Inhibition of β-Amyloid Peptide-Induced Neurotoxicity by Benzothiazoles in Neuronal Cells

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Dedicated to my parents who have encouraged me.

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ABBREVIATIONS

Aβ : Beta amyloid

AD : Alzheimer’s disease

APP : Amyloid precursor protein

DA : Dopamine

ERK : Extracellular signal-regulated kinase

GABA : Gamma-aminobutyric acid

IL-1β : Interleukin-1β

iNOS : Inducible nitric oxide synthase

LDH : Lactate dehydrogenase

MAPK : p38 Mitogen-activated protein kinases

MTT : 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NO : Nitric oxide

PD : Parkinson's disease

ROS : Reactive oxygen species

TNF-α : Tumor necrosis factor-α

TH : Tyrosine hydroxylase

ABSTRACT

In this study, I have investigated the effects of KHG21834, a benzothiazole derivative, on beta amyloid ($A\beta_{25-35}$)-induced cell death in cultured rat pheochromocytoma (PC12) cells and rat cortical and mescencephalic neuron-glia cultures. KHG21834 reduced the $A\beta_{25-35}$-induced apoptotic death in PC12 cells determined by characteristic morphological alterations and positive *in situ* terminal end-labeling. The presence of 50 μM KHG21834 rescued PC12 cells by 82% from $A\beta_{25-35}$-induced cytotoxicity. In the cortical neuron-glia cultures, KHG21834 markedly reduced the $A\beta_{25-35}$-induced terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining. $A\beta_{25-35}$-caused reduction in microtubule associated protein-2 expression was effectively prevented in the presence of KHG21834. While the average dendrite length of tyrosine hydroxylase (TH)-immunoreactive neurons in the $A\beta_{25-35}$-treated mesencephalic neuron-glia cultures was 6% of that of cultures. The cultures treated with KHG21834 after $A\beta_{25-35}$ treatment was 79% of control. Western blot analysis of rat mesencephalic neuron-glia cultures showed that $A\beta_{25-35}$ decreased the expression of TH protein by 60% and KHG21834 significantly attenuated the $A\beta_{25-35}$-induced reduction of the TH expression. KHG21834 also effectively attenuated $A\beta_{25-35}$-induced decrease in [^{3}H] gamma-aminobutyric acid uptake of cortical neuron-glia cultures and in

[^{3}H] dopamine uptake of mesencephalic neuron-glia cultures. Moreover, treatment of KHG21834 blocked Aβ-induced extracellular signal-regulated kinase1/2 (ERK1/2) phosphorylation. ERK1 was more sensitively affected than ERK2 in attenuation of $A\beta_{25-35}$-induced phosphorylation by KHG21834. These results demonstrated that KHG21834 were capable of protecting cortical and mesencephalic neurons from $A\beta_{25-35}$-induced degeneration.

Key words: benzothiazole, β-amyloid, PC12 cells, mescencephalic neurons, cortical neurons, extracellular signal-regulated kinase

Ⅰ. INTRODUCTION

Aging is a major risk factor for most of the dementing disorders predominantly represented by Alzheimer's disease (AD). The estimated number of AD patients in the world is approximately 20-25 million and is increasing as the world population ages. More than one out of every two people older than 85 years suffer from either dementia or mild cognitive impairment, a prodrome of dementia (1,2).

The clinical symptoms are progressive impairment in memory, judgment, decision making, orientation to physical surroundings, and language. Diagnosis is based on neurologic examination and the exclusion of other causes of dementia; a definitive diagnosis can be made only at autopsy. The main components of the two principal hallmarks of AD, senile plaques (typically bearing β-amyloid) and neurofibrillary tangles (mainly composed of tau protein), have helped produce hypotheses about the pathogenesis of AD that no doubt come close to reflecting the real situation (3).

AD is a multifactorial syndrome linked to abnormal metabolism of the transmembrane protein, amyloid precursor protein (4). The extracellular deposits known as neuritic plaques, a typical nuropathological feature in AD brains, contain large amounts of β-amyloid (Aβ), which is generated from amyloid precursor protein (APP) (5). In neurons, newly synthesized APP

follow the secretory pathway and is targeted into axons by fast axonal transport (6). A fraction of full-length APP reaches the plasma membrane and re-enters the cell via endocytosis (7,8). APP is degraded by several proteases, among which the catabolism reactions of β- and γ-secretases on APP lead to the production of excess Aβ peptides leading to the formation of Aβ aggregates. The excessive burden of Aβ, produced by various normal or abnormal mechanisms, may represent the starting point of neurodegenerative events. Formation of Aβ aggregates in the brain is now considered, which produces various toxic effects in neuronal cells leading to the formation of neuritic plaques (9,10).

Aβ forms fibrils that aggregate and form deposits comprising the core of senile plaques. Considerable genetic evidence has implicated Aβ in AD pathogenesis (11). Aβ formation is to cause nerve cell degeneration in AD, Both *in vitro* and *in vivo* studies have been demonstrated neurotoxic effects of Aβs including induction of apoptosis of neuronal cells. For example, exposure of cultured central nerve system neurons and neuronal PC12 cells to Aβ induces apoptotic neuronal cell death (12,13). Aβ exhibited significantly enhanced neurotoxicity toward mesencephalic or cortical neurons in the prescence of microglia. The elevated neurotoxicity was attributed to the activation of microglia and production of superoxide free radicals. Inflammation in the brain has increasingly been recognized to play an

important role in the pathogenesis of several neurodegenerative disorders, including Parkinson's disease (PD) and AD. Inflammation-mediated neurodegeneration involves activation of the brain's resident immune cells, the microglia, which produce proinflammatory and neurotoxic factors, including cytokines, reactive oxygen intermediates, nitric oxide, and eicosanoids that impact on neurons to induce neurodegeneration (14,15). Previous studies demonstrates a functional linkage between β-amyloid-dependent activation of microglia and several characteristic markers of neuronal death occurring in AD brains (16).

One therapeutic approach for treatment of the neurodegenerative disease is the use of neurotrophic factor to promote the survival and growth of dopaminergic neurons. The ultimate goal is to slow or halt neuronal degeneration at an early stage in order to preserve existing dopaminergic neurons and possibly to stimulate compensatory growth in these same cells (17, 18).

It has been reported that benzothiazole derivatives are effective treatment drug of neurodegenerative diseases (19-22). Benzothiazoles are highly interesting molecules for drug development, because they already have been shown to be useful for treating cerebrovascular, cadiovascular and neurodegenerative disorders. Benzothiazoles deriviates were identified as huntingtin aggregation inhibitors in a high-throughput *in vitro* screen and

subsequently PGL-135(2-amino-4,7-dimethylbenzothiazole) and riluzole (2-amino-6-trifluoromethoxy benzothiazole) were shown to inhibit huntingtin aggregation in cell culture and a neuroprotective agent *in vitro* and *in vivo* which prolongs survival in patients with neurodegenerative diseases such as amyotrophic lateral sclerosis (21). It potently blocks glutamate neurotransmission both at pre- and postsynaptic levels (23). Riluzole was particularly effective at blocking protein tyrosine phosphorylation stimulated by NMDA in the hippocampus. It also tested for therapy of huntington's disease (HD) patients, where treatment with riluzole has positive effects on choreatic hyperkinesia (24-26).

New therapeutic strategy for treating ischemic heart disease drug AS601245(1,3-benzothiazol-2-yl(2-{[2-(3-pyridinyl)ethyl]amino}-4-pyrimidinyl)acetonitrile) during myocardial ischemia and reperfusion significantly reduces myocardial apoptosis associated with JNK activation and improves postischemic cardiac functional recovery (27). The benzothiazole derivative AS601245 also has shown beneficial effects in models of global and focal brain ischemia (28). The new radioiodinated benzothiazole ligand might be useful as a surrogate marker for the *in vivo* quantitation of amyloid deposition in human brain for use with either positron emission tomography or single photon emission computed tomography (29). A recently published Paper indicated that 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole (5F

203, NSC 703786) exhibits selective and potent anticancer activity, and its lysylamide prodrug (Phortress, NSC 710305) entered Phase I clinical trialsin the United Kingdom (30). And, 2-(3,4-Dimethoxyphenyl)-5-fluorobenzothiazole (GW 610, NSC721648), a simple fluorinated 2-arylbenzothiazole, shows potent and selective inhibitory activity against lung, colon, and breast cancer cell lines (31). The activation of the tumor suppressor gene p53 plays a crucial role in regulating the death of neurons in vitro, following apoptotic stimuli involving molecules such as glutamate or DNA-damaging agents. The involvement of p53 in neuronal death raises the possibility that p53 inhibitors might prove effective in suppressing the degenerative processes in neurodegenerative disorders. The antiparasitic compound, PFT-α has been identified for its ability to protect cells against radiation, excitotoxic, and amyloid-induced neuronal death (32).

Although the detailed mechanism of the drugs is not fully understood, it has been suggested that the benzothiazole derivatives may react as a tyrosine kinase inhibitor (21,33-35). Tyrosine-specific protein kinase activities have been shown to be involved in the control of cell growth and differentiation. One of the main molecular mechanisms involved is the autophosphorylation of receptor tyrosine kinases in response to ligand binding (36,37). Tyrosine kinases seem also to be important in other phenomena such as ligand-induced internalization of antigen receptor in B lymphocytes, long-term potentiation in

the hippocampus, clustering of acetylcholine receptors and regulation of N-methyl-D-aspartate receptors (33,38-40). A simple way to check the involvement of these enzymes is to use specific inhibitors. This suggests that some neuronal elements involved in AD pathology may be recapitulating a developmental profile or, alternately, that elevated phosphotyrosine levels may reflect a role for tyrosine kinase/phosphatase systems in the degeneration process directly. Cells in the neuritic plaque which strongly resemble microglia also contain elevated levels of phosphotyrosine compared to non-activated ramified microglia in the same tissue section. Thus, tyrosine phosphorylation systems may be involved in the response of microglia to degeneration in AD pathology (33).

In the present study, to find more effective neuroprotective drugs against to the Aβ-induced cell death, more than 30 benzothiazole derivatives have been examined in cultured PC12 cells and mesencephalic and cortical neuron-glia cultures. The results of the present study have suggested that KHG21834, a benzothiazole derivative, is capable of neuroprotection against to the $A\beta_{25-35}$-induced degeneration of neuronal cells.

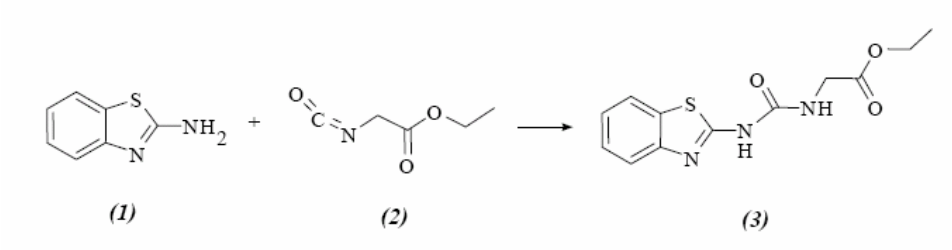
Ⅱ. MATERIALS AND METHODS

1. Reagents

β-Amyloid ($A\beta_{25-35}$), poly-D-lysine, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). $A\beta_{25-35}$ was dissolved in sterile deionized and distilled water as a stock solution (1 mM) and stored in aliquots at –70°C. For treatment, the $A\beta_{25-35}$ stock solution was diluted to the desired final concentrations in treatment medium. Laminin, RPMI 1640, Minimum Essential medium (MEM), fetal bovine serum and horse serum were obtained from Invitrogen (Carlsbad, CA, USA). Hanks' balanced salt solution (HBSS) was supplied by Sigma Chemical Co. Anti-p-ERK monoclonal and anti-ERK polyclonal antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and anti-β-actin monoclonal antibody was obtained from Sigma. Anti-tyrosine hydroxylase polyclonal antibody was obtained Calbiochem (San Diego, CA, USA). Anti-MAP2 monoclonal antibody was obtained Abcam (Cambridge, UK). [^{3}H] gamma-aminobutyric acid (GABA) (90 Ci/mmol), [^{3}H] dopamine (DA) (30 Ci/mmol) and were from PerkinElmer Life Sciences (Boston, MA).

2. Synthesis of KHG21834

More than 30 drugs including benzothiazole and riluzole derivatives such as KKK01127, KSC00024, KHG21834, KKJ00343 were kindly provide by Dr. Hoh-Gyu Hahn in KIST (Seoul, Korea). Briefly mentioned, a solution of 2-aminobenzothiazole (***1***) (1.5 g, 0.01 M) and ethyl isocyanatoacetate (***2***) (1.14 ml, 0.01 M) dissolved in ethyl alcohol (20 ml) was refluxed over 2 h (Scheme 1). The reaction mixture was cooled to room temperature and the precipitates were collected by filtration to afford the urea (***3***) as a white solid (1.47 g, 52%). Chemical properties of KHG21834 are follows; mp 282^{o}C, ^{1}H NMR (DMSO-d_6, 300MHz) δ 1.21 (t, J = 7.10 Hz, 3H, ethyl-CH_3), 3.96 (d, J = 5.8 Hz, CH_2), 4.11 (q, J = 7.10 Hz, 2H, ethyl-CH_2), 6.95 (br s. 1H, NH), 7.08-7.89 (m, 4H ArH), 11.1 (br s. 1H, NH). Drugs were freshly prepared as stock solutions (10 mM) in dimethyl Sulfoxide (DMSO) and diluted to the desired final concentrations in treatment medium.



Scheme 1. Synthesis of KHG21834

3. PC12 cell cultures

PC12 cells were maintained routinely in RPMI 1640 supplemented with 10% heat-inactivated horse, 5% fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The medium was changed every three days, and cells were plated at an appropriate density according to each experimental scale.

4. Determination of cell viability

PC12 cells were plated at a density of 5×10^4 cells in 96-well plates, and cell viability was determined by the conventional MTT reduction assay and lactate dehydrogenase (LDH) assay. The MTT assay relies primarily on mitochondrial metabolic capacity of viable cells and reflects the intracellular redox state. After incubation, cells were treated with MTT solution (final concentration, 1 mg/ml) for 4 h. The dark blue formazan crystals formed in intact cells were solubilized with lysis buffer and absorbance at 595 nm was measured with microplate reader. LDH activity released from the culture medium was measured and evaluated as an index of cell death. The culture medium was added to a test containing sodium lactate, NADH, and sodium pyruvate, after which the decrease in absorbance at 340 nm caused by NADH was measured. LDH activity in the homogenate from the untreated control was taken as 100%.

5. Rat mesencephalic neuron-glia cultures

Primary mesencephalic neuron-glia cultures were prepared from the brains of embryonic day 14 Sprague-Dawley rat following previously described protocol (41). Briefly, mesencephalic tissues were obtained and dissociated by a mild mechanical trituration. Cells were mechanically dissociated by pipetting with pasteur pipette. Cells were seeded at 7×10^5 cells to 6-well culture plate pre-coated with poly D-lysine (20 μg/ml) and laminin (1 μg/ml) and maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air in 3 ml/well maintenance medium. The medium consisted of minimum essential medium containing 20% heat-inactivated fetal bovine serum, 1 g/l glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μM nonessential amino acids, 50 U/ml penicillin and 50 μg/ml streptomycin. Five-day-old cultures were used for treatment. At the time of treatment, the composition of the mesencephalic neuron-glia cultures was 48% astrocytes, 11% micorglia, 40% neurons, and 0.8% dopaminergic neurons.

6. Rat cortical neuron-glia cultures

Rat cortical neuron-glia were prepared from the brains of embryonic day 16/17 Sprague-Dawley rats. All cell suspensions were made in serum-free Neurobasal (Nb) medium (Gibco, Gaithersburg, MD) supplemented with B27 (Gibco), 0.5 mM L-glutamine (Sigma), and antibiotic/antimycotic (Gibco).

Two protocols were used to test the effect of serum-free medium on excitotoxicity: 1) cells plated in Nb medium with 25 μM glutamate; 2) cells plated in 4-well chamber slides switched to Nb medium after 4 days *in vitro*. Cells in Nb medium were fed twice per week by exchanging half of the medium with fresh Nb/B27 without glutamate. Cells were plated at $5 \times 10^5/cm^2$ in poly D-lysine (20 μg/ml) and laminin (1 μg/ml)-coated multiwell plates or chamber slides and maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air atmosphere. Twelfth-day cultures were used for treatment. The composition of cortical neuron-glia cultures was determined by immunostaining with antibodies against MAP2. Cortical neuron-glia cultures contained 60% Neu-N-IR neurons and 15% MAP2 microglia. The remaining cells were presumed to be astroglia.

7. Confocal laser scanning fluorescence microscopy

Primary mesencephalic neuron-glia cells seeded at 3×10^6 cells to Lab-Tek Chambered Coverglass (Nunc, IL, USA) precoated with poly D-lysine (20 μg/ml) and laminin (1 μg/ml). Dopaminergic neurons were stained with an antibody against TH, the rate limiting enzyme in dopamine synthesis. After exposing $A\beta_{25-35}$ and drug, cell culture media was removed and the neurons were fixed by incubating with 4% paraformaldehyde in PBS (pH 7.5) for 10 min at room temperature. The coverslips were washed three min three times

with PBS followed by incubation for 1h at room temperature in blocking buffer. Afterwards the coverslips were incubated overnight at 4°C with the primary antibodies diluted in blocking buffer. The coverslips were washed three min three times with PBS and incubated for 1 h at room temperature with the secondary antibodies diluted in blocking buffer solution goat anti-rabbit antibody conjugated with Alexa Fluor 488 or anti-mouse antibody conjugated with Alexa Fluor 546 (Molecular Probes, Eugene, OR, USA). The coverslips were mounted on microscope slides and stored in the dark at 4°C until examination. The fluorescence microscopy imaging was performed using Zeiss 510 Meta confocal laser scanning microscope (LSM 510 META).

8. Western blotting

$A\beta_{25–35}$-treated cells (5×10^4 cells/3 ml in 6-well) were collected and washed with phosphate-buffered saline (PBS). After centrifugation, cell lysis was carried out at 4 °C by vigorous shaking for 15 min in 0.1 ml of RIPA buffer 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 7.4), 50 mM glycerophosphate, 20 mM NaF, 20 mM EGTA, 1 mM DTT, 1 mM Na_3VO_4, and protease inhibitors. After incubation for 10 min on ice, the lysate was cleared by centrifugation at 14,000 rpm 5 min. Protein (20 μg) from cell lysates was boiled and electrophoersed under reducing conditions in 12% polyacrylamide gels. Proteins were then

transferred to a nitrocellulose membrane. Nonspecific binding was inhibited by incubation in blocking buffer of 20 mM Tris, pH 7.4-buffered saline with 0.1% Tween 20 (TBST) containing 5% nonfat dried milk for 1 h. Primary antibodies anti-ERK1, anti-ERK2, anti-phospho-ERK1, and anti-phospho-ERK2 exposed to membranes for 90 min. After washing, the blots were incubated for 1 h with horseradish peroxidase-conjugated anti-mouse IgG at a dilution of 1:10,000 and detected with the chemiluminescent substrate (PIERCE) for 1 min according to the manufacturer's instructions and visualized with X-ray film.

9. DNA fragmentation

Detection of DNA fragmentation was performed using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method. Detection of DNA fragmentation was also performed using the apoptosis detection system (Promega, Madison, WI).

10. GABA uptake and DA uptake assays

[^{3}H] GABA or [^{3}H] DA uptake assays were performed as previously described (41,13). Briefly mentioned, after washing twice with warm Krebs-Ringer buffer containing 16 mM sodium phosphate, 119 mM NaCl, 4.7 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.3 mM EDTA, and 5.6 mM glucose;

pH 7.4, cultures were incubated for 20 min at 37°C with 1 μM [^{3}H] GABA or 5 μM [^{3}H] DA in Krebs-Ringer buffer for GABA uptake or DA uptake, respectively. Afterward, cultures were washed three times with ice-cold Krebs-Ringer buffer and cells were then collected in 1 N NaOH. Radioactivity was determined by liquid scintillation counting. Nonspecific GABA or DA uptake observed in the presence of 10 μM mazindol or 10 μM 1-(2-[([diphenylmethylene] imino) oxy] ethyl)-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid was subtracted.

11. Statical analysis

Changes in cell viability were analyzed by ANOVA, followed by the student's t-tests. A *p*-value less than 0.05 were considered statistically significant.

Ⅲ. RESULTS

1. KHG21834 attenuated Aβ$_{25-35}$-induced neurodegeneration in PC12 cells

Changes in cell morphology were assessed by microscopic examination. PC12 cells treated for 48 h with Aβ$_{25-35}$ at the concentration of 50 μM exhibited morphological alterations such as cell shrinkage and membrane blebbings that are normally associated with the occurrence of apoptotic cell death. Compared to the normal PC12 cells (Fig. 1A), cells treated with Aβ$_{25-35}$ became round up, detached from the bottom, and aggregated as assessed by phase-contrast microscopy (Fig. 1B). KHG21834 post-treatment mitigated such morphological features of damaged cells (Fig. 1C). The effects of KHG21834 on cell survival following exposure to Aβ$_{25-35}$ were then examined by the MTT reduction assay. Cell survival was decreased by 52% after incubation of PC12 cells for 48 h with 50 μM Aβ$_{25-35}$ and the presence of 50 μM KHG21834 rescued PC12 cells by 82% from Aβ$_{25-35}$-induced cytotoxicity (Fig. 1D). In addition to the determination of the effect of post-treatment with KHG21834 on the Aβ$_{25-35}$-induced neurodegeneration in PC12 cells, the effect of pre-treatment was also examined and very similar significant neuroprotection was observed in cultures with KHG21834 before the addition of Aβ$_{25-35}$ (data not shown).

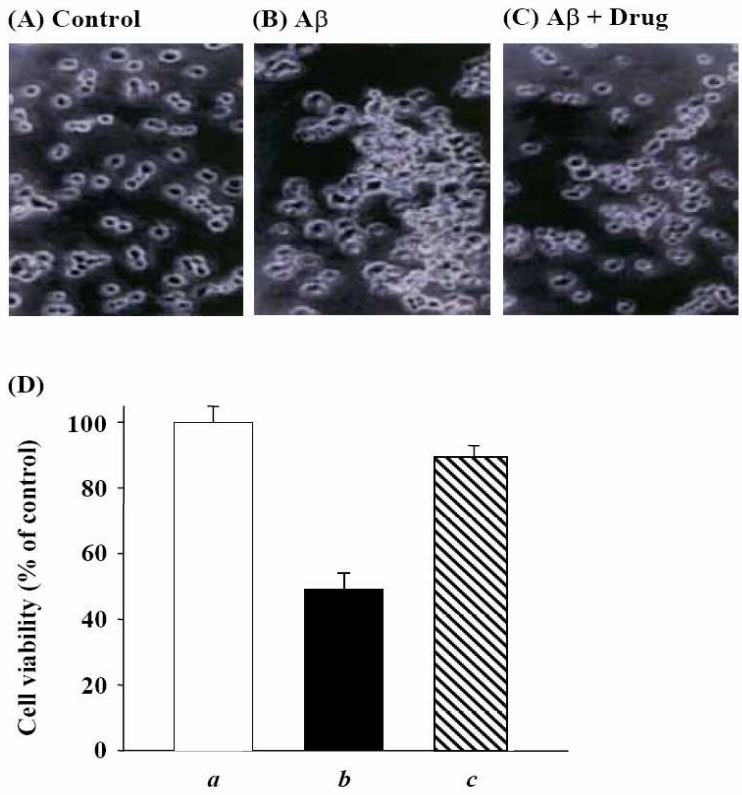


Fig. 1. Protective effect of KHG21834 on $A\beta_{25-35}$-induced cytotoxicity in PC12 cells. PC12 cells were pretreated with 50 μM $A\beta_{25-35}$ for 48 h. The $A\beta_{25-35}$ treated cells were further treated with 50 μM KHG21834 for 48 h. (A)~(C) Microscopic analysis; (D) cell viability by MTT reduction assay in (*a*) control, (*b*) $A\beta_{25-35}$, (*c*) $A\beta_{25-35}$ with KHG21834.

The effects of KHG21834 on the $A\beta_{25-35}$-induced degeneration of PC12 cells were further examined by terminal endlabeling (TUNEL staining), which is widely used in detecting DNA fragmentation in situ. In this histochemical technique, the appearance of intensely stained nucleus is indicative of terminal incorporation of labeled dUTP into the 3'-end of fragmented DNA derived from apoptotic nuclei. The effect of KHG21834 was also expressed as the percent in the number of positive neurons in the KHG21834 treated groups when compared to the control group. Results from the $A\beta_{25-35}$-treated rats were assigned a value of 100%. Exposure to 50 μM $A\beta_{25-35}$ caused widespread TUNEL staining in the PC12 cells, while no significant TUNEL staining was seen in the control group, indicating that $A\beta_{25-35}$-induced neuronal cell death proceeds via apoptotic cell death (Fig. 2A and Fig. 2B). Interestingly, KHG21834 treatment markedly reduced the $A\beta_{25-35}$-induced TUNEL staining up to 2.5-fold (Fig. 2C and Fig. 2D). Similar effects of KHG21834 on the $A\beta_{25-35}$-induced neuronal cell death were also observed in DNA ladder formation. As shown in Fig. 2E, the exposure to $A\beta_{25-35}$ caused DNA ladder formation and the $A\beta_{25-35}$-induced apoptosis was effectively prevented by the addition of KHG21834. These results indicate that KHG21834 attenuates $A\beta_{25-35}$-induced apoptotic cell death in PC12 cells.

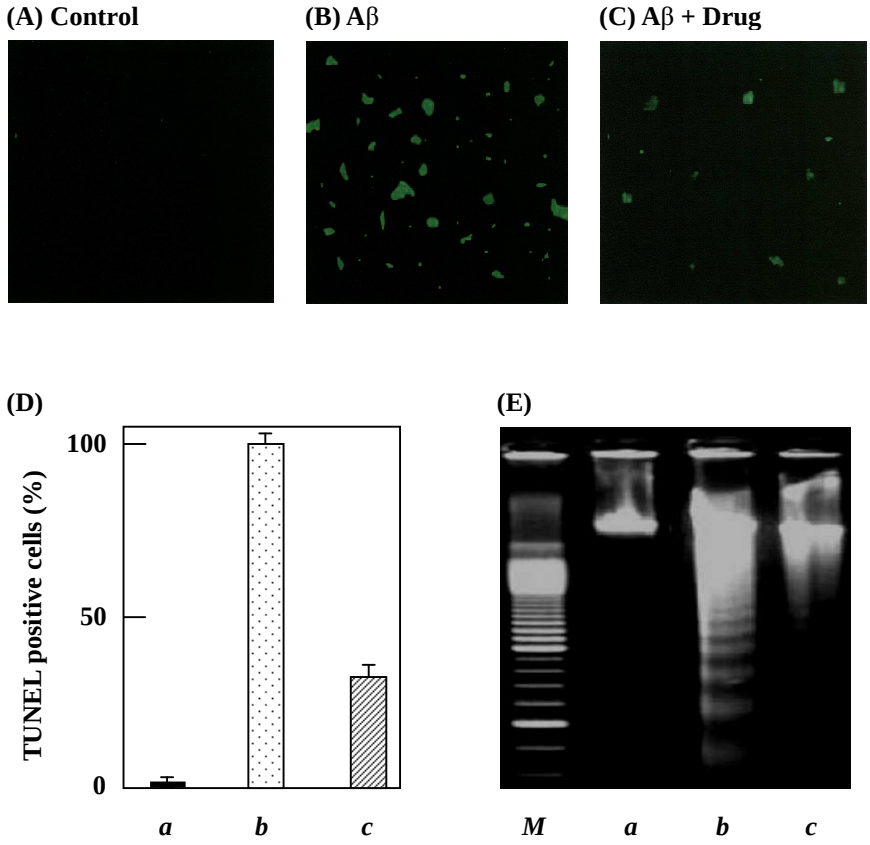


Fig. 2. Protective effect of KHG21834 on $A\beta_{25-35}$-induced TUNEL-labeling in PC12 cells. PC12 cells were pretreated with 50 μM $A\beta_{25-35}$ for 48 h. The $A\beta_{25-35}$ treated cells were further treated with 50 μM KHG21834 for 48 h. TUNEL-labeling in **(A)** control, **(B)** $A\beta_{25-35}$, and **(C)** $A\beta_{25-35}$ with KHG21834. Quantitation of TUNEL positive neurons carried out at a magnification of 400X **(D)** and DNA fragmentation **(E)** in (*a*) control, (*b*) $A\beta_{25-35}$, (*c*) $A\beta_{25-35}$ with KHG21834, and (*M*) 1 Kb plus DNA ladder (Gibco/BRL).

2. Effect of KHG21834 on $A\beta_{25-35}$-induced neurodegeneration of cortical and mesencephalic neuron-glia cultures

The neurotoxic effects of $A\beta_{25-35}$ have been reported in neuron-enriched cultures and neuron-glia cultures prepared from both mesencephalon and cortex (15,42,43). In this study, we have examined effects of KHG21834 on the $A\beta_{25-35}$ induced degeneration of cortical and mesencephalic neuron-glia cultures. Exposure to 50 μM $A\beta_{25-35}$ for 7 days caused widespread TUNEL staining in the cortical neuron-glia cultures, while no TUNEL staining was observed in the control group (Fig. 3A and Fig. 3B). When the $A\beta_{25-35}$ treated cells were further treated with 50 μM KHG21834 for 7 days, KHG21834 markedly reduced the $A\beta_{25-35}$-induced TUNEL staining (Fig. 3C). Effects of KHG21834 on the $A\beta_{25-35}$-induced neuronal cell death were also observed in microtubule associated protein-2 (MAP-2) staining. The exposure to $A\beta_{25-35}$ caused reduction in MAP-2 expression and the $A\beta_{25-35}$-induced neurodegeneration was effectively prevented by the addition of KHG21834 (Fig. 3D~3F). Results of cell viabilities determined using the LDH release assay in cortical neuron-glia cultures further suggest the protective function of KHG21834 on the $A\beta_{25-35}$-induced neuronal cell death (Fig. 3G).

For the studies of protective effects of KHG21834 in mesencephalic

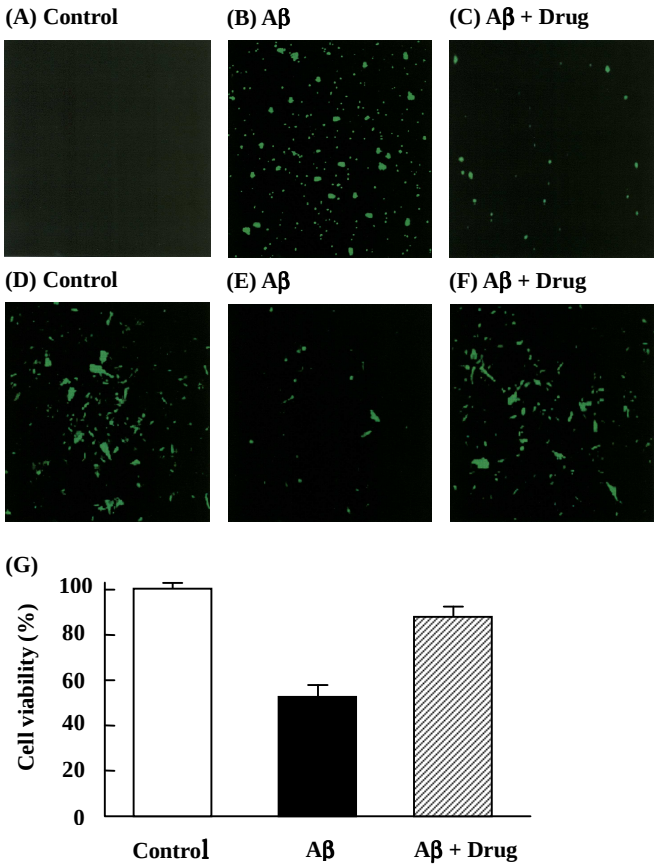


Fig. 3. Effect of KHG21834 on $A\beta_{25-35}$-induced degeneration of rat cortical neuron-glia cultures. Cortical neuron-glia cultures were pretreated with 50 μM $A\beta_{25-35}$ for 7 days. The $A\beta_{25-35}$ treated cells were further treated with 50 μM KHG21834 for 7 days. TUNEL-labeling and MAP-2 staining of $A\beta_{25-35}$-induced neurotoxicity in cortical neuron-glia cultures were performed. The images shown are representative of three experiments. TUNEL-labeling in (A) control, (B) $A\beta_{25-35}$, (C) $A\beta_{25-35}$ with KHG21834. (D)~(F) MAP-2 staining of (A)~(C), respectively. (G) Viable cells were determined using the LDH release assay and expressed as percent of control.

neuron-glia cultures, cells were pretreated with 50 μM $A\beta_{25-35}$ for 48 h. The $A\beta_{25-35}$ treated cells were further treated with 50 μM KHG21834 for 48 h.

Immunocytochemical analysis of $A\beta_{25-35}$-induced neurotoxicity in mesencephalic neuron-glia cultures with anti-TH antibody showed a typical culture of TH-immunoreactive neurons (Fig. 4A). The cells appeared with a bipolar or multipolar morphology, with long processes. When the cultures were exposed to 50 μM $A\beta_{25-35}$, the number of TH-immunoreactive cells was significantly reduced and the remaining ones had lost most of the elaborate dendrite network (Fig. 4B). However, TH-immunoreactive neurons in cultures treated with 50 μM KHG21834 after treatment of 50 μM $A\beta_{25-35}$ were markedly more healthy and had better preserved dendrite network than those in cultures treated with 50 μM $A\beta_{25-35}$ alone (Fig. 4C). Morphologically, in addition to the reduction in abundance of TH-immunoreactive neurons, the dendrites of the remaining TH-immunoreactive neurons in the $A\beta_{25-35}$-treated cultures was significantly less elaborate than that of the control cultures (Fig. 4D~4E). In cultures pretreated with KHG21834 after $A\beta_{25-35}$ treatment, TH-immunoreactive neurons were significantly more numerous with the TH-immunoreactive dendrites less affected compared with the $A\beta_{25-35}$-treated cultures (Fig. 4F). Although the average dendrite length of TH-immunoreactive neurons in the $A\beta_{25-35}$-treated cultures was 6% of that of control cultures, that of the cultures treated with KHG21834 after $A\beta_{25-35}$

treatment was 79% of control cultures. Similar results were also observed in cell viability determined using the LDH release assay (Fig. 4G). Finally, the Western blot analysis of rat mesencephalic neuron-glia cultures showed that $A\beta_{25-35}$ decreased the expression of TH protein by 60% and KHG21834 significantly attenuated the $A\beta_{25-35}$ induced reduction in the expression of TH (Fig. 4H).

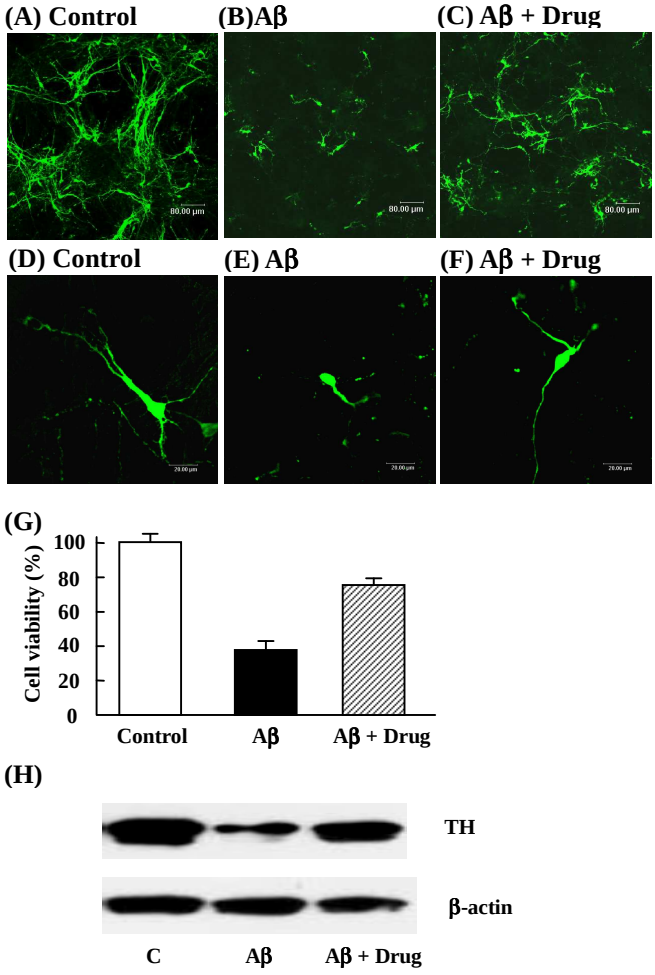


Fig. 4. Effect of KHG21834 on $A\beta_{25-35}$-induced degeneration of rat mesencephalic neuron-glia cultures. Mesencephalic neuron-glia cultures were pretreated with 50 μM $A\beta_{25-35}$ for 48 h. The $A\beta_{25-35}$ treated cells were further treated with 50 μM KHG21834 for 48 h. Immunocytochemical analysis of $A\beta_{25-35}$-induced neurotoxicity in mesencephalic neuron-glia cultures with anti-TH antibody was performed. The images shown are representative of three experiments. (A) & (D) control, (B) & (E) $A\beta_{25-35}$, (C) & (F) $A\beta_{25-35}$ with KHG21834. (G) Viable cells from (A)~(C) were determined using the LDH release assay and expressed as percent of control. (H) Western blot analysis of TH expression in cultures from (A)~(C).

3. Effect of KHG21834 on $A\beta_{25-35}$-induced decrease in GABA uptake of cortical neurons and in DA uptake of dopaminergic neurons

The $A\beta_{25-35}$-induced neurodegeneration was further examined by measuring GABA and DA uptake in the cortical and mesencephalic neuron-glia cultures, respectively. To characterize the dose and time dependence of $A\beta_{25-35}$-induced degeneration of cortical neurons, rat primary cortical neuron-glia cultures were treated for 12 days with 15 to 75 μM $A\beta_{25-35}$ or with 50 μM $A\beta_{25-35}$ for 3 to 15 days. As shown in Fig. 5A, $A\beta_{25-35}$-induced degeneration of cortical neurons was concentration-dependent. At the highest concentration used (75 μM), $A\beta_{25-35}$ resulted in a 55% decrease in [^{3}H] GABA uptake. The $A\beta_{25-35}$-induced cortical neurodegeneration was also time dependent. In cultures treated with 50 μM $A\beta_{25-35}$, significant decrease in [^{3}H] GABA uptake was observed 9 days after treatment and by 15 days, a 50% reduction in [^{3}H] GABA uptake was observed (Fig. 5B). When cortical neuron-glia cultures were treated with $A\beta_{25-35}$ in the presence of KHG21834, significantly attenuation $A\beta_{25-35}$-induced decrease in GABA uptake were observed at dose- and time-dependent manner (Fig. 5A and 5B). GABA uptake of cultures co-treated with 50 μM KHG21834 was 80% of that of control cultures. These results are consistent with those obtained in Fig. 3.

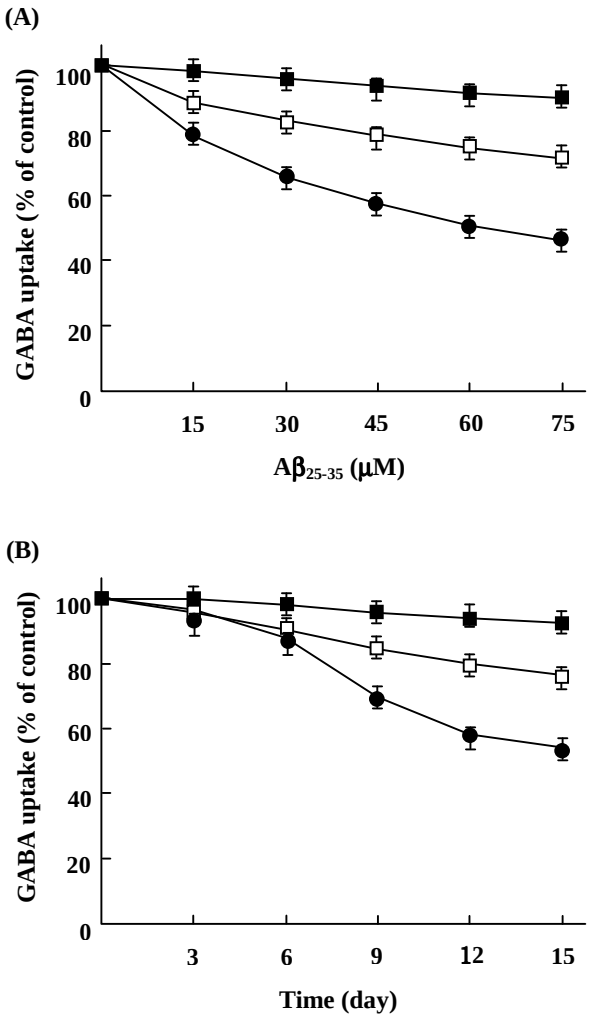


Fig. 5. Effect of KHG21834 on $A\beta_{25-35}$-induced decrease in GABA uptake of cortical neuron-glia cultures. Cultures were treated for 12 days with vehicle or indicated concentrations of $A\beta_{25-35}$ (A) or with 50 μM $A\beta_{25-35}$ for the indicated number of days (B) in the presence and absence of KHG21834. Afterwards, GABA uptake was performed as described under Materials and Methods. Results are expressed as a percentage of the control cultures and are mean ± S.E.M. of three experiments performed in triplicate. (●) 0 μM KHG21834; (□) 25 μM KHG21834; (■) 50 μM KHG21834

Next, we examined the effect of $A\beta_{25-35}$, on the degeneration of dopaminergic neurons in the mesencephalic neuron-glia cultures. As shown in Fig. 6, treatment of cultures for 9 days with 15 to 75 μM $A\beta_{25-35}$ or with 50 μM $A\beta_{25-35}$ for 3 to 15 days resulted in dose- and time-dependent decrease in DA uptake. Significant damage to dopaminergic neurons was observed in cultures treated with 50 μM $A\beta_{25-35}$ for 9 days (Fig. 6A). Furthermore, $A\beta_{25-35}$-induced dopaminergic neurodegeneration was also time dependent. In cultures treated with 50 μM $A\beta_{25-35}$, significant decrease in [^{3}H] DA uptake was observed 6 days after treatment and by 15 days, a 60% reduction in [^{3}H] DA uptake was observed (Fig. 6B). When mesencephalic neuron-glia cultures were treated with $A\beta_{25-35}$ in the presence of KHG21834, significantly attenuation $A\beta_{25-35}$-induced decrease in DA uptake were observed at dose- and time-dependent manner (Fig. 6A and 6B). DA uptake of cultures co-treated with 50 μM KHG21834 was 72% of that of control cultures. These results further support the neuroprotective effects of KHG21834 shown in the immunocytochemical analysis of dopaminergic neurons (Fig. 4). Therefore, taken together with the results of immunocytochemical studies and GABA and DA uptake analysis, it has been suggested that treatment with $A\beta_{25-35}$ is capable of inducing the degeneration of cortical and dopaminergic neurons and KHG21834 effectively can attenuate the $A\beta_{25-35}$-induced neurotoxicity.

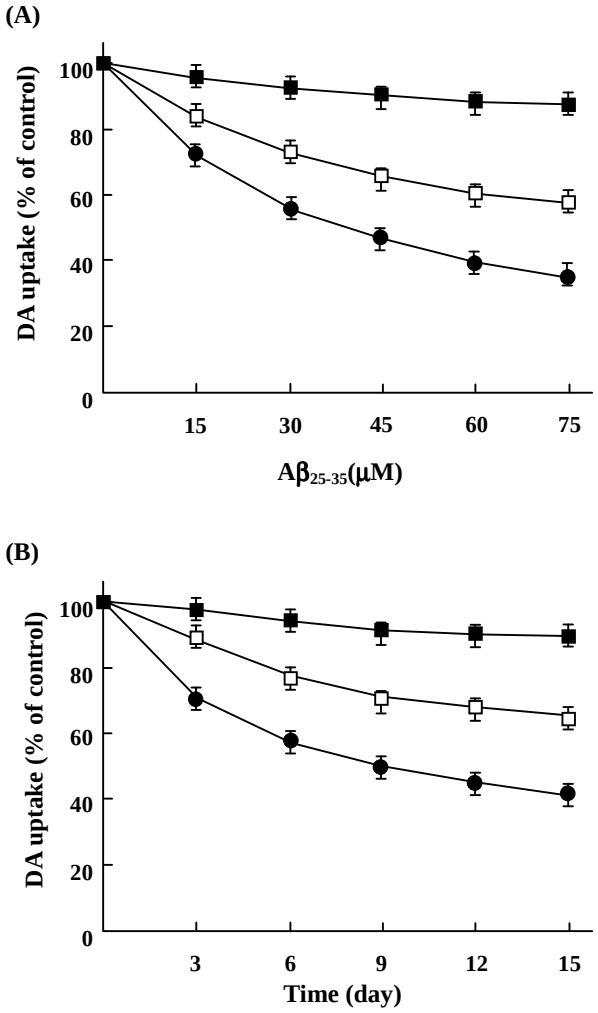


Fig. 6. Effect of KHG21834 on $A\beta_{25-35}$-induced decrease in dopamine uptake of mesencephalic neuron-glia cultures. Cultures were treated for 9 days with vehicle or indicated concentrations of $A\beta_{25-35}$ (A) or with 50 μM $A\beta_{25-35}$ for the indicated number of days (B) in the presence and absence of KHG21834. Afterwards, DA uptake was performed as described under Materials and Methods. Results are expressed as a percentage of the control cultures and are mean ± S.E.M. of three experiments performed in triplicate. (●) 0 μM KHG21834; (□) 25 μM KHG21834; (■) 50 μM KHG21834

4. Protective effect of KHG21834 on $A\beta_{25-35}$-induced ERK phosphorylation

ERKs have been shown to play an important role in Aβ neurotoxicity in various types of cultured cells via multiple mechanisms. Previous study showed that the $A\beta_{25-35}$ induced ERK1/2 phosphorylation began to occur within in 5 min, gradually increased and lasted for more than 80 h at cultured rat cortical astrocytes (44). We, therefore, attempted to examine the effects of KHG21834 on the possible involvement of ERK1/2 in $A\beta_{25-35}$-stimulated cortical and mesencephalic neuron-glia cultures. As activation of ERKs requires phosphorylation at threonine and tyrosine residues, immunoblot analysis with specific anti-phospho-ERK1/2 antibodies was performed. Unphosphorylated forms of ERK1/2 were measured as controls and relative expression level was calculated using densitometry. In cultures treated with 50 μM $A\beta_{25-35}$, significant induction in phospho-ERK1/2 was observed 24h after treatment. When mesencephalic neuron-glia cultures were treated with $A\beta_{25-35}$ in the presence of KHG21834, significantly attenuation $A\beta_{25-35}$-induced increase in phospho-ERK1/2 were observed (Fig. 7A). Densitometric analysis showed that the expression levels of phospho-ERK1 and phospho-ERK2 of cultures co-treated with 25 μM KHG21834 was 30% and 50%, respectively, of that of $A\beta_{25-35}$ treated group (Fig. 7B). At 50 μM concentration, KHG21834 reduced $A\beta_{25-35}$ induced phosphorylation of ERK1 and ERK2 to 12% and 25%

of $A\beta_{25-35}$ treated group (Fig. 7B). ERK1, therefore, was more sensitively affected than ERK2 in attenuation of $A\beta_{25-35}$ induced phosphorylation by KHG21834.

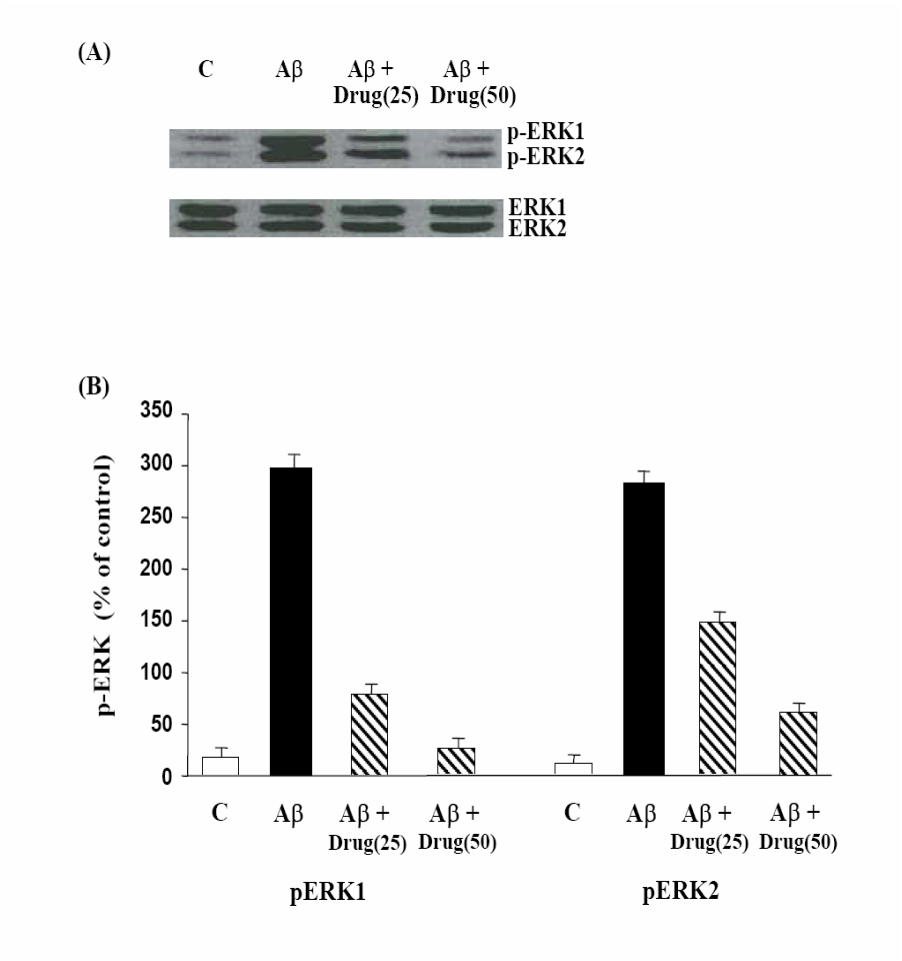


Fig. 7. Effect of KHG21834 on the activation of ERK1/2. The activation of ERK1/2 was assessed by measuring the respective phosphorylated form of ERK1/2. The cultures were treated with 25 μM to 50 μM $A\beta_{25-35}$ for 24 h in the absence or presence of the 25 μM to 50 μM KHG21834. (A) Western blot analysis was performed using primary anti-ERK1, anti-ERK2, anti-phospho-ERK1, and anti-phospho-ERK2. Unphosphorylated forms of ERK1/2 were measured as controls. (B) Relative expression level was calculated using densitometry. Values are means ± SD (n = 3).

Ⅳ. DISCUSSION

In this study, a benzothiazole derivative (KHG21834) has been examined in cultured PC12 cells, mescencephalic neuron-glia cultures and cortical neurons to find more effective neuroprotective drugs against to the Aβ-induced cell death (Fig. 2~Fig. 4). The results demonstrate that the degeneration of cortical GABAergic and mesencephalic dopaminergic neurons induced by $A\beta_{25-35}$ can be significantly attenuated by KHG21834 (Fig. 3 and Fig. 4). Our data show that Aβ is more neurotoxic to mesencephalic neuron–glia cultures than cortical neuron-glia cultures (Fig. 3G and Fig. 4G). This observation is consistent with previous finding that the mesencephalic region has a much higher density of microglia than the cortex (45). Thus, dopaminergic neurons in mesencephalic neuron-glia cultures may encounter an excessively high level of oxidative stress after Aβ treatment. Benzothiazoles are highly interesting molecules for drug development, because they already have been shown to be useful for treating cerebrovascular and neurodegenerative disorders. Previous biochemical and behavioral studies demonstrated the ability of benzothiazole partially to protect the degeneration of the nigrostriatal dopaminergic neurons induced by the toxin 6-hydroxydopamine (46). Recent studies also have reported that 2-(4'-methylaminophenyl)benzothiazole shows very good brain entry and the binding of this drug to AD brain is dominated by a specific with

Aβ amyloid deposits (47,48).

Although the detailed mechanism of benzothiazoles is not fully understood, it has been suggested the benzothiazole derivatives may react as a tyrosine kinase inhibitor (21,33-35). Tyrosine-specific protein kinase activities have been shown to be involved in the control of cell growth and differentiation. One of the main molecular mechanisms involved is the autophosphorylation of receptor tyrosine kinases in response to ligand binding (36,37). Tyrosine kinases seem also to be important in other phenomena such as ligand-induced internalization of antigen receptor in B lymphocytes (33), long-term potentiation in the hippocampus (38), clustering of acetylcholine receptors (39) and regulation of N-methyl-D-aspartate receptors (40). A simple way to check the involvement of these enzymes is to use specific inhibitors. This suggests that some neuronal elements involved in AD pathology may be recapitulating a developmental profile or, alternately, that elevated phosphotyrosine levels may reflect a role for tyrosine kinase/phosphatase systems in the degeneration process directly. Cells in the neuritic plaque which strongly resemble microglia also contain elevated levels of phosphotyrosine compared to non-activated ramified microglia in the same tissue section. Thus, tyrosine phosphorylation systems may be involved in the response of microglia to degeneration in AD pathology (33).

A key result is that KHG21834 reduces the $A\beta_{25-35}$-induced neurotoxicity

both in mesencephalic neurons and cortical neurons (Fig. 3 and Fig. 4). The results from DA and GABA uptake assay further support the effects of this drug on both GABAergic and dopaminergic neurons (Fig. 5 and Fig. 6). The similar mechanisms of toxicity by Aβ in the cortex and mesencephalon may partly explain the overlap in pathological features and clinical symptoms observed in AD and PD. Whether KHG21834 has an effect on soluble Aβ levels was not the goal of this study and remains to be determined. It has been reported that $A\beta_{1-42}$ exhibited significantly enhanced neurotoxicity toward mesencephalic or cortical neurons in the presence of microglia and the elevated neurotoxicity was attributed to the activation of microglia and production of superoxide free radicals (14,15). There is compelling evidence supporting that enhanced pro-inflammatory activities provoked by Aβ are associated with the pathogenesis and /or progression of AD, and that some anti-inflammatory agents protect against Aβ-induced neurotoxicity (49,50). $A\beta_{25-35}$ has neurotoxic effects and produces free radical spin adducts in aqueous solutions (51). One of the principal enzymes that play a pivotal role in mediating inflammatory response is inducible nitric oxide synthase (iNOS). iNOS is mainly localized in astrocytes and microglia, and catalyzes the oxidative deamination of L-arginine to produce nitric oxide (NO), a potent pro-inflammatory mediator (52). Previous studies have shown that Aβ induces iNOS expression in astrocytes through NF-κB-dependent mechanism

(53,54) and Aβ induces iNOS expression in PC12 cells through activation of AP-1 which is regulated by upstream kinases, such as ERK and p38 MAPK (51).

Numerous intracellular signal-transduction pathways converge with the activation of the MAPK family proteins, which act independently or coordinately to regulate target gene expression. The most extensively investigated MAPKs mediating intracellular signaling cascades involved in pro-inflammatory response are ERK. A number of growth factor receptor tyrosine kinases are known to be linked to the G-protein Ras and stimulate MAPK kinase kinases such as Raf, which phosphorylate and activate MAPK kinases, MEK1 and MEK2, which in turn phosphorylate and activate p44 MAPK (44 kDa; ERK1) and p42 MAPK (42 kDa; ERK2). The phosphorylated ERK1/2 translocates into the nucleus and plays a role in the regulation of gene transcription. In this way, Ras, Raf, MEK1/2 and ERK1/2 constitute a unique signal transduction cascade.

In the present study, I investigated whether ERK signaling pathways are involved in the $A\beta_{25-35}$-induced neurotoxicity. The results in Fig. 7 show that the $A\beta_{25-35}$ activated ERK1/2 and treatment of KHG21834 blocked Aβ-induced ERK1/2 phosphorylation. ERK1 was more sensitively affected than ERK2 in attenuation of $A\beta_{25-35}$-induced phosphorylation by KHG21834 (Fig. 7B). The results of ERK1/2 activation obtained with KHG21834 (Fig. 7) are internally

consistent with the previous reports and are congruent with relevant clinical findings. Results from recent studies suggested that ERK1/2-mediated tyrosine kinase activation induced secreted form of α-secretase derived APP fragment (sAPPα) production and reduced Aβ generation (55-60). Very recently, it has been reported that ERK1/2 is an endogenous regulator of γ-secretase, an essential protease in the generation of Aβ, which raises the possibility that ERK1/2 down-regulates γ-secretase activity by directly phosphorylating nicastrin (61). The ERK/MAP kinase cascade is also known to be an important signalling pathway for synaptic plasticity, and increased ERK/MAP kinase is associated with tau phosphorylation and the cytoskeletal abnormalities found in dystrophic neurites in AD. Recently, Aβ has been shown to activate the MAP kinase cascade in hippocampal neurons through the α7 nicotinic acetylcholine receptor (α7 nAChR) (62). Increased levels of activated ERK1/2 (63), p38 (64), and JNK (65) have been found in the brains of AD and Down's syndrome patients. These results suggest that the underlying mechanism of action responsible for the neuroprotective effect of KHG21834 may be related to their inhibition of $A\beta_{25-35}$-induced ERK1/2 phosphorylation.

An advantage of an integrative chemical biology approach offers a starting point for future proteomic analyses that can identify molecular differences between brain and peripheral tissues in regulating the same biological endpoint, proinflammatory cytokine upregulation. Integrative chemical biology cannot

be done in the absence of the required bioavailable compound. KHG21834 is a benzothiazole derivative with multiple positions amenable to diversification using standard chemistries. The results in this study have demonstrated that the degeneration of mesencephalic and cortical neurons induced by $A\beta_{25-35}$ can be significantly attenuated by KHG21834. The precise mechanism of action responsible for the neuroprotective effect of KHG21834 and the neuroprotective effects of this drug *in vivo* remain to be studied further.

Ⅴ. CONCLUSIONS

The major aim of this study was investigation of the effects of KHG21834, a benzothiazole derivative, on beta amyloid ($A\beta_{25-35}$)-induced cell death in cultured PC12 cells and rat cortical and mescencephalic neuron-glia cultures.

1. KHG21834 attenuated $A\beta_{25-35}$-induced neurodegeneration in PC12 cells

The presence of 50 μM KHG21834 rescued PC12 cells by 82% from $A\beta_{25-35}$-induced cytotoxicity. KHG21834 attenuated the $A\beta_{25-35}$-induced apoptotic death in PC12 cells determined by characteristic DNA fragmentation alterations and markedly reduced the $A\beta_{25-35}$-induced TUNEL staining.

2. Effect of KHG21834 on $A\beta_{25-35}$-induced neurodegeneration of cortical and mesencephalic neuron-glia cultures

In the cortical neuron-glia cultures, KHG21834 markedly reduced the $A\beta_{25-35}$-induced TUNEL staining and $A\beta_{25-35}$-caused reduction in MAP-2 expression was effectively prevented in the presence of KHG21834. While the average dendrite length of TH-immunoreactive neurons in the $A\beta_{25-35}$-treated mesencephalic neuron-glia cultures was 6% of that of cultures, that of the cultures treated with KHG21834 after $A\beta_{25-35}$ treatment was 79% of control. Western blot analysis of rat mesencephalic neuron-glia cultures showed that $A\beta_{25-35}$ decreased the expression of TH protein by 60% and KHG21834

significantly attenuated the $A\beta_{25-35}$-induced reduction in the expression of TH.

3. Effect of KHG21834 on $A\beta_{25-35}$-induced decrease in GABA uptake of cortical neurons and in DA uptake of dopaminergic neurons

KHG21834 effectively attenuated $A\beta_{25-35}$-induced decrease in [^{3}H]GABA uptake of cortical neuron-glia cultures and in [^{3}H]DA uptake of mesencephalic neuron-glia cultures.

4. Protective effect of KHG21834 on $A\beta_{25-35}$-induced ERK phosphorylation

$A\beta_{25-35}$ activated extracellular signal-regulated kinase (ERK1/2) and treatment of KHG21834 blocked Aβ-induced ERK1/2 phosphorylation. ERK1 was more sensitively affected than ERK2 in attenuation of $A\beta_{25-35}$ induced phosphorylation by KHG21834.

These results demonstrated that KHG21834, a benzothiazole derivative, is capable of neuroprotection against to the $A\beta_{25-35}$-induced degeneration of neuronal cells.

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국 문 요 약

신경세포 내의 베타-아밀로이드 펩타이드로 유도된 신경독성에 대한 benzothiazol의 억제능

알츠하이머 질병의 단백질 침착에는 신경세포 안에서 이루어지는 신경섬유 응집체(neurofibrillary tangle; NFT)와 신경세포 바깥에서 이루어지는 노인반(senile plaque)의 형성이 있다. 두 가지 경우 모두 단백질이 주성분으로 신경섬유 응집체는 미세소관 결합 단백질인 타우(tau)가, 노인반의 경우는 막 단백질인 베타-아밀로이드 펩타이드가 주성분이다. 본 연구에서는 PC12 세포와 피질신경세포와 중뇌신경세포에 베타-아밀로이드로 유도된 신경세포사에 대하여 benzothiazol의 유도체인 KHG21834의 효과를 알아보고자 한다. PC12 세포에 베타-아밀로이드(25-35) 유도의 apoptosis의 형태학적인 변화인 DNA 분절능과 TUNEL assay 변화율이 감소하였다. 50μM의 KHG21834 처리한 PC12 세포는 베타-아밀로이드(25-35) 유도 세포독성능에 대하여 82% 생존율을 보였다. 피질신경세포에서도 KHG21834 처리한 것이 베타-아밀로이드(25-35) 유도의 신경독성능에 대한 TUNEL assay 변화율과 MAP-2 발현률이 감소하였다. TH 면역세포화학 분석법을 이용하여 중간신경세포의 수상돌기의 평균길이를 측정해 보았을때 베타-아밀로이드(25-35) 처리한 것에 평균길이가 6%인 반면 베타-아밀로이드(25-35) 처리 이후에 KHG21834를 처리한 중뇌신경세포는 대조군에 비하여 독성능의 억제 통하여 79% 생존율을 보였고, Western blotting 분석법을 이용한 중뇌신경세포의 TH-단백이 베타-아밀로이드(25-35)처리한 것의 발현율이 60% 감소를 보였으며 KHG21834의 처리 이후 발현률 증가를 보였다. KHG21834를 처리하였을 때 피질신경세포의 [^{3}H]GABA와 중뇌신경세포의 [^{3}H]DA의 섭취율이 상당히 감소를 보였다. 또한, 베타-아밀로이드(25-35)로 ERK1/2가 활성되며 KHG21834의 처리로 ERK1/2 인산화가 차단되었다. 베타-아밀로이드(25-35) 유도 인산화를 KHG21834에 의해 ERK 1은 ERK 2에 비하여 민감하게 영향을 받는다. 이와 같은 결과로부터

KHG21834가 중뇌신경세포와 피질신경세포의 베타-아밀로이드(25-35) 유도된 신경퇴행현상에 대하여 보호능이 있다는 것을 증명하고자 하였다.

핵심어 : KHG21834, 베타-아밀로이드, PC12 세포, 피질신경세포, 중뇌 신경세포, ERK1/2

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