3) and brine, dried over anhydrous MgSO₄, and concentrated to give a residue, which was purified by short column chromatography (EA:hexane = 2:1) to afford the title compound as a white solid.

HPLC (0.1% TFA-water : 0.1% TFA-methanol = 30 : 70, Rt = 5 min) showed the 94% purity, ESI MS [M+Na⁺] m/z 947.0.

Fmoc-Asp(OtBu)-Met-Val-Asp(OtBu)-fmk

(16). To a cooled (-78 °C) solution of oxalyl chloride (0.010 mL, 0.12 mmol) in DCM (5 mL) was added DMSO (0.014 mL, 0.24 mmol) slowly. The reaction mixture was stirred for 15 min and the solution of Fmoc-Asp(OtBu)-Met-Val-Asp(OtBu)-fmk (0.080 g, 0.10 mmol) in DCM (1 mL) was slowly added to the reaction mixture. After the reaction mixture was stirred for another 15 min, Et₃N (0.67 mL, 0.48 mmol) was added. Stirring was continued for another 2 hr by which time the temperature changed to room temperature. The reaction mixture was diluted with DCM (10 mL) and the organic layer was washed with water (20 mL × 3) and brine. The organic phase was dried over anhydrous MgSO₄, and concentrated to give a residue, which was purified by short column chromatography (EA:hexane = 1:3) to afford two isomers of the title compound. One (A) is a yellow liquid and the other (B) is a white solid (total yield 1.43 g, total % yield 97.24%), mp 76 °C (isomer B). ¹H NMR (CDCl₃) A: δ 5.05-4.90 (m, 1H), 7.25-7.30 (m, 9H), 3.25-3.30 (m, 1H), 2.90-2.75 (m, 2H), 2.20-2.15 (m, 1H), 1.50-1.35 (s, 9H); B: δ 5.00-4.80 (m, 1H), 4.65-4.35 (m, 2H), 3.20-3.00 (m, 1H), 2.90-2.70 (m, 2H), 1.50-1.25 (s, 9H); IR (cm⁻¹) 3466.9, 1736.0, 1558.3, 1374.0. ¹³C NMR (CDCl₃) δ 277.3, 31.9, 69.8, 81.9, 168.5.

r-Butyl 5-fluoro-4-hydroxy-3-aminopentanoate

(14). A solution of r-butyl 5-fluoro-4-hydroxy-3-aminopentanoate (2.2 g, 8.7 mmol) and Raney-Ni (50% slurry in water) in MeOH (40 mL) was hydrogenated (45 psi) for 14 hr. The solution was filtered through celite pad and then the solvent was evaporated. The residue was purified by column chromatography (methanol:DCM:hexane=1:2:2) to afford the title compound as a yellow oil (1.00 g, 51.7% yield). ¹H NMR (CDCl₃) δ 4.65-4.35 (m, 2H), 3.70-3.50 (m, 1H), 2.50-2.35 (m, 2H), 1.50-1.40 (s, 9H); IR (cm⁻¹) 1740.4, 1153.9. ¹³C NMR (CDCl₃) δ 28.2, 39.3, 49.3, 71.7, 84.1, 85.8, 171.0.
Fmoc-Asp(OtBu)-Met-Val-OH (17). The compound was prepared by the similar reaction described for the synthesis of TFA-H-Asp-Met-Val-Asp-OH salt (1). White solid. HPLC (0.1% TFA-water : 0.1% TFA-methanol = 30:70, Rt = 5 min) showed the 98% purity.

Cell Culture. By following the previously reported method, mouse neocortices were obtained from embryonic day 14 fetal brains and triturated. Dissociated cells were plated in 24-well culture plates precoated with poly-D-lysine (100 µg/mL) and laminin (4 µg/mL) in Eagle’s minimal essential medium (MEM, Earle’s salts, glutamine-free) supplemented with 21 mM glucose, 5% fetal bovine serum, 5% horse serum, and 2 mM glutamine. Plating density was adjusted to 5 hemispheres per plate (approximately 2.5×10⁶ cells per culture well). The cortical cell cultures were maintained at 37 °C in a humidified 5% CO₂ incubator. For co-cultures of neurons and glia, 10 µM AraC was included to cultures at DIV 7 when glial cells were confluent under death neurons. Two days later, cultures were shifted into a growth medium identical to the plating medium but lacking fetal bovine serum and then fed every other day.

Neurotoxicity experiment. Experiments were performed in cortical cell cultures (DIV 10-12). For apoptotic injury, cultures were continuously exposed to staurosporin or okadaic acid in MEM supplemented with 21 mM glucose. Neuronal death was analyzed 24 hr later by measuring levels of LDH released into bathing media and scaled to the mean LDH value released 24 hr after continuous exposure to 500 µM NMDA (100%) or a sham wash (0%).

Acknowledgements. This work was supported by the research fund of Dankook University in 2006.

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2008, Vol. 32, No 6