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Effect of a sigma-1 receptor agonist, cutamesine dihydrochloride (SA4503), on photoreceptor cell death against light-induced damage

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ABSTRACT

Cutamesine dihydrochloride is an agonist of sigma-1 receptor, which is a ligand-operated receptor chaperone at the mitochondrion-associated endoplasmic reticulum (ER) membrane. ER stress plays a pivotal role in light irradiation-induced retinal damage. In the present study, we examined whether cutamesine is effective against experimental degenerative retinal damages *in vitro* and *in vivo*. The effects of cutamesine against white light-induced retinal photoreceptor damage were evaluated *in vitro* by measuring cell death. The expression of sigma-1 receptor after the light exposure was examined by immunoblot analysis. The disruption of the mitochondrial membrane potential and caspase-3/7 activation after excessive light exposure were also examined. In addition, retinal damage in mice induced by irradiation to white light was evaluated using histological staining and electroretinography. Cutamesine reduced the cell death rate induced by light exposure, and the protective effect was prevented by N-[2-(3,4-dichlorophenyl)ethyl]-N-methyl-2-(dimethylamino)ethylamine (BD-1047) dihydrobromide, a sigma-1 receptor antagonist. Sigma-1 receptor expression was decreased by light exposure, and cutamesine suppressed the decreased expression of sigma-1 receptor protein. Cutamesine also reduced the mitochondrial damage and reduced the elevated level of caspase 3/7 activity; this effect was attenuated by BD-1047. In *in vivo* studies, cutamesine suppressed the light-induced retinal dysfunction and thinning of the outer nuclear layer in the mouse retina. These findings indicate that cutamesine protects against retinal cell death *in vitro* and *in vivo* by the agonistic effect of sigma-1 receptor. Therefore, sigma-1 receptor may have a potential as a therapeutic target in retinal diseases mediated by photoreceptor degeneration.

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1. Introduction

Excessive light exposure induces photoreceptor degeneration (Noell et al., 1966), and the resulting retinal damage is irreversible, leading to night blindness and serious loss of visual field, and finally, the loss of central vision. In addition, several epidemiologic studies have indicated that long-term history to light irradiation may have some effects on the incidence of age-related macular degeneration (Taylor et al., 1992). Light-induced photoreceptor cell death has been reported to be induced by various factors, such as oxidative stress (Imai et al., 2010; Wenzel et al., 2005), mitochondrial damage (Donovan et al., 2001), rhodopsin mutation (Wenzel et al., 2001), and endoplasmic reticulum (ER) stress (Yamauchi et al., 2011; Yang et al., 2008). We previously reported that ER stress might play a pivotal role in light exposure-induced retinal damage (Nakanishi et al., 2013). ER stress induces apoptosis *via* mitochondrial dysfunction and loss of mitochondrial membrane potential (Deniaud et al., 2008; Gupta et al., 2010). Therefore, mitochondrial function and damages mediated by ER stress might be deeply involved in retinal damage induced by light irradiation.

1-(3,4-Dimethoxyphenethyl)-4-(3-phenylpropyl)piperazine (cutamesine dihydrochloride, SA4503) is known as a sigma-1 receptor agonist, which has high affinity for the sigma-1 receptor ($Ki = 4.6$ nM) over the sigma-2 receptor ($Ki = 63.1$ nM) in guinea pig brain homogenates (Lever et al., 2006). The sigma-1 receptor is a ligand-operated receptor chaperone that localizes specifically at the mitochondrion-associated ER membrane (Hayashi and Su, 2007). This receptor is known as a modulator of Ca^{2+} release (Monnet, 2005) that binds to inositol-1, 4, 5-trisphosphate (IP_3) receptor and enhances Ca^{2+} signaling from the ER to the mitochondria (Hayashi and Su, 2007). Several sigma-1 receptor agonists

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exert cell protective effects in various *in vitro* models, such as cerebral neurons, retinal ganglion cells, and lens cells (Ha et al., 2011; Marrazzo et al., 2005; Martin et al., 2004; Wang and Duncan, 2006), and it has been suggested that this receptor may have a protective function against ER stress (Ha et al., 2011). Cutamesine is under development for the treatment of major depression and post-stroke recovery.

A sigma-1 receptor ligand treatment in an *in vivo* model of retinal degeneration demonstrates significant neuroprotection, reduces evidence of oxidative stress, and preserves retinal architecture (Smith et al., 2008). In addition, pregnenolone, a sigma-1 receptor agonist, decreases intraocular pressure (IOP), prevents against ganglion cell loss and decreases in inner plexiform layer thickness, and increases sigma receptor 1 expression in episcleral vein-cauterized high IOP model rats (Sun et al., 2012). These reports suggest that sigma-1 receptor plays an important role in the retina and has therapeutic potential in retinal diseases. However, there are no reports regarding whether sigma-1 receptor has a protective role in photoreceptor cell death. As such, we investigated the effects of cutamesine, a sigma-1 receptor agonist, against light-induced cell death *in vitro* and *in vivo*.

2. Materials and methods

2.1. Cell culture

Murine photoreceptor-derived 661W cells were kindly gifted by Dr. Muayyad R. Al-Ubaidi (Department of Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK). The cells were maintained in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin under a humidified atmosphere of 5% CO_2 at 37 °C. These cells were passaged by trypsinization every three to four days.

2.2. Light-induced cell death model in 661W cell cultures

The 661W cells were seeded at a density of 3×10^3 cells per well in 96-well plates, and then incubated for 24 h under a humidified atmosphere of 5% CO_2 at 37 °C. Then, they were treated with 1 or 10 μM cutamesine (M's Science Corporation, Kobe, Japan) and/or 1 μM N-[2-(3,4-dichlorophenyl)ethyl]-N-methyl-2-(dimethylamino)ethylamine (BD-1047) dihydrobromide (Santa Cruz, CA) and incubated for 1 h under a humidified atmosphere of 5% CO_2 at 37 °C. The cells were exposed to 2500 lux of white fluorescent light (Nikon, Tokyo, Japan) for 24 h with each agent under a humidified atmosphere of 5% CO_2 at 37 °C. The dark control cells and light-irradiated 661W cells were all from the same stock, eliminating any preexisting bias (such as light and temperature), as previously described by Kanan et al. (2007).

2.3. Nuclear staining assays

Nuclear staining assays were performed after 24 h of light exposure. Hoechst 33342 stains the nuclei of all cells, whereas propidium iodide (PI) stains only dead cells. The cell death rate was calculated by double staining with two fluorescent dyes: Hoechst 33342 and PI (Invitrogen, CA). At the end of the light exposure, Hoechst 33342 and PI were added to the culture medium for 15 min at final concentrations of 8.1 μM and 1.5 μM, respectively. Images were collected using an Olympus IX70 inverted epifluorescence microscope (Olympus, Tokyo, Japan). The total number of cells was counted in a blind manner (by S.S.) and the rate of PI-positive cell numbers was calculated.

2.4. Western blotting analysis

The 661W cells were seeded at a density of 3×10^4 cells per well in 12-well plates, and then incubated for 24 h under a humidified atmosphere of 5% CO_2 at 37 °C. Then, they were treated with 10 μM cutamesine and incubated for 1 h. The cells were exposed to 2500 lux of white fluorescent light for 24 h, then washed with PBS, lysed in RIPA buffer (Sigma–Aldrich, St. Louis, MO) supplemented with 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktails 2 and 3 (Sigma–Aldrich), and harvested. The lysates were centrifuged at 12,000 *g* for 15 min at 4 °C. Protein concentrations were measured by comparison with a known concentration of bovine serum albumin, using a BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Sample buffer was then added, and the samples were boiled for 5 min. The samples were subjected to 5–20% SDS-PAGE gradient electrophoresis and then transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA). For immunoblotting, the following primary antibodies were used: rabbit anti-sigma-1 receptor antibody (Abcam, Cambridge, UK) and β-actin mouse monoclonal antibody (Sigma–Aldrich). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (Pierce Biotechnology, Rockford, IL, USA) and HRP-conjugated goat anti-mouse antibody (Pierce Biotechnology) were used as the secondary antibody. Immunoreactive bands were visualized using Immunostar-LD (Wako, Osaka, Japan) and a LAS-4000 luminescent image analyzer (Fuji Film Co., Ltd., Tokyo, Japan).

2.5. Mitochondrial membrane potential assay

A mitochondrial membrane potential assay was conducted after the 661W cells were exposed to light. Mitochondrial membrane potential was measured using a JC-1 Mitochondrial Membrane Potential Assay Kit (Cayman Chemical Company, Ann Arbor, MI) according to the manufacturer's protocol. Images were collected using an Olympus IX70 inverted epifluorescence microscope, which detects healthy cells with mainly JC-1 J-aggregates (excitation/emission = 540/605 nm) and apoptotic or unhealthy cells with mainly JC-1 monomers (excitation/emission = 480/510 nm). Merged cells were determined to be preapoptotic (early or middle stage of transition to cell death) cells (Tsuruma et al., 2011). The number of cells per condition was counted in a blind manner with the aid of image-processing software (ImageJ).

2.6. Caspase 3/7 assay

Caspase 3/7 activity in the 661W cells was measured after 24 h of light exposure, using Caspase-Glo 3/7 Assay (Promega, Madison, WI) according to the manufacturer's instructions. After the light exposure, caspase-Glo 3/7 Reagent was added at a 1:1 ratio of reagent volume to sample volume, and then the cells were incubated for 1 h at 37 °C. The lysated samples were transferred to a white–wall well plate. The luminescence of each sample was measured using Skanit RE for Varioslan Flash 2.4 (Thermo Scientific).

2.7. Animals

Male adult ddY mice, aged 8 weeks, were purchased from Japan SLC (Hamamatsu, Japan) and kept under controlled lighting conditions (12 h/12 h: light/dark). All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approved and monitored by the Institutional Animal Care and Use Committee of Gifu Pharmaceutical University.

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