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Research Paper

Pretreatment with the ciclosporin derivative NIM811 reduces delayed neuronal death in the hippocampus after transient forebrain ischaemia

Masaaki Hokari, Satoshi Kuroda and Yoshinobu Iwasaki

Department of Neurosurgery, Hokkaido University Graduate School of Medicine, Sapporo, Japan

Abstract

Objectives There have been several previous studies showing that ciclosporin, a ligand for cyclophilin D (CypD), reduces mitochondrial permeability transition (mPT) and ameliorates delayed neuronal death. NIM811 is a non-immunosuppressive ciclosporin derivative that also inhibits mPT, but has significantly less cytotoxicity than ciclosporin. Actually, in animal experiments, several investigators have reported that NIM811 ameliorates central nervous system disorders, such as traumatic brain injury, transient focal cerebral ischaemia and spinal cord injury. Therefore, we evaluated whether the ciclosporin derivative, NIM811 reduces mPT and ameliorates delayed neuronal death in the hippocampal CA1 sectors in mice when subjected to transient forebrain ischaemia.

Methods Male C57BL/6 mice were treated with 50 mg/kg ciclosporin, 10, 50 or 100 mg/kg NIM811 or phosphate-buffered saline. At 30 min post-injection, all mice were subjected to 20 min bilateral common carotid artery occlusion (BCCAO). To estimate delayed neuronal death, the sections were prepared for HE staining and terminal deoxynucleotidyl transferase-mediated dUTP end-labelling (TUNEL) staining at 72 h after 20 min BCCAO. Furthermore, using 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1) staining technique, we evaluated whether NIM811 (1, 10, 100 or 1000 μM) inhibited mPT in the neurons exposed to 100 μM glutamate.

Results Both delayed neuronal injury and apoptosis in the hippocampal CA1 sectors were significantly ameliorated at 72 h after transient forebrain ischaemia in the mice treated with 100 mg/kg NIM811 or 50 mg/kg ciclosporin. The treatments with 100 μM and 1000 μM NIM811 significantly inhibited the reduction of mitochondrial membrane potential in the neurons exposed to 100 μM glutamate.

Conclusions These findings strongly suggest that NIM811 inhibits mPT and ameliorates delayed neuronal death in mice subjected to transient forebrain ischaemia.

Keywords delayed neuronal death; hippocampus; NIM811; reduced mitochondrial permeability transition; transient forebrain ischaemia

Introduction

Delayed neuronal death has been attributed to many factors, including glutamate neurotoxicity, calcium influx, expression of cell suicide genes, activation of apoptotic proteins, mitochondrial dysfunction, endoplasmic reticulum dysfunction and oxygen free radicals.[1–3] Above all, many investigators have suggested that mitochondrial permeability transition (mPT) may be a final common pathway for apoptotic cell death of hippocampal CA1 neurons after transient forebrain or global ischaemia.[4–8] Thus, previous studies have shown that ciclosporin (cyclosporin A), a ligand for cyclophilin D (CypD), reduces mPT and ameliorates delayed neuronal death.[8–11] Ciclosporin is an effective immunosuppressant that has been clinically used to prevent allograft rejection and to treat autoimmune disorders. However, there is clear clinical evidence for the neurotoxic effect of ciclosporin,[12,13] so its therapeutic clinical efficacy is limited.[14]

NIM811 is a non-immunosuppressive ciclosporin derivative that inhibits mPT, but has significantly less cytotoxicity than ciclosporin.[6,15,16] Actually, in animal experiments, several investigators have reported that NIM811 ameliorated central nervous system disorders, such as traumatic brain injury,[17] transient focal cerebral ischaemia[18] and

Correspondence: Dr Masaaki Hokari, Department of Neurosurgery, Hokkaido University Graduate School of Medicine, North 15 West 7, Kita-ku, Sapporo 060-8638, Japan.
E-mail: karimasa@med.hokudai.ac.jp

spinal cord injury.[19,20] However, there have been no reports showing that NIM811 ameliorates delayed neuronal death in the hippocampal CA1 sectors.

Based on these considerations, therefore, we evaluated whether the ciclosporin derivative NIM811 reduces mPT and ameliorates delayed neuronal death in the hippocampal CA1 sectors in mice subjected to transient forebrain ischaemia. Furthermore, we ran an in-vitro experiment to assess whether NIM811 inhibits mPT in cultured neurons when exposed to the excitotoxic amino acid glutamate. The findings strongly suggest that NIM811 may ameliorate delayed neuronal death through inhibiting ischaemia-induced mPT in the hippocampal CA1 sectors.

Materials and Methods

Drug preparation in-vivo

Ciclosporin was dissolved in dimethyl sulfoxide (DMSO; Sigma, St Louis, USA) at a concentration of 5 mg/ml. NIM811 (*N*-methyl-4-isoleucine-cyclosporin) was a generous gift from Novartis Pharma Ltd, (Basel, Switzerland) and was also dissolved in DMSO at a concentration of 1, 5 and 10 mg/ml.

Surgical procedure

All animal experiments were approved by the Animal Studies Ethical Committee at Hokkaido University Graduate School of Medicine. All surgical techniques were performed under aseptic conditions and all drugs were administered in a volume of 10 ml/kg body weight (peritoneal injection). Male C57BL/6 mice (20–25 g, 10–12 weeks old) were purchased from SLC laboratories (Hamamatsu, Japan). Mice were allowed free access to food and water and housed in a climate-controlled environment (25°C). Anaesthesia was induced by inhalation of 4.0% isofulurane in N_2–O_2 (70 : 30%); a surgical procedure was performed under spontaneous ventilation in 1.5–2.0% isoflurane in N_2–O_2 (70 : 30%).

Then, 30 mice were administered 10 ml/kg dilution, including 50 mg/ml ciclosporin ($n = 7$), 10 mg/ml NIM811 ($n = 5$), 50 mg/ml NIM811 ($n = 5$), 100 mg/ml NIM811 ($n = 5$) or phosphate-buffered saline (PBS; $n = 8$). Therefore, in this study, we classified them into five experimental groups, including N10 (NIM811 10 μM), N50 (NIM811 50 μM), N100 (NIM811 100 μM), CsA50 (ciclosporin 50 μM) and vehicle groups. At 30 min post-injection, all mice were subjected to 20 min bilateral common carotid artery occlusion (BCCAO). Under a surgical microscope, the bilateral common carotid arteries were exposed after a midline cervical skin incision. The bilateral common carotid arteries were occluded with small aneurysmal clips. Aneurysmal clips were removed 20 min after the start of BCCAO. Core temperature was maintained at 36.5–37.5°C during the procedures by using a thermometer connected to a heating lamp and pad (BWT-100; Bio Research Center Co, Ltd, Tokyo, Japan). In addition, cerebral blood flow (CBF) in the PBS group was measured through the skull, using a laser-Doppler flowmetry (FLO-C1; Omega Wave, Tokyo, Japan). Also, we performed a sham operation in which the bilateral common carotid arteries were only separated and not occluded ($n = 4$).

Histological examination

At 72 h after 20 min BCCAO, the mice were deeply anaesthetized by inhalation of 4.0% isoflurane in N_2–O_2 (70 : 30%) and were transcardially perfused with heparinised saline followed by 4% buffered formalin solution (pH 7.4). The brains were removed and embedded in paraffin, and 5-μm thick coronal sections were prepared for subsequent staining.

To estimate delayed neuronal death in the hippocampal CA1 sectors, the coronal sections at 72 h after 20 min BCCAO were stained with hematoxylin–eosin (HE). Morphologically viable neurons were identified on HE staining by a large round perikaryon with a thin cytoplasm within a compact and regular neuropil, whereas non-viable ischaemic neurons had shrunken perikaryon, which were triangular in shape with vacuolation of the neuropil. The numbers of morphologically normal neurons and neurons showing the features of ischaemic cell change were counted in the CA1 pyramidal cell layer. Furthermore, to evaluate the distribution of apoptotic cells, the sections at 72 h after 20 min BCCAO were also stained using ApopTag Kit (Chemicon International, Inc, Temecula, USA), according to the manufacturer's instructions. Apoptotic neurons were identified on deoxynucleotidyl transferase-mediated dUTP end-labelling (TUNEL) sections by the deeply brown-stained cells in the hippocampal CA1 region. Owing to the possible occurrence of unilateral lesions or asymmetric density of hippocampal neuronal cell death, the values from the hemisphere with the worst damage were used for the final analysis.

Primary culture of fetal neurons

Neuronal cells were obtained by dissociating a frozen form of fetal mouse cerebral cortex (Nerve Cell CX <M>; Sumitomo Bakelite Co., Ltd, Tokyo, Japan) according to the manufacturer's protocol. The cells were seeded on poly-D-lysine-coated dishes (1.7 cm^2/well) at a cell density of $2 \times 10^5/cm^2$. According to the manufacturer's guide, these neurons can be used to evaluate glutamate cytotoxity after two weeks' culture. Therefore, they were cultured in Nerve-Cell Culture Medium (Sumitomo Bakelite Co., Ltd, Tokyo, Japan) for 14 days. The medium was replaced every three days.

Treatment with glutamate and NIM811

In our previous experiment,[21] glutamate-induced damage of cultured neurons was dose dependent, and about half of the neurons were damaged when exposed to 100 μM of glutamate for 10 min. In this study, therefore, the cultured neurons were exposed to 100 μM of glutamate for 10 min. To examine whether NIM811 reduces mPT *in vitro*, four experimental groups were studied as follows. In the control group, the neurons were exposed to 100 μM glutamate for 10 min and were cultured in culture medium lacking glutamate for 30 min. In the next group (Group N1), the neurons were incubated with 1 μM NIM811 for 30 min. Subsequently, the neurons were exposed to 10 μM glutamate for 10 min and then were cultured in culture medium lacking glutamate for 30 min. In the other groups, the neurons were pre-incubated with 10 μM (Group N10), 100 μM (Group N100) and 1000 μM

NIM811 (Group N1000) for 30 min before glutamate exposure, respectively, and then were cultured in culture medium lacking glutamate for 30 min.

Measurement of mitochondrial membrane potential

To estimate changes in mitochondrial membrane potential in cultured neurons, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1) was used, as previously reported.[22] Briefly, JC-1 (Molecular Probes, Eugene, USA) stocked in DMSO was diluted in media to a concentration of 10 μM. Cells were incubated for 30 min in the presence of 10 μM of JC-1 at 37°C, and then were washed twice. Image of JC-1 fluorescence were acquired using a confocal laser scanning microscope (MRC-1024; Bio-Rad, Hemel Hempstead, UK) 30 min after glutamate exposure with or without pre-treatment with various concentrations of NIM811. As a monomer, JC-1 emits green light, but the emission changes to red when JC-1 aggregates as JC-1 concentrates within the mitochondria. Cultured neurons were excited with 488-nm light, and the emitted fluorescence was imaged at 530 and 600 nm. Ratios of fluorescence from aggregates to that from monomers (JC-1 ratio) were calculated, and were normalized by comparison with the control red/green ratio. The average of the normalized JC-1 ratio of randomly selected 10 neurons per well was used as an estimate of the mPT of the well. The normalized JC-1 ratio in each well was quantified and values are expressed as mean ± SD of a total of 4 wells.

Statistical analysis

Continuous data were expressed as mean ± SD. We used the Kruskal–Wallis test to estimate the differences of the various treatments, and then we used Dunn's test to evaluate individual differences between the treatments. $P < 0.05$ was considered statistically significant.

Results

Physiological data during the procedure

Core temperature and CBF during and after transient forebrain ischaemia in mice are presented in Table 1. Core temperature in all groups was maintained at 36.5–37.5°C during the procedures. The CBF markedly decreased to about 10% of the baseline value immediately after the initiation of BCCAO and remained constant during ischaemia. After the start of reperfusion, CBF recovered to about 105% of the baseline value, and gradually decreased to about 90% of the baseline value.

NIM811 ameliorates delayed neuronal injury after transient forebrain ischaemia

All mice survived for 24 h following 20 min BCCAO. However, two of eight mice in the PBS group, three of seven mice in the CsA50 group and one of five mice in each of the N10, N50 and N100 groups died during the next 48 h. Mortality in the CsA50 group was higher than in the other groups, although there was no significant difference in mortality between the five experimental groups.

Representative photomicrographs on HE staining at 72 h following 20 min BCCAO are shown in Figure 1 (a–j). In the PBS group, there were numerous numbers of damaged neurons that had shrunken perikaryon and were triangular in shape with vacuolation of the neuropil, whereas such cells were fewer in the N100 and CsA50 groups. As shown in Figure 2a, the percentage of the viable neurons was 23.6 ± 22.6% in the PBS group, being significantly lower than the 83.4 ± 3.2% noted in the N100 group ($P = 0.003$) and 63.1 ± 26.4% in the CsA50 group ($P = 0.04$).

Furthermore, representative findings on TUNEL staining are presented in Figure 1 (k–o).

In the PBS group, the number of TUNEL-positive neurons was 83.0 ± 51.0 in the hippocampal CA1 sectors. However, the values were 13.5 ± 4.7 and 13.3 ± 5.4 neurons in the N100 and CsA50 group, respectively. As shown in Figure 2b, the number of TUNEL-positive cells in the hippocampus was significantly smaller in the N100 and CsA50 groups than in the PBS group, respectively ($P = 0.04$, 0.041).

On the other hand, in the sham operation group we observed no ischaemic changes on histological examination (data not shown).

NIM811 maintains mitochondrial membrane potential in glutamate-exposed neurons

The cultured neurons were exposed to 100 μM glutamate for 10 min, and the effect of NIM811 on their mPT as examined using the JC-1 staining technique. Representative confocal images of JC-1 fluorescence are shown in Figure 3. In the

Table 1 Core temperature and cerebral blood flow during and after transient forebrain ischaemia in mice

Time (min)	Core temperature(°C)						Cerebral blood flow (%)
	PBS	CsA50	N10	N50	N100	Significance	PBS
0 (just before BCCAO)	37.1 ± 0.4	37.0 ± 0.4	37.0 ± 0.2	37.0 ± 0.3	37.1 ± 0.5	none	100%
5	37.2 ± 0.1	37.1 ± 0.3	37.3 ± 0.2	37.3 ± 0.1	37.3 ± 0.1	none	9.3 ± 2.9%
10	37.3 ± 0.2	37.1 ± 0.3	37.3 ± 0.1	37.3 ± 0.1	37.2 ± 0.2	none	9.6 ± 3.3%
15	37.3 ± 0.1	37.1 ± 0.3	37.2 ± 0.1	37.3 ± 0.1	37.3 ± 0.2	none	8.9 ± 2.8%
20	37.1 ± 0.3	37.0 ± 0.3	37.2 ± 0.2	37.1 ± 0.2	37.1 ± 0.3	none	8.7 ± 2.9%
25 (5 min after BCCAO)	37.0 ± 0.3	37.0 ± 0.2	37.0 ± 0.2	37.0 ± 0.2	37.1 ± 0.2	none	104.6 ± 6.0%
30 (10 min after BCCAO)	37.0 ± 0.2	37.0 ± 0.2	37.0 ± 0.2	37.0 ± 0.2	37.0 ± 0.2	none	94.7 ± 14.3%

Mice were administered: PBS, phosphate-buffered saline ($n = 8$); CsA50, ciclosporin 50 μM ($n = 7$); N10, 10 μM NIM811 ($n = 5$); N50, 50 μM NIM811 ($n = 5$); N100, 100 μM NIM811 ($n = 5$). Data are means ± SD.

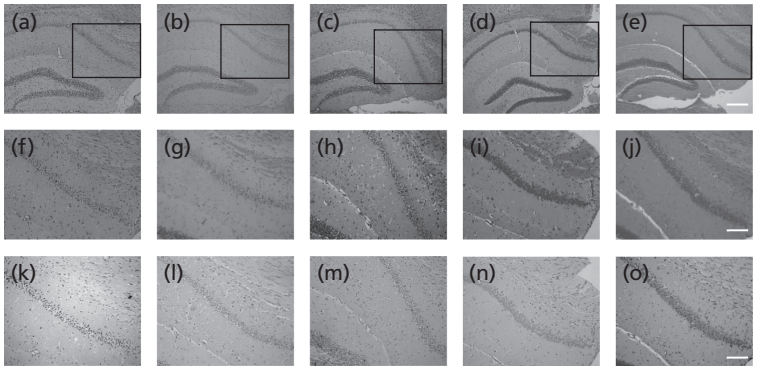

Figure 1 Representative findings on HE staining (a–j) and TUNEL staining (k–o) at 72 h after 20 min forebrain ischaemia in coronal sections of mice with bilateral common carotid artery occlusion. The sections of PBS group mice (a, f, k), CsA50 group mice (b, g, l), N10 group mice (c, h, m), N50 group mice (d, i, n) and N100 group mice (e, j, o) are shown. In the PBS group mice, there were numerous damaged neurons that had shrunken perikaryon and were triangular in shape with vacuolation of the neuropil, and TUNEL-positive cells, whereas such cells were few in the CsA50 and N100 groups. Scale bar: upper panels, 400 μm; middle and lower panels, 200 μm

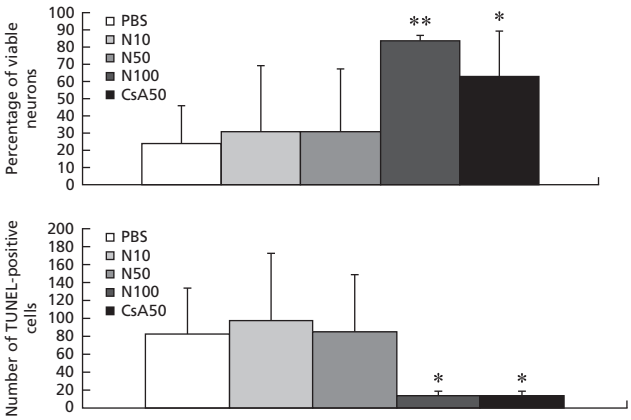

Figure 2 Percentage of viable neurons and number of TUNEL-positive cells in the hippocampus of mice with bilateral common carotid artery occlusion. Data are presented as means ± SD. The percentage of the viable neurons 72 h after 20 min forebrain ischaemia in the hippocampus was significantly higher in the CsA50 and N100 groups than in the PBS group ($^{*}P < 0.05$, $^{**}P < 0.01$). The number of TUNEL-positive cells in the hippocampus was significantly smaller in the CsA50 and N100 groups than in the PBS group ($^{*}P < 0.05$)

control group, mPT markedly decreased when exposed to 100 μM of glutamate. The normalized JC-1 ratio decreased to 0.39 ± 0.05 at 30 min after glutamate exposure (Figure 4). In Groups N1 and N10, the normalized JC-1 ratio also decreased (0.41 ± 0.14 and 0.36 ± 0.07, respectively) when the neurons were treated with 1 μM NIM811 before glutamate exposure. In Group N100, the normalized JC-1 ratio was 0.63 ± 0.06 when treated with 100 μM NIM811, this being significantly higher than the value in the control group ($P = 0.002$). In Group N1000, the normalized JC-1 ratio was 0.55 ± 0.10 when treated with 1000 μM NIM811, this being significantly higher than the value in the control group ($P = 0.02$). Consequently, treatment with 100 μM and 1000 μM NIM811 significantly inhibited the reduction of mitochondrial membrane potential due to 100 μM glutamate exposure.

Discussion

As stated, previous studies have shown that ciclosporin blocks mPT and ameliorates delayed neuronal death.[8–11] However, this is the first report showing that the ciclosporin derivative NIM811 ameliorates delayed neuronal death in the hippocampal CA1 sectors in mice subjected to transient forebrain ischaemia. In-vitro experiments support the speculation that NIM811 may do this through preventing glutamate-induced reduction of mPT.

Many investigators have suggested that mPT may be implicated in the intrinsic cell death pathway that contributes to injury after transient forebrain or global ischaemia.[4–8] It is suggested that Ca^{2+} overload, inorganic phosphates, reactive oxygen species (ROS), loss of mitochondrial membrane potential and changes in mitochondrial matrix pH could all promote mPT,[23–29] which was defined as a sudden increase in permeability of the mitochondrial inner membrane to solutes with molecular weights of 1.5 kDa or less.[4,24,30] Szabo *et al*. reported that mPT was due to the opening of a nonselective megachannel called the mPT pore.[31–33] Several proteins are thought to comprise the mPT pore, including the adenine nucleotide translocator (ANT) in the inner mitochondrial membrane and the voltage-dependent ion channel in the outer mitochondrial matrix.[34–36] Ciclosporin is believed to inhibit mPT by binding to CypD and preventing the opening of the mPT pore.[30,37] Actually, ciclosporin has been shown to provide significant protection in several models of transient cerebral ischaemic injury,[10,38–40] although there is clear clinical evidence for the neurotoxic effect of ciclosporin.[12,13]

NIM811 inhibits Ca^{2+}-induced mitochondrial swelling and mPT with significantly less cytotoxity than ciclosporin.[15,16,41] NIM811 is a ciclosporin derivative with a substitution of *N*-methyl-leucine in position 4 by *N*-methyl-isoleucine.[15] Unlike ciclosporin, its neuroprotective actions appear to be specific to its effects on mPT as NIM811 does not inhibit calcineurin, and its immunosuppressive activity is > 3000 times less than ciclosporin.[42] Recently, Argaud *et al*. have shown that specific inhibition of mPT pore opening by NIM811 at reperfusion following acute myocardial infarction ameliorates

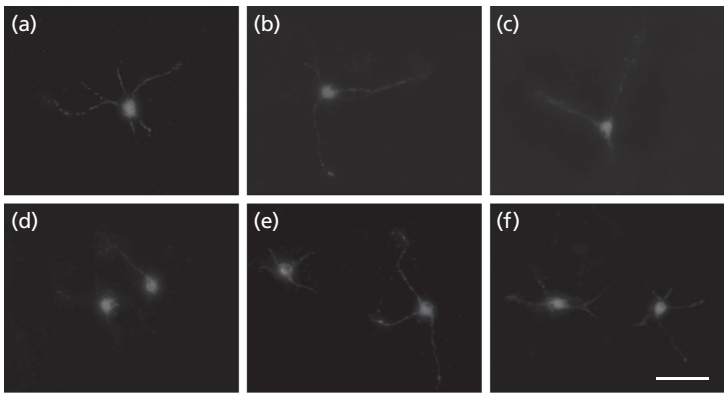

Figure 3 Representative JC-1 fluorescence overlay image of cultured neurons (at 530 and 600 nm) exposed to 100 μM glutamate for 10 min and the effect of NIM811 on their mitochondrial permeability transition. (a) JC-1 fluorescence overlay image of control. (b–f) Overlay image 30 min after glutamate exposure. (b) NIM811 0 μM. (c) NIM811 1 μM. (d) NIM811 10 μM. (e) NIM811 100 μM. (f) NIM811 1000 μM. Note that the R/G ratio in b, c and f are remarkably deteriorated, whereas those in d and e are relatively maintained. Scale bar, 50 μm

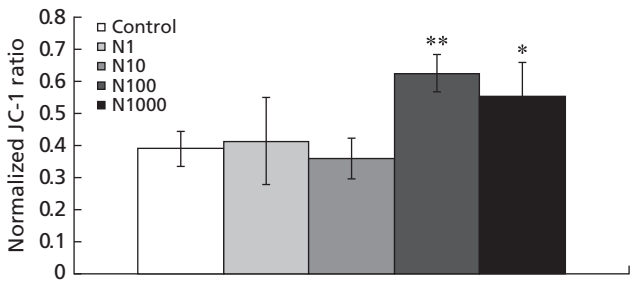

Figure 4 Normalized JC-1 ratio in each condition 30 min after exposure of cultured neurons to glutamate. Data are presented as means ± SD. The treatment with 100 μM and 1000 μM of NIM811 produced significantly less deterioration in normalized JC-1 ratio when the neurons were exposed to 100 μM glutamate ($^{*}P < 0.05$, $^{**}P < 0.01$)

necrotic and apoptotic cell death.[43] Because mPT has been implicated in the mechanisms of apoptotic delayed neuronal death,[4–8] NIM811 has been suggested as a novel agent against central nervous system disorders. Several investigators revealed the neuroprotective effect of NIM811 in several central nervous system disorders in animal experiments.[17–20] Actually, spinal cord contusion was ameliorated by pre-treatment with 40 mg/kg NIM811[20] and post-treatment with 20 mg/kg NIM811.[19] Furthermore, Mbye *et al.* reported that acute mitochondrial dysfunction after traumatic injury in mice was attenuated by NIM 811 (10 mg/kg, i.p.) and ciclosporin (20 mg/kg, i.p.).[17] Concerning focal transient cerebral ischaemia, Korde *et al.* revealed the neuroprotective effect of post-treatment with 50 mg/kg NIM811.[18] They reported that the concentration of NIM811 achieved after administration of 50 mg/kg NIM811 was ~40 μM. This dose of NIM811 was based on the identical molecular weight and similar structure to ciclosporin and the observation that treatment with 50 mg/kg ciclosporin conferred neuroprotection against transient focal cerebral ischaemia, albeit with high mortality.[40,44] Regarding transient forebrain ischaemia, Kobayashi *et al.* reported that simple injection of 50 mg/kg ciclosporin 30 min before ischaemic insult showed significantly reduced CA1 hippocampal neuron injury in gerbils.[8] A recent report showed that the concentration of ciclosporin in the brain after injection of 50 mg/kg ciclosporin was approximately 10 μM, which is known to be a sufficient concentration to prevent mPT *in vitro*.[45] Based on these considerations, therefore, we administered 10, 50 and 100 μM NIM811, and we expected that the effective dose of NIM811 would be equivalent to that of ciclosporin or a little lower. However, our results were the opposite. The present study confirmed that 50 mg/kg of ciclosporin and 100 mg/kg of NIM811 ameliorated delayed neuronal death in the hippocampal CA1 sectors in mice when subjected to transient forebrain ischaemia. The reason for this discrepancy is probably because three of seven mice in the ciclosporin 50 group were excluded (dying), or this result may be biased by the immunosuppressive effect of ciclosporin.

Conclusions

In conclusion, we demonstrate that NIM811 markedly inhibits mPT and ameliorates delayed neuronal death in the hippocampal CA1 sectors in mice. Further studies are necessary to clarify the role of NIM811 in maintaining brain mitochondrial function for clinical application.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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