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SP-8203 shows neuroprotective effects and improves cognitive impairment in ischemic brain injury through NMDA receptor

Su-Jin Noh [a], Jong Min Lee [b], Ki Sung Lee [b], Hyun Su Hong [b], Chul Kyu Lee [b], Il Hwan Cho [b], Hye-Sun Kim [a], Yoo-Hun Suh [a,*]

[a] Department of Pharmacology, College of medicine, National Creative Research Initiative Center for Alzheimer's Dementia and Neuroscience Research Institute, MRC, Seoul National University, Seoul, Republic of Korea
[b] Central Research Institute, Shinpoong Pharmaceutical Company, Ltd, 434-4, Moknae-dong, Danwon-Gu, Ansan, Kyunggi-Do, Republic of Korea

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ABSTRACT

The extracts of earth worms, *Eisenia andrei*, have been used as a therapeutic agent for stroke in the traditional medicine. It is also reported that the protease fraction separated from the extracts has strong anti-thrombotic activity. Besides anti-thrombotic actions, we found that SP-8203, N-[3-(2,4-dioxo-1,4-dihydro-2H-quinazolin-3-yl)propyl]-N-{4-[3-(2,4-dioxo-1,4-dihydro-2H-quinazolin-3-yl)propylamino]butyl}acetamide, derived from the extracts of earth worms blocked *N*-methyl-$_D$-aspartate (NMDA) receptor-mediated excitotoxicity in a competitive manner. The neuroprotective effects of SP-8203 were attributable to prevention of Ca^{2+} influx through NMDA receptors. The systemic administration of SP-8203 markedly reduced neuronal death following middle cerebral artery occlusion in rats. SP-8203 significantly improved spatial learning and memory in the water maze test. These results provided strong pharmacological basis for its potential therapeutic roles in cerebral ischemia.

1. Introduction

Excessive activation of *N*-methyl-D-aspartate (NMDA) receptor produces neuronal death and has been implicated as a major cause of ischemic brain injuries (Choi 1988; Rothman and Olney 1986). Therefore, the NMDA receptor is considered as a target for the prevention of neuronal death after cerebral ischemia (Brauner-Osborne et al. 2000; Wong and Kemp 1991). Several NMDA antagonists, including MK-801, provide substantial protection against ischemic injuries induced by oxygen-glucose deprivation in vitro or occlusion of middle cerebral artery (MCAO; Goldberg et al. 1987; Simon et al. 1984).

Physiological NMDA receptor activity, however, is also crucially involved in the induction of long-term potentiation (LTP) in many brain areas (Collingridge and Bliss 1995). LTP has been proposed as a neural mechanism of memory, and NMDA receptor antagonists might therefore be expected to interfere with memory acquisition. Non-competitive NMDA-receptor antagonist MK-801 has been shown side effects such as psychosis and hyperlocomotion (Ginsberg 2008; Ellison 1995; Ouagazzal et al. 1993) and learning and memory deficits in rodents (Bardgett et al. 2003; Upchurch and Wehner 1990; Wozniak et al. 1996). Thus, some researchers are making great efforts to find a therapeutic agent which has dual effects that can protect neuronal cell death against excitotoxicity and improve memory impairment.

We isolated a single compound, N-[3-(2,4-dioxo-1,4-dihydro-2H-quinazolin-3-yl)propyl]-N-{4-[3-(2,4-dioxo-1,4-dihydro-2H-quinazolin-3-yl)propylamino]butyl}acetamide (SP-8203; Fig. 1), from the coelomic fluid of the live earth worms, *Eisenia andrei*, after electric shock. It can also be organically synthesized. Earth worms extract, a kind of traditional medicine, has been used for treatment for stroke. It is also reported that the protease fractionated from the earth worms, *E. andrei*, appeared to be very stable and showed greater antithrombotic activity than other currently used antithrombotics (Lee et al. 2007). In previous report, we also reported that SP-8203 has anti-oxidant effect via SOD activity and is suggested as a potential drug for brain ischemic injury (Noh et al. 2011), but its action was rather indirect and secondary. Furthermore, we didn't test whether SP-8203 has ameliorating effect on learning and memory deficits. Therefore we wondered whether SP-8203 derived from earth worms has beneficial effects on neuronal cell death and memory impairment in cerebral ischemia as an NMDA antagonist.

In this study, we examined whether SP-8203 could displace glutamic acid binding to the NMDA receptor. We also checked if SP-8203 could block NMDA-induced excitotoxicity and protect neurons against cell death in vitro and in vivo model of cerebral ischemia and improved ischemic injury-induced memory impairments in rats.

* Corresponding author. Tel.: +82 2 740 8285; fax: +82 2 745 7996.
E-mail address: yhsuh@snu.ac.kr (Y.-H. Suh).

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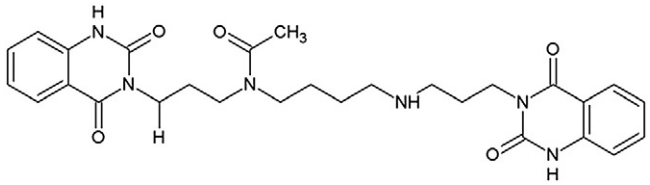

Fig. 1. The structure of SP-8203. The structure of SP-8203 (N-[3-(2,4-dioxo-1,4-dihydro-2H-quinazolin-3-yl)propyl]-N-{4-[3-(2,4-dioxo-1,4-dihydro-2H-quinazolin-3-yl)propylamino] butyl}acetamide) was shown.

2. Materials and methods

2.1. Chemicals

SP-8203 was supplied by the Central Research Institue, Shinpoong Pharmaceutical Company (Ansan, South Korea). It was discovered by purifying the coelomic fluid of the live earth worm, *E. Andrei*, after electric shock. It can also be organically synthesized and we used synthesized form of SP-8203. MK-801, NMDA, AMPA, kainic acid, glutamic acid and quisqualic acid were purchased from Sigma (MO, USA). [^{14}C]L-glutamic acid (Specific activity 250 mCi/mmol) was purchased from PerkinElmer (MA, USA). SP-8203, MK-801 and NMDA were dissolved in phosphate-buffered saline (PBS) and prepared just before use.

2.2. Primary cortical neuron cultures mixed with glial cells

Cortical cell cultures containing neuronal and glial cells were obtained as previously described (Choi et al. 1987). Briefly, first glial cell cultures were established from newborn mice (postnatal day 1–3), then 3 weeks later neurons were isolated from cerebral cortices of ICR mouse embryos at 13–14 day of gestation and seeded on top of the glial cell cultures.

For glial cell culture, dissociated cortical cells from newborn mice were plated in 6-well plate at a density of 1×10^6 cells/well. The cells were seeded in Dulbecco's Modified Eagle Medium (DMEM, WelGENE Inc., Daegu, South Korea) supplemented with 10% heat-inactivated horse serum (WelGENE Inc., Daegu, South Korea), 10% fetal bovine serum (FBS, GibcoBRL, NY, USA) and media replacement was carried out twice a week. The culture was incubated at 37 °C in a humidified 5% CO_2 atmosphere.

For neuronal cell culture, dissociated cortical cells isolated from mouse embryos were seeded on top of the glial cell cultures at a density of 1×10^6 cells/well. After overnight incubation in DMEM supplemented with 10% FBS and 10% horse serum, the media was replaced by FBS-free medium containing 10% horse serum. The culture was incubated at 37 °C in a humidified 5% CO_2 atmosphere and the media replacement was carried out twice a week. Experiments were performed after 12–13 days.

2.3. LDH assay

Cell death was determined by measuring LDH release from the damaged cells, which is a marker of cell necrosis. Primary cortical neurons mixed with glial cells were pre-treated with 87.5, 175, or 350 μM of SP-8203, or 3.3, 10, 30 μM of MK-801 and then co-treated with 100 μM or 0, 100, 200, 300, 400, 500 μM of NMDA. After 20 min, cell death was determined by LDH release. LDH was measured in culture medium (lacking serum) at room temperature using a Cytotox96 nonradioactive cytotoxicity assay kit (Promega, WI, USA) according to the manufacturer's instructions. The percent of neuronal death was normalized to the mean LDH value released after 80% death of neurons on 20 min exposure to 100 μM NMDA as 100%.

2.4. Radioligand binding assay

2.4.1. Crude synaptic membrane preparation

Membrane preparation for NMDA receptor was obtained from rat brain cortex. Brain tissues were homogenized in a Potter-Elvehjem homogenizer with 10 volumes of 20 mM HEPES buffer (pH 7.4) containing 1 mM EDTA at 4 °C and centrifuged at 40,000 *g* for 10 min. Supernatant was decanted and then added homogenization buffer to the pellet. The mixture were resuspended and recentrifuged at 40,000 *g* for 10 min. The pellet was resuspended again in homogenization buffer, and the suspension was incubated for 30 min at 37 °C. The suspension was centrifuged for 10 min at 40,000 *g* and resuspended in HEPES buffer without EDTA. The final pellets were stored at −70 °C.

2.4.2. Receptor binding assay

L-[^{14}C]glutamic acid was used to measure competitive binding of SP-8203 with NMDA receptor. Assay tube contained 100 μl L-[^{14}C] glutamic acid (final concentration 1 μM), 100 μl SP-8203 (0.1–1000 μM) and 750 μl membranes (200 μg of protein). 20 mM HEPES buffer was added to each tube till a final volume of 1 ml. The mixture was incubated for 1 h at room temperature. Incubation was terminated by rapid filtration using Whatman GF/B filters. Radioactivity was measured using MicroBeta^2 (PerkinElmer, MA, USA). Non-specific binding was determined in the presence of 1 mM unlabeled L-glutamic acid. For specific binding of NMDA receptor, the radioligand binding assay were performed using L-[^{14}C]glutamic acid in presence of quisqualic acid 5 μM and kainic acid 10 μM to block AMPA, kainate and metabotrophic Glutamate receptor. All individual assays were carried out in replicates of three. Data analysis and plotting was conducted using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA).

2.5. Calcium imaging

Fluo-4 AM was used to measure intracellular calcium levels. Primary cortical neurons mixed with glial cells were loaded with 1 μM Fluo-4 AM (Molecular Probes, USA) for 5 min at room temperature. Cells were superfused with Hanks' Balanced salt solutions (HBSS, pH 7.1, Invitrogen co., USA), gassed with oxygen at 37 °C. For imaging of the Fluo-4 fluorescence, excitation light was provided by an argon laser at 488 nm and the emission was filtered with a 515 nm long pass filter. Images were acquired using the photomultiplier of the Zeiss LSM 510. For continuous monitoring of Fluo-4 fluorescence, time series of images were obtained at regular intervals (60 s). After the injections of vehicle (HBSS), 350 μM SP-8203 or 30 μM MK-801 in media, the change of fluorescence of each experiment was measured for 4 min. After 4 min of the measurement, 1.2 mM $CaCl_2$ and 100 μM NMDA were inserted in media and the change of fluorescence of each experiment was measured for another 6 min. Analysis of fluorescence intensity was performed off-line after the image acquisition by averaging the fluorescence intensity values using the imaging software of Zeiss LSM.

2.6. Animals

120 rats were used in this study. Nine week-old male Wistar rats weighing 280–310 g (Central Laboratory Animal incorporation) were used in this study and allowed to acclimatize for at least 5 days prior to experimentation. Animals were housed in clear Plexiglas cages containing sawdust at a room temperature of 22 ± 2 °C and a relative humidity of 50 ± 10% on a 12-h light/dark cycle and given food and water ad libitum. All animals were handled in accordance with the Guidelines for Animal Experiments of Ethics Committee of Seoul National University (Publication No. 1653, revised 2008).

2.7. Focal cerebral ischemia surgery

Transient middle cerebral artery (MCA) occlusion was induced by using a suture-occlusion technique (Nagasawa and Kogure 1989) as described previously (Noh et al. 2011). Briefly, after pentobarbital injection (25 mg/kg, i.p.), the left common carotid artery was exposed after a midline cervical incision. The external carotid artery and the extra-cranial branch of the internal carotid artery were ligated. The origin of MCA was then occluded by introducing 4–0 black silk suture with a tip through the stump of the external carotid artery. The black silk was secured in place with a ligature, and the wound was closed. After 1 h of occlusion, the silk was removed to restore blood flow.

2.8. Administration with SP-8203

One group of MCA-occluded rats was injected intraperitoneally (i.p.) with 10 or 20 mg/kg of SP-8203 before 30 min and after an hour of the MCA-occlusion operation. Timing of drug administration was determined considering pharmacokinetics of SP-8203 (Lee et al. 2010). Another group of rats was injected i.p. with 3 mg/kg of MK-801. Normal saline were injected with the same volume of SP-8203 injection in the other group.

2.9. Estimation of cerebral infarct size

Animals were sacrificed at day 14 after MCA-occlusion operation and infarct volume of each focal ischemia model rat was analyzed as previously described (Noh et al. 2011). After anesthetization (3 mg/kg, i.p.) with pentobarbital, the brain of each rat was isolated, coronally sectioned into 1 mm thick, and placed in 2% 2,3,5-triphenyltetrazolium chloride (TTC) at 37 °C for 15 min and immersion-fixed in a 4% paraformaldehyde. TTC-stained sections were photographed and the digital images were analyzed using image analysis software (Image J 1.42q). The lesion volume was calculated by multiplying the area by the thickness of slices. To compensate for the effect of brain edema, the corrected infarct volume was calculated by the following equation: corrected infarct area = contralateral hemisphere area − (ipsilateral hemisphere − infarction area) (Swanson et al. 1990).

2.10. Morris water-maze task

The experimental apparatus, which is a circular water tank (140 cm in diameter, 45 cm high) was filled with opaque water made by adding dry milk powder to water at the temperature of 21–23 °C and located in a laboratory that contained prominent extra-maze cues. Animals are required to find a submerged platform (15 cm in diameter, 35 cm high) in the pool using the spatial cues. The two starting points were changed daily. Spatial training consisted of five sessions, (two trials per session per day) during which the platform was left in the same position. In each training session, the latency to escape on to the hidden platform was recorded. After final train session, a single probe trial was conducted. The escape platform was removed, and each rat was allowed to swim for 90 s in the maze. The number of times the rat crossed the annulus where the platform had been located was recorded. Data collection was automated by a video image motion analyzer (Ethovision, Noldus Information Technology h.v., Netherlands).

2.11. Statistical analysis

Data were analyzed using SAS 9.1 (Cary, NC, USA). Two comparisons were made using Student *t*-test and multiple comparisons were made using one-way ANOVA and two-way ANOVA tests followed by the Newman–Keuls multiple comparison test. All data were presented as means ± S.E.M. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ were considered to be significant.

3. Results

3.1. SP-8203 blocks NMDA neurotoxicity in a competitive manner

We checked whether SP-8023 could protect neuronal cells against NMDA-induced cell death. The structure of SP-8203 is shown in Fig. 1. Local toxicity in the brain was associated with not only neuron but also glial cell, we applied co-culture system to take condition similar with human body. We treated NMDA just for 20 min to know the effect of SP-8203 on short-term action of NMDA. The percent of LDH release was obtained by comparing to the maximal release of only NMDA-treated group as 100%.

The pretreatment with SP-8203 at 87.5, 175 or 350 μM significantly attenuated neuronal death induced by NMDA at 100 μM in neuronal cultured cells compared with NMDA alone-treated cells (Fig. 2A). SP-8203 at 87.5, 175 or 350 μM decreased the LDH release (54, 61 and 65% respectively, $p<0.05$). Pretreatment with MK-801 at 3.3, 10 or 30 μM also significantly decreased the LDH release induced by NMDA 100 μM (57, 54 and 64% respectively, $p<0.05$).

To know whether the action of SP-8203 could relate to the NMDA antagonism, the effect of SP-8203 was compared to that of MK-801 on NMDA-induced toxicity. In neuronal cultured cells, LDH release was measured after treatment with NMDA at 0, 100, 200, 300, 400 or 500 μM. MK-801, at 3.3, 10, 30, 90 μM maintained the inhibition of LDH release even at a high dose of NMDA in neuronal cultured cells compared to NMDA alone (Fig. 2C). It indicated that MK-801 inhibited NMDA in a non-competitive manner. SP-8203, however, could not inhibit the LDH release at a high dose of NMDA (Fig. 2B). In order to show competitive interaction we perform a Schild Plot using the existing data of Fig. 3A, plotting Log (dose ratio – 1) on y axis against Log concentration of SP-8203 on the x axis. Dose ratio is the ratio of the IC_{50} of NMDA dose–response curve with blocker over the IC_{50} of NMDA with no blocker. The plot gave a straight line with slope of 1.0 (Fig. 2D), suggesting that SP-8203 competitively inhibits NMDA receptor function.

To investigate the effect of SP-8203 on the other excitatory amino acid receptors, we performed LDH assay using AMPA and kainate. 1.75 μM of SP-8203 also blocked AMPA-induced cell death by 53.4% comparing with only AMPA group as a 100%, but it did not block kainate-induced cell death by 24.3% comparing with only kainate group as a 100% that was statistically not significant (data not shown). 10 μM of NBQX known as an AMPA/kainate antagonist blocked AMPA-induced cell death by 91.5%, and kainate-induced cell death by 90.9%. It is indicating that SP-8203 may act as NMDA/AMPA antagonist.

3.2. SP-8203 competes with glutamic acid for the binding site of NMDA receptors

To elucidate whether SP-8203 could bind to NMDA receptor as a competitive antagonist, we conducted receptor binding assay using L-[^{14}C]glutamic acid. Nonlinear curve-fitting analysis demonstrated that specific L-[^{14}C]glutamic acid binding to rat cortical membranes was decreased by SP-8203 (Fig. 3A). It shows that SP-8203 directly compete with L-glutamic acid for the binding site of NMDA receptor. Scatchard plot of L-[^{14}C]glutamic acid binding showed two class binding sites of NMDA receptor, high affinity and low affinity ones (Fig. 3B), and Kd value of SP-8203 was 0.2 μM and 28 μM, respectively. Hill plot analysis in high dose of SP-8203 gave a straight line with a slope of 1.0 (Fig. 3C), suggesting that SP-8203 does not affect affinity in the other receptor. In a low dose of SP-8203, the slope was lower than 1.0, suggesting that SP-8203 decreases the affinity of another receptor.

We also performed radioligand binding assay using L-[^{14}C] glutamic acid in presence of quisqualic acid 5 μM and kainic acid 10 μM to block AMPA, kainate and metabotrophic Glutamate receptor for specific binding of NMDA receptor. As a result, SP-8203 also shows specific binding to the NMDA receptor (Fig. 3D).

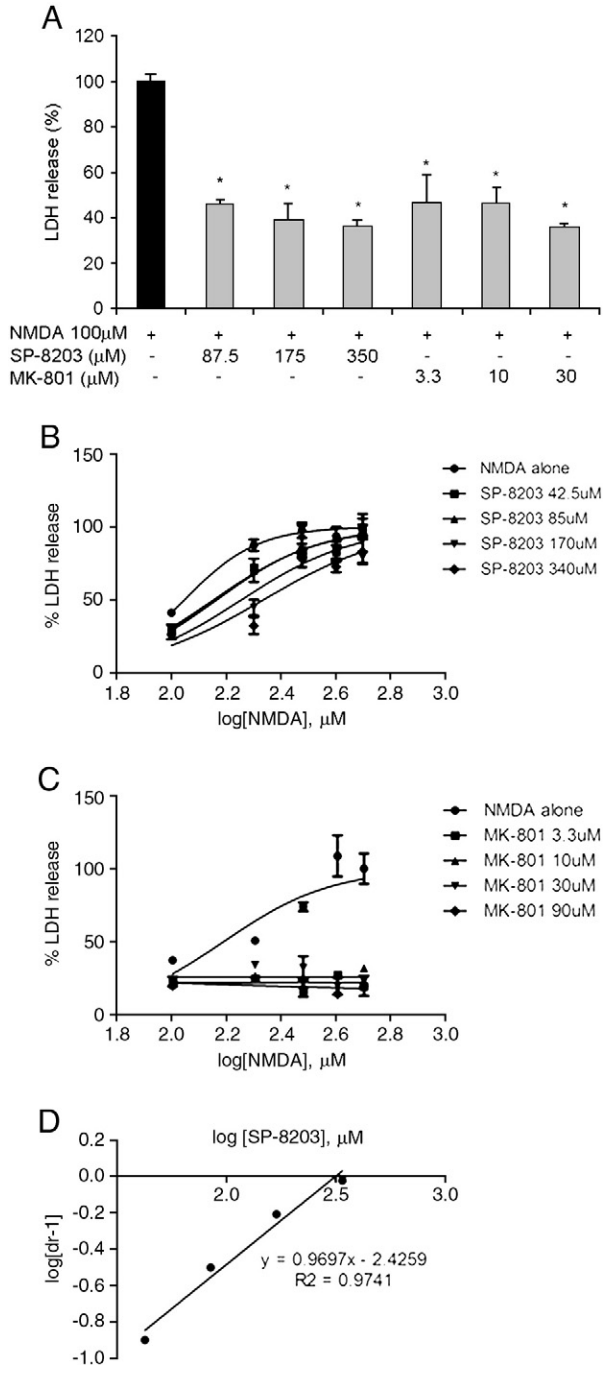

Fig. 2. Competitive blockade of NMDA-mediated excitotoxicity by SP-8203. Primary cortical neurons mixed with glial cells were pre-treated with 87.5, 175, or 350 μM of SP-8203, or 3.3, 10, 30 μM of MK-801 and then treated with 100 μM (A), 0–500 μM (B) and (C) of NMDA. After 20 min, cell death was determined by LDH release. Results were expressed as percentages of peak in three set of independent experiments compared with NMDA alone. Schild Plot for SP-8203 as a competitive antagonist of NMDA (D). Log (dose ratio – 1) on y axis against Log concentration of SP-8203 on the x axis. Dose ratio is the ratio of the IC_{50} of NMDA dose–response curve with blocker over the IC_{50} of NMDA with no blocker. $^{*}p<0.05$ compared with NMDA alone, one-way ANOVA.

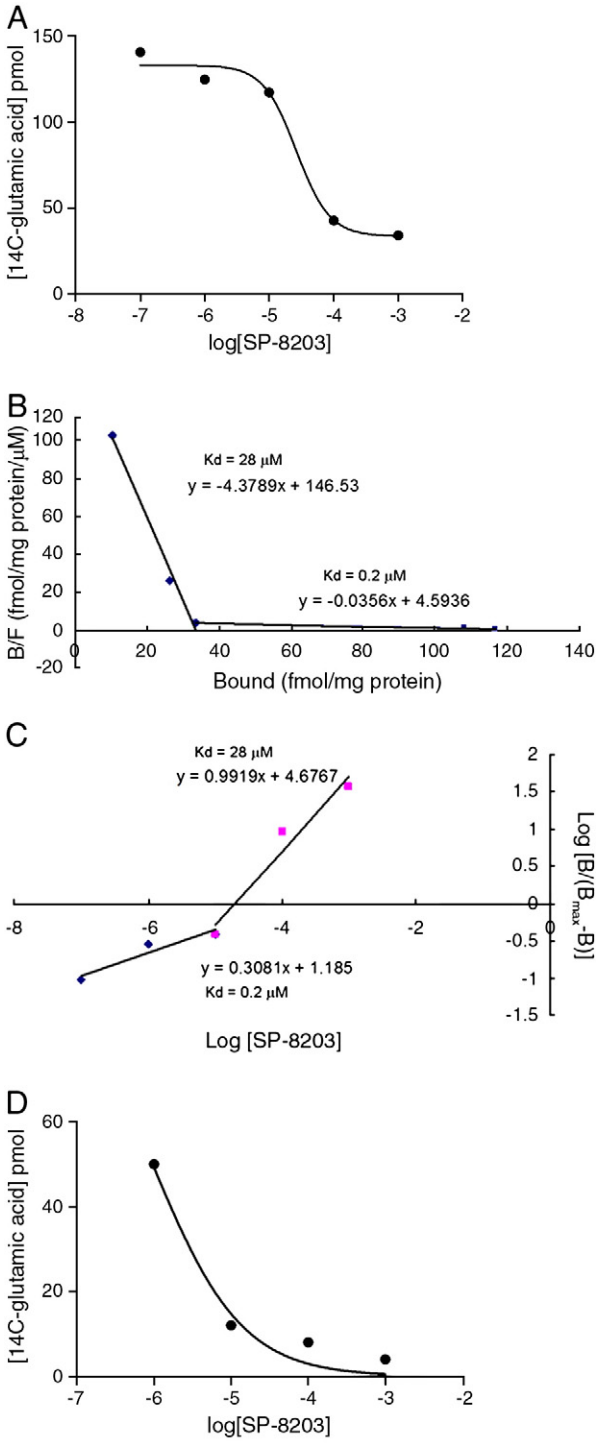

Fig. 3. SP-8203 competitively binds the glutamic acid binding site of NMDA receptor. 1 mM L-[^{14}C]glutamic acid and SP-8203 were co-incubated with rat cortex membranes, and filtrated in GF/B filters. Displacement curve of L-[^{14}C]glutamic acid binding from membranes by varying concentrations of SP-8203 (A), Scatchard plot of L-[^{14}C]glutamic acid binding to rat cortex membranes in the presence of SP-8203 (B) and Hill plot of SP-8203 displacement of L-[^{14}C]glutamic acid binding (C). Specific binding of SP-8203 to the NMDA receptor (D). 1 mM L-[^{14}C]glutamic acid, SP-8203 and quisqualic acid 5 μM and kainic acid 10 μM were co-incubated with rat cortex membranes, and filtrated in GF/B filters. Displacement curve of L-[^{14}C]glutamic acid binding from membranes by varying concentrations of SP-8203. All individual assays were carried out in replicates of three.

3.3. SP-8203 attenuates Ca^{2+} influx following activation of NMDA receptors

Excessive activation of NMDA receptors results in massive influx of Ca^{2+} that causes delayed neuronal death (Choi et al. 1987). We reasoned that the neuroprotective effect of SP-8203 against NMDA would involve intracellular Ca^{2+} influx. Intracellular Ca^{2+} levels increased 5 times after the exposure of neuronal cell cultures to 100 μM NMDA. This NMDA-induced influx of Ca^{2+} was markedly decreased by SP-8203 pre-treatment (Fig. 4). MK-801 also decreased NMDA-induced influx of

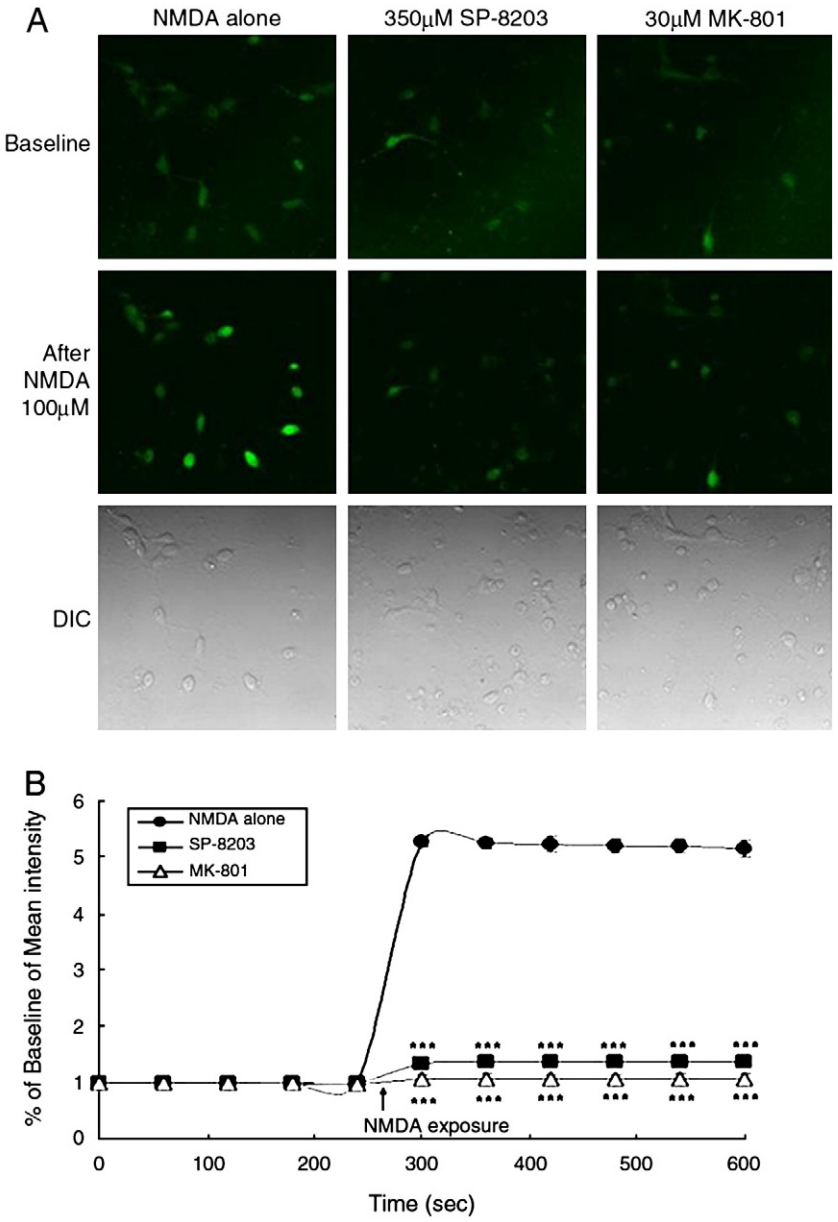

Fig. 4. SP-8203 inhibits NMDA-induced influx of Ca^{2+} in primary cultured neuron. 100 μM NMDA and 1.2 mM $CaCl_2$ were treated in primary cultured neuron mixed with glial cells with pretreatment of vehicle (HBSS), SP-8203 or MK-801. Confocal micrograph (A) and quantitative analysis of Fluo-4 AM intensity (B). Data represents mean ± S.E. Results were expressed as percentages of peak in three set of independent experiments compared with NMDA alone. ***$p<0.001$ compared with NMDA alone, two-way ANOVA.

Ca^{2+}. These results imply that SP-8203 most likely decreased the influx of Ca^{2+} ions through activation of the NMDA receptors.

3.4. SP-8203 prevents ischemic neuronal death in vivo

We next examined effects of SP-8203 on ischemic brain injuries that were known to cause neuronal death primarily through excessive activation of NMDA receptors. Approximately 250 mm^3 of cerebral infarct was observed 14 days following occlusion of middle cerebral artery (MCAO) for 60 min. When 20 mg/kg SP-8203 was injected intraperitoneally 30 min before occlusion and 1 h after reperfusion, the infarct volume was significantly reduced ($p<0.01$; Fig. 5). 3 mg/kg MK-801 also significantly reduced infarct size ($p<0.01$), and its dosage was determined by Gerasimov et al. 2004. The ED_{50} (effective dose 50) of MK-801 for neuroprotection was 0.3 mg/kg and the majority of the animals were protected against the ischemia-induced damage at doses greater than or equal to 3 mg/kg, when MK-801 was given 1 h prior to the occlusion of the carotid arteries.

3.5. SP-8203 improves spatial learning and memory impairments in MCAO rats

Finally, we assessed effects of SP-8203 on memory impairment in MCAO rats using the Morris Water Maze test 6 days after MCAO operation. At the third, fourth and fifth learning session day, 20 mg/kg SP-8203-treated group showed a shorter latency time than the vehicle-treated group ($p<0.01$, $p<0.001$; Fig. 6A) but MK-801-treated group did not show it.

To confirm whether memory impairment shown in the vehicle-treated rats was attenuated by SP-8203 treatment, we performed the probe test and recorded average staying time in zone 4 without platform. 20 mg/kg SP-8203-treated rats stayed significantly longer in zone 4 than in the other zones (zone 1, 2 and 3) ($p<0.001$; Fig. 6B), suggesting that SP-8203 significantly attenuates memory impairment by MCAO. However, there were no differences between stays in zones 1, 2, 3 and 4 in the vehicle-treated, MK-801 treated and 10 mg/kg SP-8203-treated rats, suggesting that MK-801 cannot reduce memory

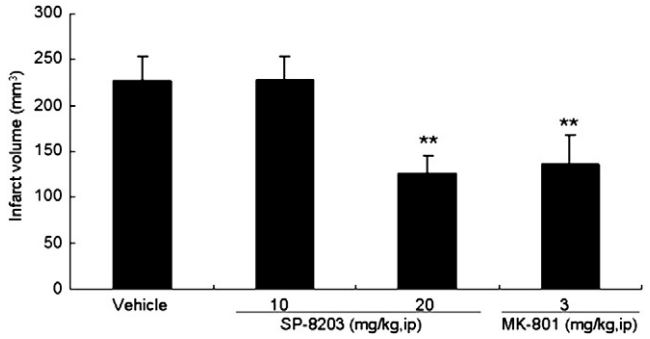

Fig. 5. SP-8203 protects neurons against ischemia in the occlusion model of MCA. Infarct volume was measured 14 days after MCAO with vehicle, SP-8203 (10 mg/kg, 20 mg/kg) or MK-801 (3 mg/kg). Mean volume ± SEM after edema correction (vehicle, n = 6; SP-8203 10 mg/kg, n = 6; SP-8203 20 mg/kg, n = 8; MK-801 3 mg/kg, n = 4). $^{**}p<0.01$ compared with the vehicle-treated control, one-way ANOVA.

impairment. Daily injection of 10 mg/kg SP-8203 also increased spatial memory in Y-maze and 0.5 mg/kg MK-801 decreased memory, but its difference was not significant (data not shown).

Although Morris water maze is memory test based on locomotor activity, it is mostly used in tMCAO model (Yonemori et al. 1999) and there was no significant difference in swim speed and distance of water maze among the different groups (Supplementary Fig. 1). However, they appeared motor deficits in Rotarod test, SP-8203 showed ameliorating effect but MK-801 didn't (Supplementary Fig. 2).

To investigate the mechanism of memory improvement by SP-8203 in pathological study, we performed hematoxylin and eosin staining but did not find any difference because MCAO model did not show any

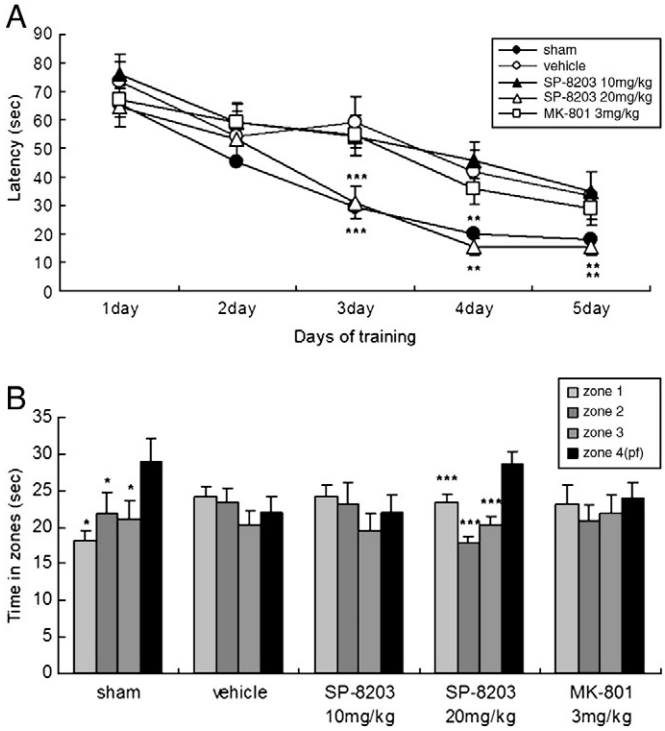

Fig. 6. SP-8203 attenuates learning and memory impairment in the occlusion model of MCA. (A) 6 days after MCAO, training session was performed over five sessions. (sham, n = 6; vehicle, n = 13; SP-8203 10 mg/kg, n = 13; SP-8203 20 mg/kg, n = 12; MK-801 3 mg/kg, n = 15) $^{**}p<0.01$ compared with the vehicle-treated control, two-way ANOVA, $^{***}p<0.001$ compared with the vehicle-treated control, two-way ANOVA. (B) The probe test was performed after the final training session. The times that rats of the SP-8203 20 mg/kg treated group stayed in zones 1, 2, 3 and 4 were compared with those of the vehicle-injected group (sham, n = 6; vehicle, n = 13; SP-8203 10 mg/kg, n = 13; SP-8203 20 mg/kg, n = 12; MK-801 3 mg/kg, n = 15) $^{*}p<0.05$, $^{***}p<0.001$ compared with the vehicle-treated control, two-way ANOVA.

pathological changes in hippocampus as previously described (Yonemori et al. 1999). Cell death was not affected in the hippocampus in MCAO model, but significantly increased in parietal cortex and piriform cortex and SP-8203 ameliorates the cell death in parietal cortex and piriform cortex, but MK-801 didn't (data not shown).

4. Discussion

The present study strongly suggests that SP-8203 blocks NMDA receptor-mediated excitotoxicity in a competitive manner through replacing glutamic acid at excitatory amino acid receptor and attenuated Ca^{2+} influx through NMDA receptors and thereby protects primary cultured neuron from NMDA-induced cell death. Systemic administration of SP-8203 also reduces infarction volume and improves memory deficits in cerebral ischemia model.

SP-8203 showed notable features of neuroprotection against excitotoxicity. This molecule purifed from earthworms, *E. andrei*, blocked NMDA-induced neuronal death at doses of 87.5 to 350 μM in a competitive manner. In addition, SP-8203 attenuates the influx of Ca^{2+} ions through activation of the NMDA receptors. These results imply that SP-8203 could inhibit Ca^{2+} current of channel function and the neuroprotective effects of SP-8203 were attributable to inhibition of Ca^{2+} influx through NMDA receptors. SP-8203 also blocked NMDA-induced cell death by 47.4% and AMPA-induced one by 53.4%, while NBQX known as an AMPA/kainate antagonist blocked AMPA-induced cell death by 91.5% comparing with AMPA or Kainate-induced cell death as 100%, indicating that SP-8203 may act as NMDA/AMPA antagonist.

We examined the neuroprotective effects of SP-8203 against brain injuries that were shown to exert neuronal death primarily through activation of ionotropic glutamate receptors (Rothman and Olney 1986). Antagonists of NMDA receptors are reported to reduce brain injury from hypoxic ischemia, especially focal ischemia (Park et al. 1988). Systemic administration of 20 mg/kg of SP-8203 markedly reduced infarct volume 14 days after occlusion of MCA. In the previous study we showed that daily injection of 10 mg/kg SP-8203 for 10 days significantly reduced infarct size in same model (Noh et al. 2011). In this study, however, 10 or 20 mg/kg SP-8203 was only given before and after MCA occlusion, only 20 mg/kg SP-8203 showed significant neuroprotective effect.

Taken together cell death and receptor binding studies with L-[^{14}C] glutamic acid, SP-8203 acts as a competitive NMDA antagonist. In the Hill plot analysis, SP-8203 can bind to high affinity and low affinity binding site of NMDA receptor as previously described (Biziere et al. 1980).

NMDA-receptor antagonist such as MK-801 has not been shown to improve neurological outcome in clinical trials for treatment of ischemic stroke. Furthermore, they elicited side effects such as psychosis and hyperlocomotion (Ginsberg 2008; Ellison 1995; Ouagazzal et al. 1993). Moreover, learning and memory deficits are shown by systemic administration of MK-801 in rodents (Bardgett et al. 2003; Upchurch and Wehner 1990; Wozniak et al. 1996) and therefore MK-801 has been used as a memory impairment model because of NMDA blockade property (Bardgett et al. 2003). However, there are some reports that competitive NMDA antagonist administration does not affect learning and memory (Gutnikov and Gaffan 1996; Villarreal et al. 2002; Bischoff and Tiedtke 1992). For example, AP5, a competitive NMDA antagonist, administration into the entorhinal cortex and basolateral amygdala does not alter the memory of fear (Schenberg et al. 2005; Maren et al. 1996). Polyamines, a NMDA modulator, have stimulatory and inhibitory effect of NMDA receptor and improve memory of aversive and non-aversive tasks as well as spatial memory (Camera et al. 2007; Velloso et al. 2009; Gomes et al. 2010; Conway 1998).

As shown in this study, quinazolione region of SP-8203, especially aromatic ring-excluded form, is similar 3 dimensional structure of glutamic acid or NMDA, strongly suggests that SP-8203 could be a competitive antagonist of NMDA. Many reports about competitive NMDA

antagonists demonstrated that they have neuroprotective effects but do not produce memory deficit (Schenberg et al. 2005; Maren et al. 1996), unlikely uncompetitive and noncompetitive NMDA antagonists. Its stem structure is similar to spermine, a kind of polyamine, which is known as NMDA receptor modulator. The polyamine spermine and its derivatives bind polyamine binding site (Han et al. 2008) and have inhibitory effects on NMDA receptor (Igarashi and Williams, 1995). Polyamines also have neuroprotective effect in a rat model of cerebral ischemia (Shirhan et al. 2004; Gilad and Gilad 1991; Clarkson et al. 2004) and memory enhancing effect in the other models such as Huntington's disease (Camera et al. 2007; Velloso et al. 2009; Gomes et al. 2010). Polyamines are effective in memory improvement but they are known to have very limited access to the brain (Shin et al. 1985; Shimada et al. 1994). When transport of SP-8203 across the blood–brain barrier of adult rats was examined by measuring amount of SP-8203 in the cerebral spinal fluid by Liquid Chromatography, ng/ml range of SP-8203 is detected, which is relatively higher than spermine administration. Taken these together, it is possible that SP-8203 may act as a competitive NMDA antagonist or NMDA modulator and may have favorable effects on neuronal cell death and memory impairment in cerebral ischemia via NMDA receptor.

SP-8203 may act through NMDA receptor as a competitive NMDA antagonist or modulator, thereby it is possible that SP-8203 could improve memory deficits. Thus, we investigated whether SP-8203 could ameliorate memory deficits in cerebral ischemia model. MCAO model in rats was known to present impairment of spatial cognitive performance (Yonemori et al. 1996; Yonemori et al. 1999). Administration of 20 mg/kg SP-8203 significantly restored memory impairment induced by MCAO in both training session and probe test of water maze. MK-801, however, does not attenuate memory impairment. Daily injection of 10 mg/kg SP-8203 also increased spatial memory in Y-maze.

Previous report suggests that reductions in infarct volume after MCAO positively correlate with improvement in memory impairments (Ellsworth et al., 2003). In the present result, however, it seems that there is no difference between the effects of SP-8203 and MK-801 on infarct volume of MCAO rats. To investigate the mechanism of memory improvement by SP-8203 in pathological study, we performed hematoxylin and eosin staining but did not find any difference because MCAO model did not show any pathological changes in hippocampus as previously described (Yonemori et al. 1999). Cell death was not found in the hippocampus in MCAO model, but significantly increased in parietal cortex and piriform cortex and SP-8203 ameliorates the cell death in parietal cortex and piriform cortex, but MK-801 didn't. Therefore, we could guess decrease of cell death in cortex region is one of plausible mechanisms of memory improvement of SP-8203.

In conclusion, SP-8203, as a NMDA antagonist, has dual effects that protect cells against excitotoxicity and improve memory impairment in brain ischemic injuries. Taking these in vitro and in vivo results together, our study suggests that SP-8203 plays potentially therapeutic roles in cerebral ischemia or brain ischemic injuries.

Supplementary materials related to this article can be found online at doi:10.1016/j.pbb.2011.07.018.

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