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INVITED REVIEW

Modulation of valosin-containing protein by Kyoto University Substances (KUS) as a novel therapeutic strategy for ischemic neuronal diseases

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Abstract
Retinal ischemia causes several vision-threatening diseases, including diabetic retinopathy, retinal artery occlusion, and retinal vein occlusion. Intracellular adenosine triphosphate (ATP) depletion and subsequent induced endoplasmic reticulum (ER) stress are proposed to be the underlying mechanisms of ischemic retinal cell death. Recently, we found that a naphthalene derivative can inhibit ATPase activity of valosin-containing protein, universally expressed within various types of cells, including retinal neural cells, with strong cytoprotective activity. Based on the chemical structure, we developed novel valosin-containing protein modulators, Kyoto University Substances (KUSs), that not only inhibit intracellular ATP depletion, but also ameliorate ER stress. Suppressing ER stress by KUSs is associated with neural cell survival in animal models of several neurodegenerative diseases, such as glaucoma and retinal degeneration. Given that a major pathology of ischemic retinal diseases, other than intracellular ATP depletion, is ER stress-induced cell death, KUSs may provide a novel strategy for cell protection in ischemic conditions. Hence, we investigated the efficacy of KUS121 in a rat model of retinal ischemic injury. Intravitreal injections of KUS121, which is clinically preferable route of drug administration in retinal diseases, significantly suppressed inner retinal thinning and retinal cell death, and maintained visual functions. Valosin-containing protein modulation by KUS is a promising novel therapeutic strategy for ischemic retinal diseases.

Key Words: *adenosine triphosphatase; C/EBP homologous protein; central retinal artery occlusion; endoplasmic reticulum stress; neuroprotective therapy; retinal ganglion cell*

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Introduction
Of all human tissues, the retina has the highest oxygen demands, and retinal ischemia contributes to the pathologies of several vision-threatening diseases, including diabetic retinopathy, glaucoma, retinal artery occlusion, and retinal vein occlusion (Osborne et al., 2004; Zheng et al., 2007). These retinal diseases account for a large proportion of blindness. Among these ischemic conditions, central retinal artery occlusion (CRAO) causes sudden severe vision loss, resulting in permanent blindness (Hayreh, 2011). The incidence of CRAO is estimated to be around 1.9/100,000 in the United States (Leavitt et al., 2011), but is increasing due to population aging and increased lifestyle-related diseases (Park et al., 2014). Patients with CRAO typically present with sudden deterioration of vision without pain, and 80% of affected patients have a final visual acuity of counting fingers or worse (Hayreh and Zimmerman, 2005). The most common cause of CRAO is thromboembolus in the dural sheath of the optic nerve, the narrowest part of the CRA (Hayreh, 2011). CRAO can also occur due to occlusive thrombus immediately posterior to the lamina cribrosa (Mangat, 1995).

Given that the CRA perfuses the inner retinal layers, namely the retinal ganglion cell (RGC) layer and inner nuclear layer, visual loss in the CRAO occurs due to loss of blood supply to the inner retina (Hayreh et al., 2004). Deficiency of oxygen and glucose supply to retinal cells hinders adenosine triphosphate (ATP) production, which is necessary for nervous conduction in RGCs, resulting in immediate visual dysfunction. Retinal ischemia lasting more than 6 hours causes neural cell death in the RGC layer and inner nuclear layer, and subsequent nerve fiber dropouts (Hayreh et al., 2004). After thickening of the inner retina in the acute phase of CRAO, inner retinal atrophy gradually progresses, resulting in permanent visual loss. However, in most cases of CRAO, spontaneous partial recanalization of the occluded CRA occurs within 48 to 72 hours. Spontaneous reperfusion is important for continued blood supply to the surviving neural cells, and reperfusion injury may contribute to addi-

tional neural cell death (Szabo et al., 1991).

Current strategies for the management of acute CRAO focus on inducing reperfusion of the retina, ocular massage, anterior chamber paracentesis, and fibrinolysis. However, these treatments have limited or no efficacy for improving vision, given the short retinal tolerance time frame (potentially within 6 hours of symptom onset) for any therapy to be effective (Schrag et al., 2015). Moreover, as spontaneous reperfusion occurs within a few days, therapeutic strategies for delaying neural cell death may be important components of CRAO treatment. There is no clinically effective neuroprotective treatment for CRAO, but some neuroprotective approaches have been trialed, especially for glaucoma (Sena and Lindsley, 2017). The latter is characterized by progressive RGC apoptosis and possibly associated with compromised blood flow in the optic nerve.

In addition to intracellular ATP depletion, the involvement of subsequent induced endoplasmic reticulum (ER) stress

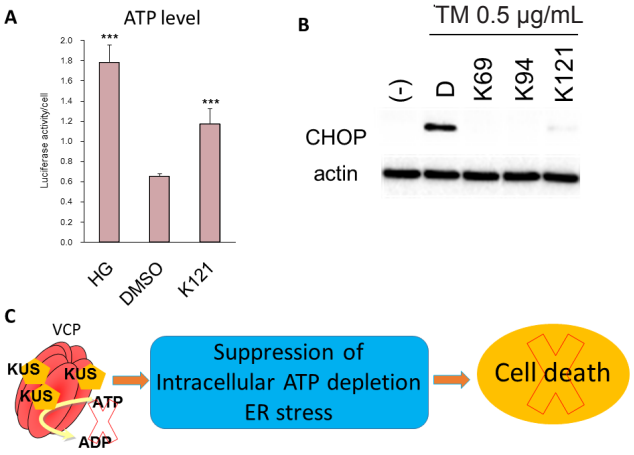

Figure 1 Kyoto University Substances (KUS) suppress adenosine triphosphate (ATP) depletion and endoplasmic reticulum (ER) stress in tunicamycin-treated cells.
(A) HeLa cells were cultured in a low glucose medium (0.25 g/L) for 20 hours, with or without KUS121. ATP levels were measured with luciferase assays. Error bars indicate standard deviation. ***$P < 0.001$ *vs.* Dimethyl sulfoxide (DMSO) control (Dunnett's test, $n = 3$). HG = high glucose. (B) Western blot analyses for C/EBP homologous protein (CHOP). HeLa cells were treated with 0.5 μg/mL of tunicamycin (TM) as an ER stress inducer for 5 hours in the presence of KUSs (50 μM). Cells were then harvested and subjected to western blot analyses. Actin served as a loading control. D = DMSO, (–) = no treatment of tunicamycin (C) Schema of the mechanisms of the neuroprotective effects of KUSs.

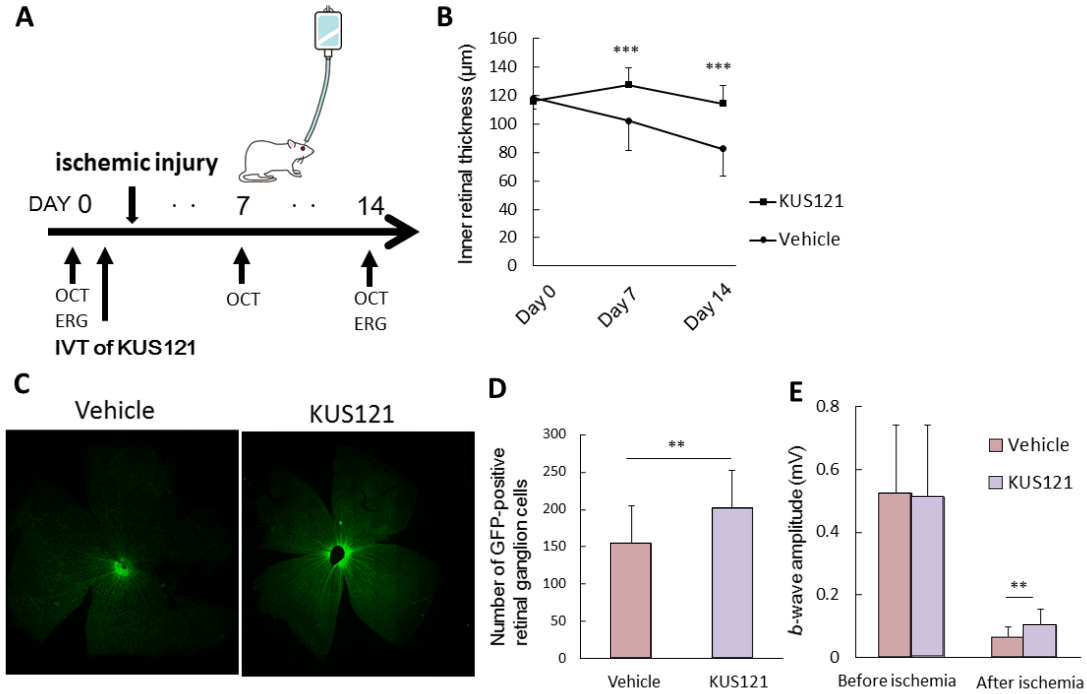

Figure 2 Effects of intravitreal injections of Kyoto University Substance 121 (KUS121) on a retinal ischemic rat model.
(A) Experimental system of the retinal ischemic rat model treated with KUS121. OCT = optical coherence tomography, ERG = electroretinography. (B) Longitudinal changes in inner retinal thickness in the control ($n = 13$, circles) and intravitreal (IVT)-KUS121-treated ($n = 11$, squares) groups. Inner retinal thickness was significantly greater in the IVT-KUS121-treated group than in the control group 7and 14 days after ischemic retinal injury. Error bars indicate standard deviation. ***$P < 0.001$ *vs.* control (Student's *t* test). (C) Images of flat-mounted retinas of rats treated with KUS121 or not after ischemic retinal injury. (D) Retinal ganglion cell (RGC) number of flat-mounted retinas in the control ($n = 15$) and IVT-KUS121-treated ($n = 20$) groups, confirming significant preservation of RGC numbers in the IVT-KUS121-treated group compared with the control group after ischemic retinal injury. Error bars indicate standard error. **$P < 0.01$, *vs.* control (Student's *t*-test). (E) The *b*-wave amplitudes of electroretinography recordings of rats in the control ($n = 21$) and IVT-KUS121-treated ($n = 24$) groups before and 14 days after ischemic injury. Rats treated with IVT-KUS121 showed a significant preservation of *b*-wave amplitude 14 days after ischemic injury. Error bars indicate standard deviation. **$P < 0.01$, *vs.* control (Student's *t*-test).

has been proposed as the underlying mechanism of ischemic neuronal cell death, which is probably apoptosis (Tajiri et al., 2004). Neuronal cells have a highly-developed ER. Ischemia is an ER stressor that induces cell death in neural cells. C/EBP homologous protein (CHOP) is an ER stress-induced molecule that is reported to be a major mediator of apoptosis in neural ischemia. New drugs or compounds with ER stress-reducing activities may have neuroprotective functions in ischemic diseases, and investigation into their ability to prevent or delay neural cell death induced by ischemic injury might be useful.

Kyoto University Substances Protect Cells under ER Stress-Inducing Conditions

Valosin-containing protein (VCP) is an AAA (ATPase Associated with diverse cellular Activities)-type ATPase protein, ubiquitously expressed within various types of cells, including retinal neural cells (Higashiyama et al., 2002; Watts et al., 2004; Johnson et al., 2010). In several neurodegenerative diseases, such as Inclusion Body Myopathy with Paget's disease of bone and Frontotemporal Dementia (IBMPFD) and familial amyotrophic lateral sclerosis, VCP has been proposed to be a major contributor. Pathogenic VCPs show elevated ATPase activities in these conditions, compared with wild-type VCPs, indicating that the constitutive elevation of ATPase activity may be pathogenic. On the other hand, in addition to ATPase activity, there are many proposed cellular functions of VCP, such as proteasome-mediated protein degradation, ER-associated degradation, cell cycle control, membrane fusion, protein trafficking, and autophagy (Meyer et al., 2012; Wolf and Stolz, 2012). Knockdown of VCP, as well as overexpression of dominant-negative forms of VCP showing cellular toxicity, indicate that some VCP functions are crucial for cell survival (Ikeda et al., 2014).

Recently, we found that a naphthalene derivative can inhibit ATPase activity of VCP and show cytoprotective activity, thereby enabling the development of novel VCP modulators, KUSs, or small chemical compounds derived from naphthalene, selected from approximately 200 newly synthesized compounds based on VCP inhibition of ATPase activity (Ikeda et al., 2014). KUSs do not significantly impair reported cellular functions of VCP (*e.g.*, ER-associated degradation or proteasome-mediated protein degradation (Ikeda et al., 2014)) but do suppress the decrease in cellular ATP levels. These "VCP modulators" have strong neuroprotective effects, both morphologically and functionally, *in vivo* on various types of retinal cells, including retinal photoreceptor cells and RGCs (Ikeda et al., 2014; Hasegawa et al., 2016; Nakano et al., 2016). We showed that KUSs not only inhibit intracellular ATP depletion, but ameliorate ER stress in Hela cells treated with tunicamycin (Ikeda et al., 2014) (**Figure 1**). Among several KUSs, KUS121 had a strong cellular protective effect during *in vitro* experiments using culture cells, including neural cells (Ikeda et al., 2014; Nakano et al., 2016), and were easily synthesized. Therefore, we mainly used KUS121 in subsequent experiments. ER stress can induce apoptosis in a diverse range of cell types, contributing to various diseases. Suppressing ER stress by KUSs was associated with enhanced neural cell survival in several neurodegenerative ocular diseases (Ikeda et al., 2014; Hasegawa et al., 2016; Nakano et al., 2016).

VCP Modulation by KUSs as a Promising Novel Therapeutic Strategy for Ischemic Neuronal Diseases

Given that the major pathology of ischemic neural diseases is ER stress-induced cell death (Li et al., 2014), KUSs may provide a novel strategy for cell protection in ischemic conditions. Hence, we investigated the neuroprotective effect of a KUS on the retina after ischemic injury, using a rat model of ischemic retinal injury (Hata et al., 2017). The intraocular pressure of *Thy1*-green fluorescent protein (GFP) rats, which express GFP in RGCs (Magill et al., 2010), was increased to 120 mmHg for 60 minutes to induce retinal ischemia (**Figure 2A**). We evaluated the efficacy of intravitreal (IVT) injections of KUS, given that IVT is the clinically preferred route of drug administration in retinal diseases. In the group treated with IVT injections, KUS121 (25 μg/eye) or phosphate buffer saline (PBS) was injected into the vitreous of *Thy1*-GFP rats, using a 33-gauge needle, two hours before ischemic retinal injury.

We examined the time-dependent changes in inner retinal thickness in the eyes of *Thy1*-GFP rats with ischemic retinal injury using spectral domain-optical coherent tomography (SD-OCT), to detect retinal damage or atrophy caused by ischemic retinal injury. The inner retina, composed of the retinal nerve fiber layer, RGCs, inner plexiform layer, and inner nuclear layer, located under the retinal vessel supply, was primarily impaired by ischemic insult (Murata et al., 2008). Notably, compared to the control group, the KUS121-treated group showed significant suppression of inner retinal thinning 7 and 14 days after ischemic insult (day 7, $P = 0.0008$; day 14, $P = 0.0001$; **Figure 2B**) (Hata et al., 2017). To evaluate the neuroprotective effects of KUS121 on the RGC layer, we used a scanning laser ophthalmoscope (SLO) to measure the number of RGCs remaining after ischemic insult. IVT injections of KUS121 showed a preservation of GFP-positive RGCs 14 days after the insult ($P = 0.0078$ **Figure 2C** and **D**) (Hata et al., 2017). For functional analysis, we performed electroretinography (ERG) and assessed *b*-wave amplitudes to confirm whether the neuroprotective effects of KUS121 were mirrored by the preservation of visual function after ischemic retinal injury (Block and Schwarz, 1998). KUS121 preserved *b*-wave amplitude 14 days after the insult (before the insult, $P = 0.861$; after the insult, $P = 0.0018$; **Figure 2E**) (Hata et al., 2017). To examine the mechanisms underlying the pathological and KUS treatment processes of ischemic retinal injury, we examined ER stress and apoptosis markers after ischemic insult (Hata et al., 2017). CHOP and cleaved caspase-3 proteins were induced after ischemic injury. The CHOP and cleaved caspase-3 proteins levels were significantly decreased in the ischemic retinal-injured rats treated

with KUS121 (Hata et al., 2017). TdT-mediated dUTP Nick-end Labeling (TUNEL) staining of retinal sections confirmed that there were fewer apoptotic cells in the RGC layer and inner nuclear layer of the KUS121-treated group than in the control group (Hata et al., 2017). Therefore, KUS121 exerts not only morphological but also functional neuroprotective effects on the inner retina during retinal ischemia, thereby demonstrating the potential of KUS121 in the treatment of ischemic retinal diseases. To test the safety and efficacy of KUS121 on CRAO patients, we are now performing an investigator-initiated clinical trial.

Statistical analyses were performed using SPSS Statistics version 17.0 (SPSS Inc, Chicago, IL, USA). Variables among cells or rats treated with or without KUSs were compared with the Dunnett's test or Student's *t*-test.

Conclusion

We showed that the VCP modulator KUS121 exerts an anti-apoptotic effect on ischemic retinal injury by suppressing ER stress, resulting in morphological and functional neuroprotection. Our findings may provide a novel therapeutic strategy for the treatment of neural ischemic diseases. Furthermore, given that ER stress is also the major underlying pathology of many other incurable disorders, such as neurodegenerative diseases, KUSs may provide a novel strategy for cell protection in such conditions.

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Conflicts of interest: *Kyoto University has applied for patents related to this study (PCT/JP2015/055619 & PCT/JP2011/073160), with Masayuki Hata and Hanako Ohashi Ikeda listed as inventors. Masayuki Hata and Hanako Ohashi Ikeda are currently performing a joint research project in preparation for an investigator-initiated clinical trial with Kyoto Drug Discovery and Development Co., Ltd.*
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