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A novel RIPK1 inhibitor that prevents retinal degeneration in a rat glaucoma model

Yun-Ju Do[a,1], Jee-Won Sul[a,1,2], Ki-Hong Jang[a], Nam Sook Kang[b], Young-Hoon Kim[c], Young-Gwan Kim[c], Eunhee Kim[a,*]

[a] Department of Biological Sciences, Chungnam National University, Daejeon 34134, Republic of Korea
[b] Graduate School of New Drug Discovery and Development, Chungnam National University, Daejeon 34134, Republic of Korea
[c] Kukjepharma R & D Center, Sanseong-ro 47, Ansan, Gyeonggi-do 15437, Republic of Korea

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ABSTRACT

In glaucoma, retinal ganglion cells (RGCs) are exposed to ischemic stress with elevation of the intraocular pressure and are subsequently lost. Necroptosis, a type of regulated necrosis, is known to play a pivotal role in this loss. We observed that receptor-interacting protein kinase 1 (RIPK1), the key player of necroptosis, was activated by diverse ischemic stresses, including TCZ, chemical hypoxia (CH), and oxygen glucose deprivation (OGD). In this study, we introduce a RIPK1-inhibitory compound (RIC) with a novel scaffold. RIC inhibited downstream events following RIPK1 activation, including necrosome formation and mitochondrial dysfunction in RGC5 cells. Moreover, RIC protected RGCs against ischemic injury in the rat glaucoma model, which was induced by acute high intraocular pressure. However, RIC displayed biochemical characteristics that are distinct from those of previous RIPK1 inhibitors (necrostatin-1; Nec-1 and Compound 27; Cpd27). RIC protected RGCs against OGD insult, while Nec-1 and Cpd27 did not. Conversely, Nec-1 and Cpd27 protected RGCs from TNF-stimulated death, while RIC failed to inhibit the death of RGCs. This implies that RIPK1 activates alternative pathways depending on the context of the ischemic insults.

1. Introduction

Glaucoma is a leading cause of irreversible blindness and a progressive neurodegenerative disease with an estimated seventy million people affected in the world [1]. Loss of retinal ganglion cells (RGCs) by ischemic injury following intraocular pressure (IOP) elevation is the predominant cause of glaucoma-related blindness although some RGCs are also lost in normal tension glaucoma [2,3]. Therefore, lowering the IOP is the common treatment for glaucoma. However, a decrease in the IOP only slows the progression of retinal degeneration, but it does not prevent the death of RGCs and visual impairment. Therefore, development of a disease-modifying drug with neuroprotective potential is needed.

Ischemic injury leads to both apoptotic and necrotic types of cell death in the retina [4–6]. However, recent studies show that necroptosis, a type of regulated necrosis, predominates under retinal ischemic conditions [7–9]. Moreover, when apoptosis is inhibited by ATP depletion that often occurs under ischemic conditions, the type of death in photoreceptors shifts from apoptosis to necroptosis mediated by receptor interacting protein kinases 1 (RIPK1) [10–15]. Therefore, the inhibition of necroptosis upon ischemia in RGCs might be a new strategy for treating ophthalmic disorders, including glaucoma. In this regard, we focused on the regulation of necroptosis through controlling RIPK1 activation.

The catalytic activity of RIPK1 is required for triggering necroptosis [16]. Phosphorylated forms of RIPK1 interact with RIPK3, and assemble necrosomes that initiate necroptosis [17,18]. Activation of RIPK1 has been detected in ganglion cells of ischemia-insulted mice [8]. Additionally, pharmacological inhibition of RIPK1 delayed the death of cone cells in pde6c^{w59} mutant zebrafish [19]. Moreover, the RIPK1 kinase-dead mice, *Ripk1*^{K45A}, were protected from necroptotic stress *in vitro* and *in vivo* [20]. Collectively, these studies indicate that

Abbreviations: CH, chemical hypoxia; CHX, cycloheximide; Cpd27, compound 27; DAPI, 4,6-diamidino-2-phenylindole; GCL, ganglion cell layer; H_2O_2, hydrogen peroxide; MNNG, 1-methyl-3-nitro-1 nitrosoguanidine; Nec-1, necrostatin-1; OGD, oxygen glucose deprivation; PI, propidium iodide; RGCs, retinal ganglion cells; RIC, RIPK1-inhibitory compound; RIPK, receptor interacting protein kinases; t-BHP, tert-butyl hydroperoxide; and TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling

* Corresponding author.
E-mail address: eunhee@cnu.ac.kr (E. Kim).
[1] These authors contributed equally to this work.
[2] Present address: Department of Medicine, University of Ulsan College of Medicine, Asan Medical Center, 88, Olympic-ro 43-gil, Seoul 05505, Republic of Korea.

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the kinase activity of RIPK1 plays a crucial role in retinal degeneration.

To date, a number of RIPK1 inhibitors have been reported, including Nec-1 [21,22], compound 27 (Cpd27) [23] and compound 4b [24]. These inhibitors have protective effects against TNF-induced necroptosis [21–24]. However, Nec-1 has modest cellular potency with a short *in vivo* half-life as well as inhibits indoleamine-2,3-dioxygenase, compromising its target specificity to RIPK1 [25,26]. Although optimized necrostatin (Nec-1s) improved the pharmacokinetic properties and minimized off-target effects, Nec-1s still has modest potency [27]. Cpd27 and compound 4b had higher potencies and selectivities for RIPK1 than Nec-1 *in vitro*. Moreover, compound 4b demonstrated protective potential *in vivo* against L-arginine induced pancreatitis and Cpd27 had protective potential against TNFα/z-VAD-induced hypothermia. However, their protective potentials in the retina have yet to be examined [23,24].

Here, we introduce a novel RIPK1-inhibitory compound (RIC) [28,29] with a biochemical profile distinct from those of previously developed compounds. RIC inhibited RIPK1 catalytic activity *in vivo* and *in vitro* as well as suppressed necroptosis through blocking the necrosome assembly and mitochondrial dysfunction in RGC-5 cells. However, the sensitivity profile of RIC to various ischemic stimuli was different from other RIPK1 inhibitors. Collectively, we present RIC with novel glaucoma therapeutic potential through necroptosis inhibition. Our findings suggest the presence of differential pathways mediated by RIPK1 with ischemic stresses.

2. Materials and methods

2.1. Reagents

RIC, Nec-1, and Cpd27 were synthesized at Kukjepharma, Inc. (Ansan, Korea). The antibodies were purchased as follows: mouse anti-β-actin (Sigma-Aldrich, Saint Louis, MI, USA), rabbit anti-RIPK3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-RIPK1 (BD Biosciences, Franklin Lakes, NJ, USA), horseradish peroxidase (HRP)-conjugated anti-mouse (Thermo Fisher Scientific, Inc., Rockford, IL, USA), HRP-conjugated anti-rabbit (Thermo Fisher Scientific, Inc.), anti-HSP60 (Santa Cruz Biotechnology) and protein A/G agarose beads (Santa Cruz Biotechnology). Chemical reagents were purchased as follows: TNF-α (AbFrontier, Seoul, Korea), cycloheximide (Sigma-Aldrich), zVAD-fmk (Adipogen, San Diego, CA, USA), dimethyl sulfoxide (Sigma-Aldrich), propidium iodide (Sigma-Aldrich), 50% glutaraldehyde (Sigma-Aldrich), polyethylene glycol 400 (Sigma-Aldrich) and [γ-^{32}P]-ATP (PerkinElmer, Waltham, MA, USA). The MitoSOX-Red fluorescent probe was purchased from Thermo Fisher Scientific, Inc. The TUNEL assay was performed using an *in situ* cell death detection kit (Roche, Basel, Switzerland)

2.2. Cell culture

Retinal ganglion cell line (RGC-5) obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and mouse embryonic fibroblasts (MEFs; RIPK1 wild-type and RIPK1 knockout) provided by Dr. Gang Min Hur (Chungnam National University, Daejeon, Korea) [30,31] were cultured in Dulbecco's modified Eagle's medium (WelGENE, Daegu, Korea) supplemented with 10% fetal bovine serum (Atlas Biologicals, Fort Collins, CO, USA) and 1% penicillin/streptomycin in a humidified atmosphere (37 °C, 5% CO_2).

2.3. Oxygen-glucose deprivation

The medium of RGC-5 cells and MEFs was replaced with glucose-free deoxygenated medium containing HEPES (10 mM), NaCl (116 mM), KCl (5.4 mM), NaH_2PO_4 (0.8 mM), sodium bicarbonate (25 mM), sucrose (25 mM), $CaCl_2$ (1.8 mM), and phenol red (0.04%; pH 7.3) and then incubated within anaerobic chamber (Thermo Fisher Scientific, Inc.) with CO_2 (5%), H_2 (10%) and N_2 balanced at 37 °C for the indicated experimental time.

2.4. Immunoprecipitation

RGC-5 cells were lysed in lysis buffer (containing Tris-HCl (20 mM; pH 7.5), NaCl (150 mM), EDTA (2 mM), NP-40 (1%), PMSF (0.4 mM), β-glycerophosphate (25 mM), Na_3VO_4 (1 mM), DTT (1 mM), and NaF (1 mM)), vortexed and then centrifuged at 13,000 rpm for 10 min at 4 °C. The resulting whole-cell lysates (WCLs) were incubated with anti-RIPK1 or anti-RIPK3 antibodies and protein A/G agarose beads for 12 h at 4 °C. The immunocomplexes were washed three times with mammalian lysis buffer and sample buffer was added. The collected supernatants were subjected to SDS–PAGE and immunoblotted with the indicated antibodies.

2.5. SDS-PAGE and western blot analysis

RGC-5 cells were lysed with lysis buffer; then, the protein concentration was determined by the Bradford method. WCLs were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked with 5% skim milk and incubated with the indicated antibodies at 4 °C for 12 h. After washing three times for 10 min, membranes were incubated with HRP-conjugated secondary anti-mouse or HRP-conjugated secondary anti-rabbit antibodies for 1 h. Protein bands were visualized by ECL detection kits (YounginFrontier, An-Yang, Korea)

2.6. Kinase assay

RGC-5 cells were lysed in lysis buffer. WCLs were immunoprecipitated with anti-RIPK1 or anti-RIPK3 antibodies and protein A/G agarose beads for 12 h at 4 °C. The resulting complexes were washed with lysis buffer and kinase buffer A containing HEPES (20 mM; pH 7.5), $MgCl_2$ (10 mM) and $MnCl_2$ (10 mM). Finally, they were washed with kinase buffer B containing HEPES (20 mM; pH 7.5), $MgCl_2$ (10 mM), $MnCl_2$ (10 mM) and DTT (1 mM). The complexes were incubated in kinase buffer B (15 μl) for 5 min. After incubation, ATP (2 μl; 500 μM) and [γ-^{32}P]-ATP (1 mCi) were added and incubated for 30 min at 30 °C. Afterwards, the mixture was washed with kinase buffer B and subjected to SDS-PAGE [32,33]. The resulting PAGE gel was dried on 3 M paper for 1 h and exposed to a Hypercassette for 12 h. The image of the radioactive labeled specimen was obtained and quantified by Typoon FLA-7000 (GE healthcare, Fairfield, CT, USA).

2.7. Flow cytometry

After OGD treatment, RGC-5 cells were collected and washed three times with PBS. Following centrifugation, the PBS was discarded and the cells were stained with 5 μg/ml propidium iodide (PI) for 10 min at 37 °C in the dark. Flow cytometric analysis was performed using a Guava easycyte flow cytometer (Millipore, Billierica, MA, USA). A total of 10,000 cells were analyzed per sample. Analysis was described as the percentage of PI uptake in RGC-5 cells. All results were repeated three times.

2.8. Measurement of ROS

To quantify the production of mitochondrial superoxide, RGC-5 cells grown on coverslips were treated with OGD for 1 h. Afterwards, cells were loaded with 5 μM fluorescent probe MitoSOX- Red (Ex/Em, 510/580 nm) in Hanks' balanced salt solution (HBSS) at 37 °C and 5% CO_2. Nuclei were stained with 4′, 6′-diamidino-2-phenylindole (DAPI) (Ex/Em, 364/461 nm). Fluorescence was observed under a confocal microscope (Ziess, Oberkochen, Germany).