Neuroscience 242 (2013) 28–38

IMM-H004, A NOVEL COUMARIN DERIVATIVE COMPOUND, PROTECTS AGAINST AMYLOID BETA-INDUCED NEUROTOXICITY THROUGH A MITOCHONDRIAL-DEPENDENT PATHWAY

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Abstract—We have investigated the effect of IMM-H004 (7-hydroxy-5-methoxy-4-methyl-3-(4-methylpiperazin-1-yl)-2H-chromen-2-one), a coumarin derivative, on the amyloid beta (Aβ)-induced neurotoxicity in primary culture cortical neurons and pheochromocytoma (PC12) cells. Our results showed that treatment with IMM-H004 markedly reduced the number of apoptotic cells after exposure to $A\beta_{25-35}$ or $A\beta_{1-42}$, determined by MTT, TUNEL staining and Flow cytometry. Further study indicated that IMM-H004 significantly inhibited Aβ-induced cytotoxicity and apoptosis by reversing Aβ-induced mitochondrial dysfunction, including MMP (mitochondrial membrane potential) decrease, reactive oxygen species production, and mitochondrial release of cytochrome *c*. IMM-H004 can regulate the interaction between Bax and Bcl-2, decreased levels of p53 and active caspase-3 protein induced by $A\beta_{25-35}$. Furthermore, IMM-H004 also reduced translocation of AIF (apoptosis-inducing factor) induced by $A\beta_{25-35}$. These results demonstrated that IMM-H004 was capable of protecting neuronal cells from Aβ-induced degeneration through a mitochondrial-dependent apoptotic pathway. The results of this study lend further credence to the notion that IMM-H004 is a 'multipotent therapeutic agrent' that reduces toxic levels of brain Aβ, and holds the potential to protect neuronal mitochondrial function in Alzheimer's disease.

Key words: IMM-H004, amyloid beta, neurotoxicity, apoptosis, mitochondrial-dependent pathway, Alzheimer's disease.

INTRODUCTION

Alzheimer's disease (AD), a progressive degenerative disorder of the brain, is the most common cause of dementia among elderly people. The deposition of amyloid beta (Aβ) within the senile plaques that are a hallmark of AD is thought to be a primary cause of the cognitive dysfunction that occurs in AD (Basun et al., 2008). Both *in vitro* and *in vivo* studies have reported the toxic effects of Aβ suggesting an important role in the pathogenesis of AD (Cotman et al., 1992; Iversen et al., 1995; Kaneko et al., 2000; Wang et al., 2007). Thus, identifying a way to reduce the Aβ-induced neurotoxicity would be of help in AD treatment.

Although the precise mechanism underlying Aβ-induced neurotoxicity remains obscure, several lines of evidence suggest that it is an apoptotic process (Cotman and Anderson, 1995). Recent studies have shown that in AD brains and in cultured neurons exposed to Aβ, the dying cells display the characteristics of apoptosis (Abdul et al., 2006; Majd et al., 2008; Tillement et al., 2011; Xuan et al., 2012). Therefore, reagents that suppress neuronal apoptosis induced by Aβ may be promising candidates for AD therapy.

Although $A\beta_{1-40}$ and $A\beta_{1-42}$ are the predominant forms, other fragments can be present as well: in the brains of aged patients, $A\beta_{25-35}$, a stretch of 11 amino acid-long residues of the full length from position 25 to 35, is produced by the proteolysis of full-length $A\beta_{1-40}$ (Kubo et al., 2002; Millucci et al., 2010). Reported evidence suggests that the fraction 25–35 of Aβ ($A\beta_{25-35}$) is the functional neurotoxic domain, where the methionine residue located at the position 35 is responsible for the neurotoxic properties (Clark and Vulliet, 1993; Casley et al., 2002; Andersen et al., 2003; Butterfield and Boyd-Kimball, 2005).

IMM-H004 (7-hydroxy-5-methoxy-4-methyl-3-(4-methylpiperazin-1-yl)-2H-chromen-2-one) is a 3-piperazinylcoumarin compound derived from compound 41, which we have already reported to be

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Abbreviations: Aβ, amyloid beta; AD, Alzheimer's disease; AIF, apoptosis-inducing factor; ANOVA, analysis of variance; CCN, cultured cortical neurons; COX-IV, cytochrome *c* oxidase-IV; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulphoxide; ECL, enhanced chemiluminescent; ES, horse serum; FBS, fetal bovine serum; HFIP, hexafluoroisopropanol; IMM-H004, 7-hydroxy-5-methoxy-4-methyl-3-(4-methylpiperazin-1-yl)-2H-chromen-2-one; MMP, mitochondrial membrane potential; MTT, 3-(4,5)-dimethylthiahiazo-(-z-y1)-3,5-diphenytetrazoliumro-mide; PBS, phosphate buffered saline; PC12, pheochromocytoma; PI, propidium iodide; PLL, poly-L-lysine; ROS, reactive oxygen species; TUNEL, TdT-mediated dUTP nick-end labeling.

0306-4522/13 $36.00
http://dx.doi.org/10.1016/j.neuroscience.2013.02.049

effective in blocking the calcium mobilization and chemotaxis induced by CKLF1-C27 and lessening the asthmatic pathologic changes in the lung tissue of human CKLF1-transfected mice (Li et al., 2010). In our preliminary study, IMM-H004 showed better activity than compound 41 among kinds of derivative compounds against several neurotoxic agents *in vitro*, especially in Aβ-induced neuron death. Then it was also found to be a well-free radical scavenger. Thereby, it is vitally necessary to research the protective effect of IMM-H004 in Aβ-induced neurotoxicity.

EXPERIMENTAL PROCEDURES

Materials

The compound IMM-H004 was provided by the department of chemosynthesis, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College (Beijing, China). The chemical structure was shown in Fig. 1. $A\beta_{25-35}$ was obtained from the Yansheng Biochemical Company (Shanghai, China). All experiments conformed to a named local as well as International Guidelines on the ethical use of animals and that all efforts were made to minimise the number of animals used and their suffering.

Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), horse serum (ES), penicillin/streptomycin were purchased from Gibco BRL (New York, NY, USA). Anti-Bcl-2, anti-Bax, anti-cytochrome *c*, anti-COX-IV (cytochrome *c* oxidase-IV) primary antibodies and anti-rabbit/mouse IgG secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibodies against Caspase-3 and p53 were obtained from Cell Signaling Biotechnology (Hertfordshire, England). Anti-AIF (apoptosis-inducing factor) primary antibody was purchased from Abcam (Cambridge, CB, UK). Anti-β-actin primary antibody, anti-rabbit IgG secondary antibody conjugated with Alexa Fluor 488, MTT (3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-diphenytetrazoliumro-mide) hexafluoroisopropanol (HFIP) and DMSO (dimethyl sulphoxide) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Enhanced chemiluminescent (ECL) substrate was from Pierce (Rockford, IL, USA). JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolyl-carbocyanine iodide), DCFH-DA (Mitochondria/cytosol Fractionation Kit, 2,7-dichlorofluorescein diacetate), Cell lysis buffer for Western/PMSF was purchased from Beyotime Institute of Biotechnology (China). FragEL DNA Fragmentation detection kit was provide by Merk Biosciences (Calbiochem, San Diego, CA, USA).

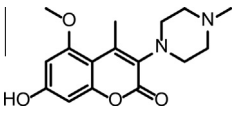

Fig. 1. The chemical structure of IMM-H004.

Aβ preparation and IMM-H004 treatment

$A\beta_{25-35}$ or $A\beta_{1-42}$ was dissolved in HFIP and HFIP was removed by N2 gas. Then $A\beta_{25-35}$ was suspended at 20 mM into DMSO, while $A\beta_{1-42}$ was dissolved at 2.5 mM in DMSO, brought to 100 μM in DMEM, and incubated at 4 °C for 24 h as stock solution (Stine et al., 2003). Before treatment, the Aβ stock solution was diluted to the desired final concentrations in treatment medium. 10 μM Aβ was used in cultured cortical neurons (CCN), and 15 μM in PC12 (pheochromocytoma) cells.

IMM-H004 was freshly prepared as stock solutions (10 mM) in DMSO and diluted to the desired final concentrations in treatment medium. For the determination of the protective effect of IMM-H004 on Aβ-induced cytotoxicity, cultures treated with Aβ were further administrated with various concentrations of IMM-H004 for 24 h as described in each figure legend. The concentration of IMM-H004 was 0.1, 1 and 10 μmol/L (μM), respectively.

Rat cortical neurons culture and treatment

Rat cortical neurons were prepared from the brains of newborn Sprague–Dawley rats. All cell suspensions were grown in DMEM/F12 medium with 10% ES, 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were plated in poly-L-lysine (PLL, 0.1 mg/mL) coated multi-well plates or chamber slides and maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air atmosphere. After 2 days 10 μg/mL of cytarabine (Ara-C) was added to the culture for 48 h to inhibit the outgrowth of glial cells, mechanocytes and nerve stem cells, etc. Seven-day cultures were used for treatment.

The cortical neurons were then exposed to 10 μM $A\beta_{25-35}$ in the presence or absence of IMM-H004 (given immediately before $A\beta_{25-35}$) for 24 h in all experiments. In viability assay and morphological observation, cells were seeded into 96-well culture plates at a density of 1×10^5 per well. As for intracellular ROS (reactive oxygen species), MMP (mitochondrial membrane potential) determination and TUNEL (TdT-mediated dUTP nick-end labeling) staining, cells were plated onto 12-well plates at a density of 1×10^6 per well. For Western blot analysis, cells were plated onto 6-well culture plates at a density of 2×10^6 per well.

PC12 cell culture and treatment

PC12 cells were maintained routinely in DMEM supplemented with 5% ES, 5% FBS, at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The medium was changed every 2 days, and cells were seeded on PLL-coated plates at an appropriate density according to each experimental scale. After 24 h subculture, cells were switched to fresh medium for treatment.

The attached cells were then exposed to 15 μM $A\beta_{25-35}$ or $A\beta_{1-42}$ in the presence or absence of IMM-H004 (given immediately before $A\beta_{25-35}$) for 24 h in all