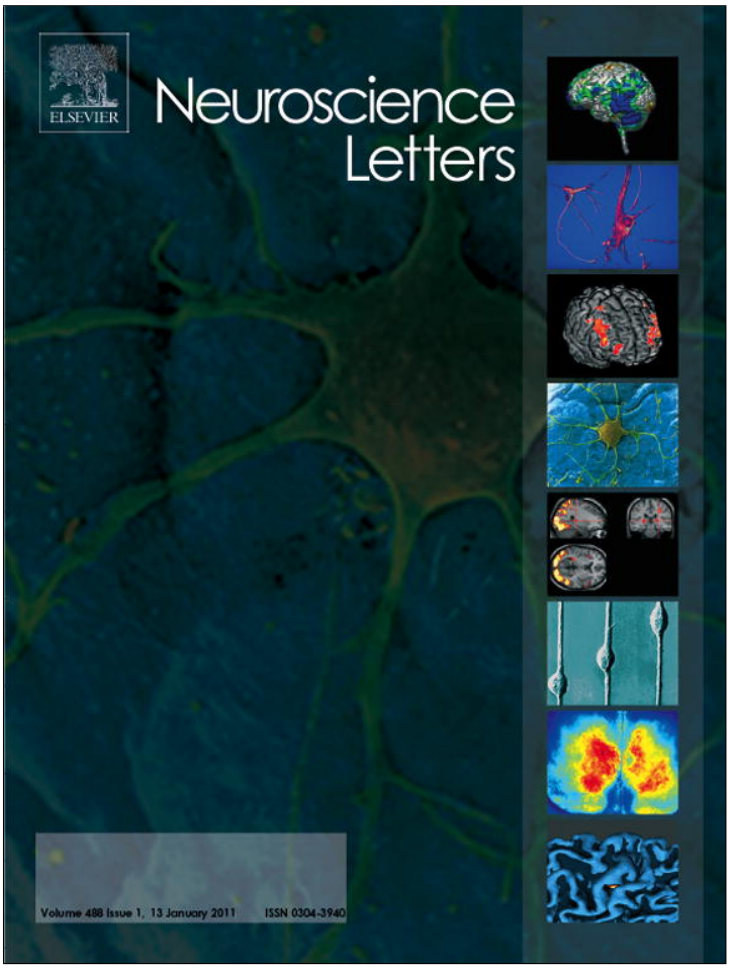

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# SUN N8075, a novel radical scavenger, protects against retinal cell death in mice

Mai Akane, Masamitsu Shimazawa, Yuta Inokuchi, Kazuhiro Tsuruma, Hideaki Hara *

*Molecular Pharmacology, Department of Biofunctional Evaluation, Gifu Pharmaceutical University, 1-25-4 Daigaku-nishi, Gifu 501-1196, Japan*

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ABSTRACT

In this study, we examined the effect of SUN N8075, a radical scavenger with neuroprotective properties, on murine retinal damage induced by intravitreous injection of *N*-methyl-D-aspartate (NMDA) or high-intraocular pressure (IOP). In both models, systemic administration of SUN N8075 decreased the cell loss in the ganglion cell layer (GCL) after retinal damage occurred. Moreover, SUN N8075 reduced the number of apoptotic cells and the expression of an oxidative stress marker in GCL in the NMDA model. These findings suggest that SUN N8075 has a neuroprotective effect against retinal damage, presumably via the radical scavenging effect.



Retinal ganglion cell (RGC) death is a serious and common clinical problem: it leads to visual loss in several retinal diseases, including glaucoma, optic neuropathies, and various retino-vascular diseases (diabetic retinopathy and retinal vein occlusions). RGC death may occur via a variety of mechanisms involving, for example, oxidative stress [3], excitatory amino acids [2], nitric oxide [20], and apoptosis [17]. Of these mechanisms, oxidative stress is considered to be a critical mediator in RGC injury of various etiologies and has been implicated in RGC death in the eye [16]. Oxidative stress is associated with various retinal disorders such as diabetic retinopathy, glaucoma, age-related macular degeneration, and retinitis pigmentosa. Thus, we evaluated the effects of SUN N8075, a radical scavenger, on retinal damage in mice.

SUN N8075 was synthesized at Asubio Pharma Co. Ltd. (Osaka, Japan), in its continuous endeavor to develop agents for acute ischemic stroke, based on molecular modification of flunarizine, a vasodilator and a $Ca^{2+}$ channel blocker. Flunarizine selectively dilates retinal blood vessels without affecting blood pressure or heart rate [19]. However, flunarizine has the binding affinity for dopamine $D_2$ receptor [7], and it exerts Parkinsonism as an adverse side effect in clinical [6]. SUN N8075 was synthesized as a dual inhibitor for $Na^+$ and T-type $Ca^{2+}$ channels with a potent antioxidative effect and without binding affinity for dopamine $D_2$ receptor ($IC_{50}$ > 10 μM) [1]. SUN N8075 is suppressing lipid peroxidation of brain membranes in the rat model of transient middle cerebral artery occlusion (MCAO) and is currently in clinical trials for stroke [1]. The antioxidative action of SUN N8075 contributes to attenuation of the damage induced by reactive oxygen species (ROS) during cerebral ischemia and reperfusion [22].

In our previous study, in the murine permanent MCAO model, SUN N8075 significantly reduced the number of cells that were immunopositive for 4-hydroxy-2-nonenal (4-HNE), reflecting lipid peroxidation, in the cortical core and penumbra regions [13]. Furthermore, SUN N8075 showed antioxidant properties in the murine Parkinson disease model performed by intraperitoneal injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), suppressing the accumulation of 8-hydroxy-2-deoxy-guanosine (8-OHdG), an oxidative stress marker, in the striatum [21]. However, there has been no report about the effects of SUN N8075 on retinal cell death. The purpose of this study was, therefore, to examine the effects of SUN N8075 on retinal cell death induced by intravitreous injection of NMDA and high-intraocular pressure (IOP), which are glaucoma-like animal models.

Adult male ddY mice (Japan SLC, Hamamatsu, Japan) were kept under controlled lighting conditions (12 h:12 h light/dark). All experiments were approved by the Institutional Animal Care and Use Committee of Gifu Pharmaceutical University and performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society. SUN N8075, (2s)-1-(4-amino-2,3,5-trimethylphenoxy)-3-{4-[4-(4-fluorobenzyl)phenyl]-1-piperazinyl}-2-propanoldimethanesulfonate was kindly gifted from Asubio Pharma. In this study, total 72 mice were used, and NMDA and IOP elevation were induced only on unilateral eyes of each mouse. Retinal damage induced by NMDA (Sigma–Aldrich, St. Louis, MO) or high-IOP was produced as previously reported [24]. SUN N8075 (10 or 30 mg/kg) or vehicle [6% captisol in 0.48% (w/v) NaCl] was administered subcutaneously (s.c.) three times at 30 min before, just, and at 1 h after an intravitreal injection of NMDA at 5 nmol/eye or IOP elevation at 100 mmHg for 40 min. In this study, we used 2 types of animal models for retinal injury as above mentioned. Both of them are partly similar in down-stream signals

* Corresponding author. Tel.: +81 58 230 8126; fax: +81 58 230 8126.
*E-mail address:* hidehara@gifu-pu.ac.jp (H. Hara).

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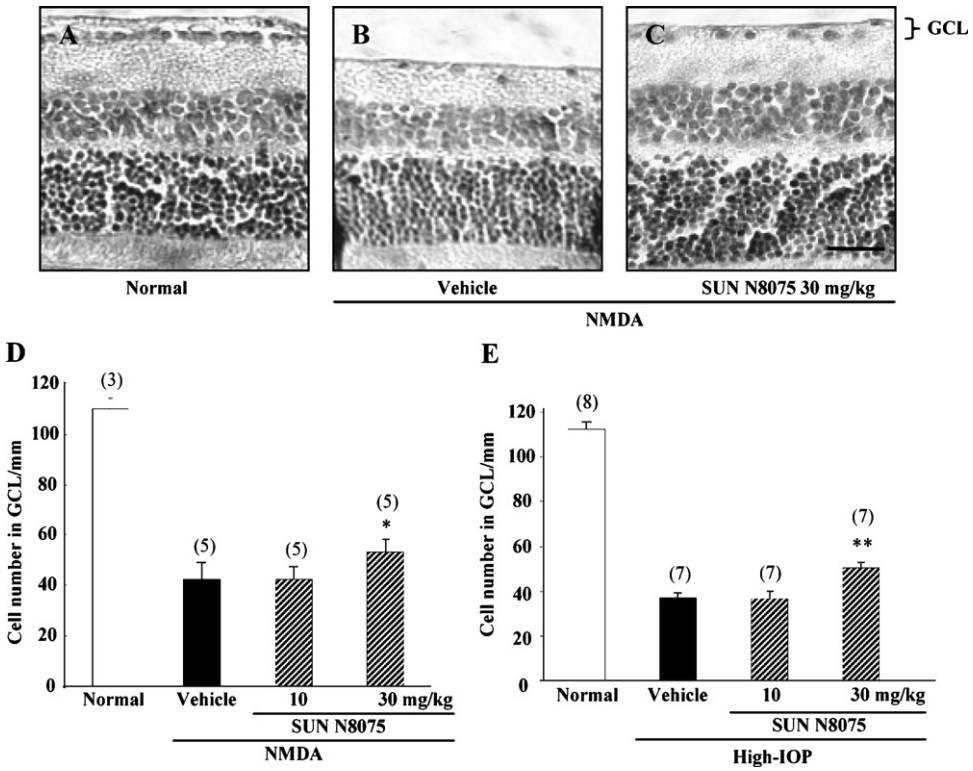


**Fig. 1.** Effects of SUN N8075 on retinal damage at 7 days after intravitreous injection of *N*-methyl-D-aspartate (NMDA) and high intraocular pressure (IOP) in mice. (A) Non-treated normal retina, (B) vehicle plus NMDA (5 nmol)-treated retina, (C) SUN N8075 (30 mg/kg, s.c.) plus NMDA-treated retina. (D) Cell number in GCL/mm after NMDA injection. (E) Cell number in GCL/mm after high-IOP. Data are shown as mean ± SEM, $n = 3$ to 8, $^{*}p < 0.05$ vs. vehicle plus NMDA-treated group, $^{**}p < 0.01$ vs. vehicle plus high-IOP-treated group (Dunnett's test). The number in parentheses above each column represents the number of animals. GCL: ganglion cell layer. Scale bar represents 25 μm.

after NMDA receptor activation. In the ischemia/reperfusion-induced retinal damage, endogenous glutamate activates NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)/kainate receptors in retinal ganglion cells resulting in apoptotic cell death, and MK801, an NMDA receptor antagonist markedly protects retinal cells against the ischemic damage [24]. Therefore, the activation of NMDA receptor plays a key role to initiate retinal cell death after ischemia/reperfusion. Accordingly, we examined whether SUN N8075 would be effective in the two types of animal models, and then apoptotic cell death and oxidative stress were evaluated in NMDA model.

In the evaluation of histological damage at 7 days after NMDA and high-IOP treatments, each eye was enucleated and kept immersed for 24 h at 4 °C in 4% paraformaldehyde. Six paraffin-embedded sections (thickness: 5 μm) were gained by cutting parallel with the maximum circle of the eyeball through the optic disc and stained with hematoxylin and eosin. A digital camera (COOLPIX 4500; Nikon, Tokyo, Japan) was used to photograph light-microscope images were photographed. The cell counts in the ganglion cell layer (GCL) at a distance between 375 and 625 μm from the optic disc were measured on the photographs in a masked fashion by a single observer (MA). Data from three sections selected randomly from the six sections were averaged for each eye, and used to evaluate the cell count. At 24 h or 12 h after NMDA injection, the eyes were enucleated, fixed overnight in 4% paraformaldehyde, immersed for 48 h in 25% sucrose with PBS, and embedded in a supporting medium for frozen-tissue specimens, optimum cutting temperature (OCT) compound (Tissue-Tek, Waukegan IL) for terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining, or immunostaining, respectively. Retinal sections of 10-μm thicknesses were cut on a cryostat at −25 °C. TUNEL staining was performed according to the manufacturer's protocols (In Situ Cell Death Detection Kit; Roche Biochemicals, Mannheim, Germany), and developed using fluorescent microscope images were photographed. In immunostaining, tissue sections were washed in 0.01 M PBS for 10 min, and endogenous peroxidase was quenched by 3% hydrogen peroxide in absolute methanol followed by pre-incubation with 10% normal goat serum. 8-OHdG mouse monoclonal antibody (Japan Institute for the Control of Aging, Fukuroi, Shizuoka, Japan) was diluted in PBS (1:1000), incubated with the tissue sections primary antibodies overnight at 4 °C, washed, and incubated with biotinylated anti-mouse IgG. They were subsequently incubated with the avidin–biotin-peroxidase complex for 30 min, and then developed using diaminobenzidine (DAB) peroxidase substrate. We confirmed the staining by comparing with the negative control. Data are presented as the means ± SEM. Statistical comparisons were made using Dunnett's test or Student's *t*-test. $p < 0.05$ was considered to indicate statistical significance.

In the present study, histological analysis was performed to examine the effects of SUN N8075 on retinal damage induced by intravitreous injection of NMDA and high-IOP. Intravitreous injection of NMDA decreased the cell number in the GCL of the murine retina (Fig. 1A, B, and D). SUN N8075 (30 mg/kg, s.c.) significantly suppressed cell loss in the GCL compared with the vehicle-treated group (Fig. 1A–D). On the other hand, elevating IOP at 100 mmHg for 40 min decreased the cell number in the GCL of the murine retina at 7 days after high-IOP (Fig. 1E). SUN N8075 (30 mg/kg, s.c.) significantly inhibited cell loss in the GCL, compared with the vehicle-treated high-IOP group (Fig. 1E). Thus, SUN N8075 suppressed the cell loss in the GCL in both models. These results suggest that SUN N8075 has a neuroprotective effect on histological damage induced by NMDA and high-IOP in the murine retina.

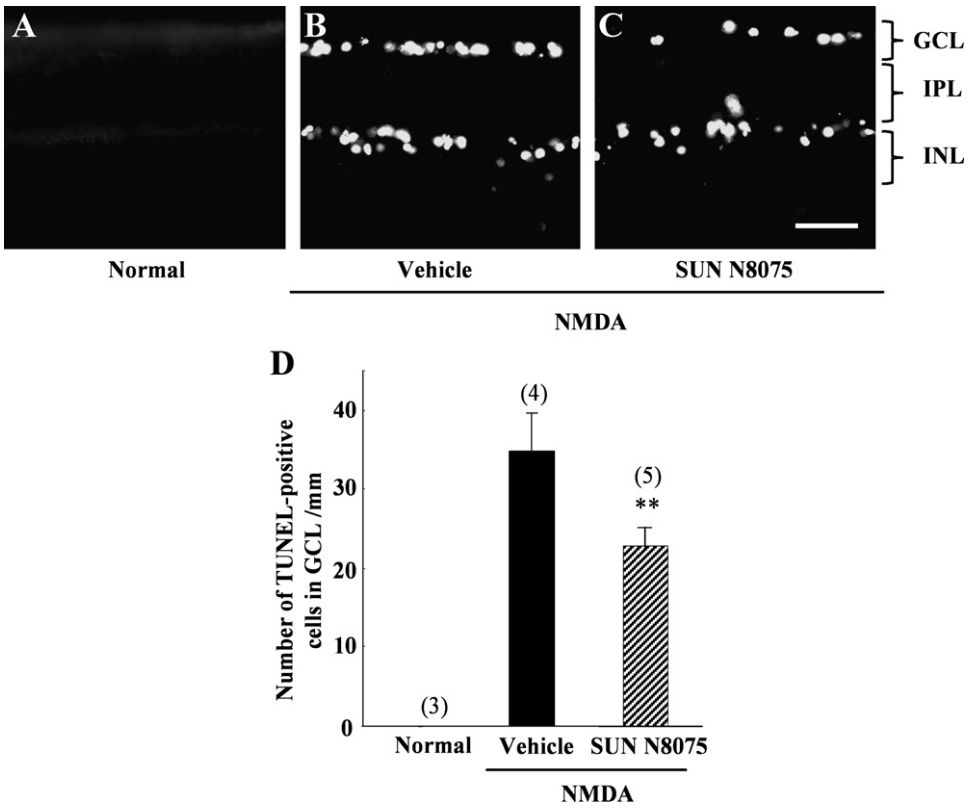


**Fig. 2.** Effects of SUN N8075 on the expression of TUNEL-positive cells at 24 h after intravitreous injection of NMDA in mice. (A) Non-treated normal retina, (B) vehicle plus NMDA (5 nmol)-treated retina, and (C) SUN N8075 (30 mg/kg, s.c.) plus NMDA-treated retina. (D) Number of TUNEL-positive cells in GCL. Data are shown as mean ± SEM, $n = 3$ to 5. $^{*}p < 0.05$ vs. vehicle plus NMDA-treated group ($t$-test). The number in parentheses above each column represents the number of animals. GCL: ganglion cell layer, IPL: inner plexiform layer, INL: inner nuclear layer. Scale bar represents 25 μm.

In the GCL of mice, about 60% of the cells are displaced amacrine cells, and the remaining cells are RGCs; amacrine cells are mostly located at the inner part of the INL [11]. In this study, we evaluated the cells in the GCL, not only RGCs but also amacrine cells. TUNEL-positive cells, indicative of apoptotic cell death, were observed in the GCL and the inner part of the INL at 24 h after intravitreous NMDA injection (Fig. 2B), but almost none were seen in the untreated retina (Fig. 2A). SUN N8075 (30 mg/kg, s.c.) significantly reduced the number of TUNEL-positive cells in the GCL (Fig. 2B–D), but not in the INL (versus the vehicle-treated group, data not shown). These findings support the idea that SUN N8075 has protective effects on the cells in the GCL, mainly RGCs.

One of the factors causing RGC death is ischemia/reperfusion to the retina. Actually, retinal ischemia is closely related to pathologic processes in many retinal diseases such as glaucoma and diabetic retinopathy. During ischemia and subsequent reperfusion, glutamate is released from neuronal terminals and Müller cells to extracellular spaces and/or vitreous body [14,15,18]. High-IOP, of which we used a model, leads to an increase in glutamate in the vitreous body with ischemia/reperfusion because of the elevating IOP [4]. Levels of glutamate are elevated in the vitreous in patients with glaucoma and in animals subjected to experimental ocular hypertension [12,23]. Moreover, excessive glutamate activates glutamate receptors such as NMDA and AMPA/kainite receptors. Activation of the NMDA receptor leads to an intracellular $Ca^{2+}$ influx, and the subsequent ROS increase may be detrimental to cell viability [5]. Furthermore, activation of this receptor depletes intracellular glutathione, which could decrease the intracellular capacity for ROS inactivation [25]. In this study, we used NMDA, an agonist of NMDA receptors, to generate these events to the death of retinal neuronal cells, especially RGC, sensitive to the effects of both glutamate and its analog NMDA. Oxidative stress, leading to the formation of ROS, has been implicated as a stage in the final common pathway for neurotoxicity in various retinal diseases such as glaucoma, age-related macular degeneration, and retinitis pigmentosa. Thus, it is important for the protection of retinal cells to reduce oxidative stress.

In the present study, to investigate the effects of SUN N8075 on ROS generation, 8-OHdG, which is an oxidative by-product, was measured using immunostaining techniques. 8-OHdG-positive cells were observed in GCL at 12 h after intravitreous NMDA injection (Fig. 3B), but almost none were seen in the untreated retina (Fig. 3A) and vehicle-treated retina (data not shown). SUN N8075 (30 mg/kg, s.c.) significantly reduced the number of 8-OHdG-positive cells (versus the vehicle-treated group) (Fig. 3B–D). These results indicate that the protective effects of SUN N8075 on NMDA-induced retinal damage may be mediated by a reduction in oxidative stress.

SUN N8075 has inhibitory activities on $Na^{+}$ ($IC_{50} = 0.42$ μM) and T-type $Ca^{2+}$ ($IC_{50} = 0.37$ μM at −70 mV/2.0 μM at −100 mV) channels in addition to the potent antioxidant activity ($IC_{50} = 0.31$ μM) [1], but it has lower binding activity on L-type $Ca^{2+}$ channel ($IC_{50} = 3.4$ μM) than the others (Tamura S. et al., unpublished data). Previously, we have reported that lomerizine, a $Ca^{2+}$ channel blocker, reduces glutamate-induced neurotoxicity and ischemia/reperfusion damage in rat retina [24]. Lomerizine has a potent binding activity on L-type $Ca^{2+}$ channel ($IC_{50} = 0.086$ μM) in synaptosomal membranes prepared from the guinea pig cerebral cortex [10] and, therefore, the protective effect of lomerizine on retinal cell death after retinal ischemia/reperfusion and glutamate-induced neurotoxicity may be due to its blocking effect on excessive $Ca^{2+}$ influx through L-type $Ca^{2+}$ channel in retinal neurons. Furthermore, we have reported that an antioxidative agent, edaravone, protects retinal cells against NMDA-induced retinal damages in

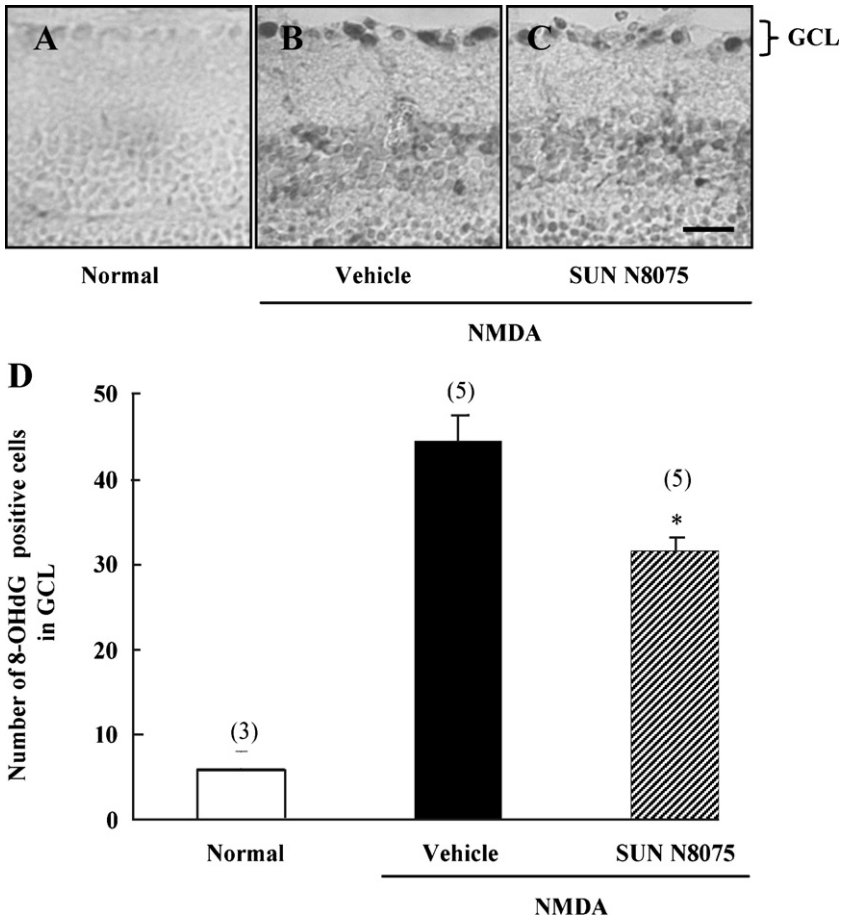


**Fig. 3.** Effects of SUN N8075 on expression of 8-OHdG-positive cells at 12 h after intravitreous injection of NMDA in mice. (A) Non-treated normal retina, (B) vehicle plus NMDA (5 nmol)-treated retina, and (C) SUN N8075 (30 mg/kg, s.c.) plus NMDA-treated retina. (D) Number of 8-OHdG-positive cells in GCL. Data are shown as mean ± SEM, $n = 3$ to 5. $^{**}p < 0.01$ vs. vehicle plus NMDA-treated group ($t$-test). The number in parentheses above each column represents the number of animals. GCL: ganglion cell layer. Scale bar represents 25 μm.

mice [8]. When SUN N8075 was administered by subcutaneous injection at 10 and 30 mg/kg, s.c. for mice, the maximum concentrations of SUN N8075 at 10 and 30 mg/kg, s.c. in plasma were approximately 0.6 and 2 μM, respectively, and it was decreased to less than 0.1 and 0.3 μM, respectively, within 8 h (Tamura S. et al., unpublished data). Therefore, its antioxidative effect and inhibitory effects on $Ca^{2+}$ and $Na^{+}$ channels were expected at 30 mg/kg, s.c., On the other hand, the plasma concentration of SUN N8075 at 10 mg/kg is insufficient to exert the pharmacological effects. The pharmacokinetics and pharmacological profiles of SUN N8075 support that protective effects of SUN N8075 at 30 mg/kg, s.c. may be mediated by antioxidative effect and inhibitory effects on $Na^{+}$ and/or T-type $Ca^{2+}$ channels.

Depolarization, triggered by increased IOP or NMDA, can activate $Na^{+}$ channel and subsequent $Ca^{2+}$ overload through reversed route of $Na^{+}/Ca^{2+}$ exchanger and voltage-dependent $Ca^{2+}$ channel resulting in apoptotic cell death. Previously, we demonstrated that SEA0400, a selective $Na^{+}/Ca^{2+}$ exchanger inhibitor, protected retinal cell death after NMDA injection or ischemia/reperfusion [9]. Furthermore, we have reported that an antioxidative agent, edaravone, protects retinal cells against NMDA-induced retinal damages in mice [8]. Therefore, antioxidative effect and blockade of $Na^{+}$ channel by SUN N8075 may attenuate these signaling processes after NMDA receptor activation or ischemia/reperfusion.

In conclusion, we investigated the effects of SUN N8075 on retinal damage and found that SUN N8075, a potent radical scavenger, has neuroprotective effects in both NMDA- and high-IOP-induced retinal damaged models. These findings indicate that SUN N8075 may be a potential candidate for therapeutic drugs for retinal diseases.

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