European Journal of Pharmacology 723 (2014) 259–266

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Neuropharmacology and analgesia

IMM-H004, a novel courmarin derivative, protects against oxygen-and glucose-deprivation/restoration-induced apoptosis in PC12 cells

Hai-jie Ji [a], Dong-mei Wang [a], Jin-feng Hu [a], Ming-na Sun [a], Gang Li [a], Zhi-peng Li [a], Dong-hui Wu [a], Gang Liu [a,b,c,*], Nai-hong Chen [a,**]

[a] State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, 2A Nanwei Road, Xicheng District, Beijing 100050, PR China
[b] Tsinghua-Peking Center for Life Sciences, School of Medicine, Tsinghua University, Haidian District, Beijing 100084, PR China
[c] Department of Pharmacology and Pharmaceutical Sciences, School of Medicine, Tsinghua University, Haidian District, Beijing 100084, PR China

ARTICLE INFO

Article history:
Received 7 June 2013
Received in revised form
25 September 2013
Accepted 5 November 2013
Available online 28 November 2013

Keywords:
IMM-H004
Coumarin derivative
Oxygen- and glucose-deprivation/restoration
Apoptosis
Stroke
PC12 cells

ABSTRACT

7-Hydroxy-5-methoxy-4-methyl-3-(4-methylpiperazin-1-yl)-coumarin (IMM-H004) is a novel coumarin derivative synthesized in our laboratory. The purpose of the current study was to determine the neuroprotective effects of IMM-H004 on PC12 cells and its potential mechanism of action. PC12 cells were subject to oxygen and glucose deprivation (OGD) followed by the restoration of oxygen and glucose (R), which mimics ischemia and reperfusion in vivo. Cell viability was determined by MTT assay. DNA fragmentation was analyzed by DNA ladder. ROS and mitochondrial membrane potential were measured by fluorescent microscope and quantified by Image-Pro Express 6.0 software. ATP was measured by luciferin–luciferase assay. The activation of signal-regulated molecules was assessed by the Western blot analysis. ·OH formation was determined using the Electron Spin Resonance (ESR) trapping technique in combination with 5, 5-dimethyl-1-pyrroline-N-oxide. OGD/R reduced cell viability and induced cell apoptosis, which were both dose-dependently attenuated by IMM-H004. The accumulation of intracellular reactive oxygen species (ROS) and reduced mitochondrial membrane potential observed in PC12 cells treated with OGD/R, which switch on the mitochondrion-dependent apoptotic pathway, were reversed by IMM-H004. ATP production in OGD/R-treated PC12 cells was elevated by IMM-H004, which suggests that it restored the functions of the mitochondria. OGD/R-induced cytochrome c release from the mitochondria reduced the ratio of apoptotic proteins, Bcl-2/Bax, and induced caspase-3 activation and DNA fragmentation. These changes were significantly inhibited by IMM-H004. IMM-H004 also significantly inhibited ·OH formation, determined by electron spin resonance, which indicates that it is a potent free-radical scavenger. This study has demonstrated that IMM-H004 protects PC12 cells against OGD/R-induced apoptosis, at least in part, by scavenging excessive ROS and inhibiting the mitochondrion-dependent apoptotic pathway.

1. Introduction

Stroke is the third most frequent cause of adult death in most industrialized countries, after cardiovascular disease and cancer (Flynn et al., 2008; Doyle et al., 2008). Ischemic stroke, which results from the occlusion of an artery in the brain, accounts for more than 80% of all strokes (Flynn et al., 2008). According to the degree of hypoperfusion and the duration of the ischemic insult, it is possible to identify an area with complete absence of flow, the "core", where neuronal death occurs within a few minutes, and a surrounding area, the "penumbra", which suffers from a moderate reduction in blood flow and contains functionally impaired but still viable brain tissue (Lipton, 1999). If the blood flow is not restored within a relatively short time, the neurons in the penumbra undergo apoptosis as their final fate (Endres et al., 2008).

A large body of evidence supports the increased production of free radicals during ischemia and reperfusion, and oxidative stress is suggested to be a fundamental mechanism of brain damage under these conditions (Cuzzocrea et al., 2001). During ischemia, mitochondria generate huge amounts of reactive oxygen species

Abbreviations: BBB, blood–brain barrier; DCFH-DA, 2,7-dichlorfluorescein diacetate; DMEM, dulbecco's modified Eagle medium; DMPO, 5, 5-dimethyl-1-pyrroline-N-oxide; EB, ethidium bromide; ES, equine serum; ESR, electron spin resonance; FBS, fetal bovine serum; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide; HPLC, high performance liquid chromatography; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; OGD, oxygen- and glucose-deprivation; R, reoxygenation; ROS, reactive oxygen species; PI, propidium iodide

* Corresponding author. Tel.: +86 10 62797740; fax: +86 10 62797740.
** Corresponding author. Tel./fax: +86 10 63165177.
E-mail addresses: gangliu27@tsinghua.edu.cn (G. Liu), chennh@imm.ac.cn (N.-h. Chen).

0014-2999/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ejphar.2013.11.023

(ROS). The overproduction of ROS results in damage to the cellular lipids, proteins, and DNA, so ROS have been associated with both necrosis and apoptosis (Noh et al., 2006). Apoptosis is biochemically activated via the intrinsic and extrinsic pathways. The intrinsic pathway is regulated by members of the Bcl-2 family of proteins and involves the release of proteins such as cytochrome c and apoptosis-inducing factor from the mitochondria (Bredesen et al., 2006). These proteins lead to the activation of caspases, particularly caspase 3, which in turn induce DNA fragmentation (Galluzzi et al., 2009; Krantic et al., 2007). Thus, reducing oxidative stress by suppressing ROS production should provide a specific therapeutic target for ischemic stroke.

The antioxidant mechanisms in neurons can prevent the cell apoptosis mediated by ROS (Ferrari et al., 1995; Greenlund et al., 1995). Antioxidant strategies have had very encouraging results in numerous studies of experimental stroke. For instance, Edaravone inhibits lipid peroxidation by scavenging free radicals, such as O^{2-}, NO, and $ONOO^{-}$, and has been used in the treatment of cerebral infarction (Eiichi, 2003). Coumarins, which comprise a group of phenolic compounds, are widely distributed in natural plants. It has recently been reported that esculetin, a coumarin derivative with xanthine-oxidase-inhibiting and free-radical-scavenging activities, exerts neuroprotective effects (Lin et al., 2008; Siân et al., 2009). 7-hydroxy-5-methoxy-4-methyl-3-(4-methylpiperazin-1-yl)-coumarin (IMM-H004, Fig. 1(B)) is a 3-piperazinylcoumarin compound derived from compound 41(Fig. 1(A)), which is a potent antagonist of chemokine-like factor 1 (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 (Song et al., 2013). Furthermore, the compound effectively protected rats against ischemic brain injuries, with clear evidence of its capacity to cross the blood–brain barrier (BBB) in rats (Sun et al., 2013). In this article, we focus on the mechanistic role of IMM-H004 against OGD-induced apoptosis in PC12 cells when it scavenges excessive ROS and inhibits the mitochondrion-dependent apoptotic pathway.

2. Materials and methods

2.1. IMM-H004 preparation

IMM-H004 was synthesized in department of medicinal chemistry, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College. HPLC analysis indicates that the purity of IMM-H004 is more than 99%. IMM-H004 was dissolved in dimethyl sulfoxide as a stock solution kept in −20° C.

2.2. PC12 cell culture and OGD/R establishment

Undifferentiated PC12 cells, rat pheochromocytoma cell line, were cultured in DMEM with 10% FBS, 5% ES, 100 U/ml penicillin and 100 U/ml streptomycin at 37 °C under a humidified atmosphere containing 5% CO_2. To initiate OGD, cell culture media was removed and cells were washed twice with glucose-free DMEM. Then cells were incubated in the glucose-free medium in an oxygen-free incubator (95% N_2 and 5% CO_2) in absence or presence of indicated concentrations of IMM-H004 for a duration of 1–5 h. After that, the cells were incubated with conditioned DMEM in absence or presence of indicated concentrations of IMM-H004 for 24 h.

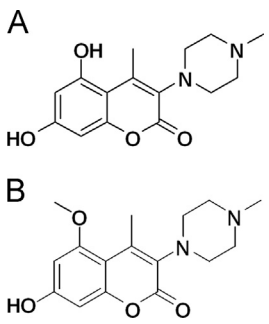

Fig. 1. Chemical structure of compound 41 (A) and IMM-H004 (B).

2.3. Morphological observation and determination of cell viability

Treated cells were visualized by phase-contrast microscopy using an inverted microscope (Nikon TE 2000) connected to a digital camera. Cells were cultured at density of 5×10^3/well in 96-well plates. MTT solution was added to each well at a final concentration of 0.5 mg/ml and the incubation was continued for 4 h at 37 °C in dark, following incubation with lysing buffer containing 5% (v/v) isobutanol and 10% (g/v) sodium dodecyl sulfate (pH 4.8) for overnight at room temperature. The absorbance was measured at 570 nm. Cell viability was expressed as a percentage of vehicle control.

2.4. Annexin V staining

Approximately 1 × 106 cells were centrifuged (1200*g*, 5 min) to remove the medium, washed 3 times with binding buffer (10 mM HEPES/NaOH, PH 7.4, 140 mM NaCl, 5 mM $CaCl_2$) and stained with final concentration of 1 μg/ml Annexin V-FITC. After 15 min of incubation, cells were washed with binding buffer, and incubated with a final PI concentration of 10 μg/ml for 10 min. Then the cells were washed with binding buffer again and kept on ice without exposure to light prior to analysis. Annexin V-FITC and PI emissions were detected by flow cytometry (BECKMAN, USA), using emission filters of 525 nm and 575 nm, respectively.

2.5. DNA fragmentation analysis

Briefly, treated cells were harvested and centrifuged at 3500*g* for 5 min to collect cell pellets. The pellet was lysed in DNA extraction buffer (100 mM Tris–HCl, pH 8.0, 200 mM NaCl, 5 mM EDTA, 0.2% sodium dodecyl sulfate, 0.2 mg/ml proteinase K) for at least 3 h at 56 °C. After digestion with RNase A at 37 °C for 30 min, the sample was subjected to electrophoresis in a 2% agarose gel and visualized by ethidium bromide staining. Soluble DNA from equal numbers of cells was loaded in each lane.

2.6. Measurement of ROS

DCFH-DA diffuses through the cell membrane and is enzymatically hydrolyzed by intracellular esterases to the nonfluorescent DCFH, which can be rapidly oxidized to the highly fluorescent DCF, the fluorescent product, in the presence of ROS. Briefly, treated cells were incubated with DCFH-DA at a final concentration of 20 μM for 30 min at 37 °C in darkness and then were washed with PBS for 3 times. Cells were imaged using a fluorescent microscope (Nikon TE 2000). Cells were located under bright-field optics and then scanned once with the laser (488 nm for excitation and 525 nm for emission). The green fluorescence intensity was analyzed by Image-Pro Express 6.0 software.

2.7. Measurement of mitochondrial membrane potential ($\Delta\Psi m$)

Mitochondrial membrane potential was determined using a fluorescent probe JC-1. JC-1 is a cationic dye that diffuses into cytoplasm in monomer and accumulates at mitochondria in aggregate by a potential dependent manner. When illuminated with