### Abstract
The antioxidant MCI-186 eliminates radicals in the brain and has been used to treat brain ischemia under the name of Edaravone (Radicut®). Here, we examined its ability to prevent the lens opacity and DNA damage that occur upon UV radiation. For this purpose, we used organ-cultured rat lenses. We examined the effect of co-culturing the lenses with MCI-186 on the degree of UV light-induced lens opacity. We also measured the levels of the reduced form of glutathione (GSH) as well as the amount of DNA damage via the comet assay. When the lenses were exposed to 5 kj/m² UV radiation, MCI-186 had no significant effect on lens opacity or GSH levels. However, the comet assay revealed MCI-186 slightly but significantly prevented DNA damage. In addition, when the lenses were exposed to 2 kj/m² UV radiation, a more palliative dose, DNA damage was strongly inhibited by the drug.
Ability of the Free Radical Scavenger MCI-186 to Protect the Lens from Ultraviolet Radiation-induced Damage

Mikako Oka,¹ Norio Sugama,¹ Eiichi Shoji,² Shizuko Kobayashi,¹ Makoto Takehana¹

¹Department of Molecular Physiology, Kyoritsu University of Pharmacy, ²Department of Ophthalmology, Koshigaya Hospital Dokkyo University, School of Medicine.

Abstract
The antioxidant MCI-186 eliminates radicals in the brain and has been used to treat brain ischemia under the name of Edaravone (Radicut®). Here, we examined its ability to prevent the lens opacity and DNA damage that occur upon UV radiation. For this purpose, we used organ-cultured rat lenses. We examined the effect of co-culturing the lenses with MCI-186 on the degree of UV light-induced lens opacity. We also measured the levels of the reduced form of glutathione (GSH) as well as the amount of DNA damage via the comet assay. When the lenses were exposed to 5 kJ/m² UV radiation, MCI-186 had no significant effect on lens opacity or GSH levels. However, the comet assay revealed MCI-186 slightly but significantly prevented DNA damage. In addition, when the lenses were exposed to 2 kJ/m² UV radiation, a more palliative dose, DNA damage was strongly inhibited by the drug.

Introduction
Ultraviolet (UV) light is classified into three types according to its wavelength, namely, UVA, UVB, and UVC. These have wavelength zones that range from 320-400 nm, 290-320 nm, and 190-290 nm, respectively. UVA accounts for about 90% of the UV light emanating from the sun, while UVA and UVB together form near-ultraviolet rays. The latter primarily affect the living body, as UVC is completely absorbed by the ozone layer in the stratosphere of the earth.

Free radicals generated by exposure to UV rays or lipid peroxide are believed to be one of the causes of cataracts (1,2). Free radicals include superoxide, hydrogen peroxide, hydroxy radicals, and lipid hydroperoxy radicals. These molecules have the ability to steal electrons from other compounds, thereby stabilizing themselves. Once they are generated, a chain reaction of free radical production can occur. In the lens, free radicals generated by UV rays can oxidize the thiol-groups of proteins in the lens, thus removing hydrogen and generating a disulfide bond. This induces the protein to aggregate (3,4). In addition, oxygen radicals produced in the eye generate lipid peroxide from unsaturated lipid via lipid peroxidation. The resulting lipid peroxides have a potent oxidative force and induce the degeneration and aggregation of proteins in the lens. These protein-damaging mechanisms ultimately lead to the development of cataracts.

All organisms have a variety of mechanisms that prevent free radicals from being generated or reacting with surrounding compounds. Important antioxidants in
the lens include the reduced form of glutathione (GSH), superoxide dismutase (SOD) and ascorbic acid (5). While these antioxidants and enzymes block the oxidation of the lens, when their bioactivity is reduced, the lens becomes oxidated and opaque.

It may be possible to prevent the development of cataracts by topically applying antioxidant drugs that eliminate radicals. One such drug is MCI-186, which has been used to treat brain ischemia and exerts a protective action by mopping up radicals in the brain (6,7). Here, we tested the efficacy of MCI-186 in preventing lens damage by culturing rat lenses with or without the drug under 2 or 5 kJ/m² UVB radiation (275 ~375 nm, max. 312 nm). These radiation conditions resulted in lens opacity within the following 48 hours of culture. We examined the ability of MCI-186 to prevent lens opacity, elevate GSH levels, and reduce DNA damage.

Experimental materials and methods

Lens culture

Wistar rats were purchased from Sankyo Lab Service Co. Inc. (Tokyo, Japan). Animal experimental procedures conformed to the guidelines of the Committee of the Ethics of Animal Experiments at the Kyoritsu University of Pharmacy. The lenses were obtained by sacrificing the rats and enucleating the eyes. After the eyes were cleaned with phosphate buffer solution (PBS) and sterilized with 75% ethanol for 10 seconds, the lenses were removed and cultured in 5% CO₂ at 37°C in 3.5 mm petri dishes containing 4 mL culture medium composed of DMEM (GIBCO BRL), 10% FBS, and 1% antibiotic-antimycotic (GIBCO BRL).

Drug treatment, UV radiation, and determination of opacity

MCI-186 was supplied by Mitubishi Pharma Corp. (Osaka Japan). An aliquot from Lot. 99001 was weighed and dissolved in 0.5 mL 1 N NaOH, after which Milli-Q was added to yield a volume of 8 mL. The pH was adjusted to 7.0 with 1 N hydrochloric acid and then Milli-Q was added to increase the volume to 10 mL. The solution was subsequently filtered (0.22 mm). The lenses that had been cultured for 24 hours and had maintained their transparency were then cultured further for 1 hour with various concentrations of the drug delivered as a 40 μL aliquot. Thereafter, the lenses were placed in PBS and subjected to a total dose of 2 or 5 kJ/m² UVB radiation (275~375 nm, max. 312 nm). The lenses were irradiated with 2 mW/cm² ultraviolet rays for 250 seconds and with 333 μW/cm² UVB rays for 600 seconds to provide a total radiation dose of 5 kJ/m² and 2 kJ/m² respectively. The lenses were then placed back in MCI-186-containing medium and cultured for an additional 48 hours. The opacity of the lens 48 hours after UV radiation was visually scored, as described in Table 1 and Figure 1.

<table>
<thead>
<tr>
<th>Opacity score</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Transparent</td>
</tr>
<tr>
<td>1</td>
<td>Fair opacity on the surface</td>
</tr>
<tr>
<td>2</td>
<td>Opacity in the overall cortex</td>
</tr>
<tr>
<td>3</td>
<td>Severe opacity in the cortex</td>
</tr>
<tr>
<td>4</td>
<td>Severe opacity in the cortex and fair opacity in the nucleus</td>
</tr>
<tr>
<td>5</td>
<td>Severe opacity in the cortex and severe opacity in the nucleus</td>
</tr>
</tbody>
</table>

The Journal of Kyoritsu University of Pharmacy 2006. 3 Vol. 1
Measurement of lens opacity.

Shown are lenses with opacity scores of 0 (transparent) (a), 1 (fair opacity on the surface) (b), 2 (opacity in the overall cortex) (c), and 3 (severe opacity in the cortex) (d).

Measurement of GSH

The amount of GSH in the lenses was assayed by reacting the homogenate of the lens with dithionitrobenzoic acid [5,5'-dithio-bis-(2-nitrobenzoic acid), DTNB] as described by Sedlak (8). Briefly, after measuring opacity, the lenses were homogenized in 800 μL of 0.2 M sodium phosphate buffer (pH8.0) and centrifuged at 9000 x g for 10 minutes. 150 μL of 50% TCA solution was added to 600 μL of the supernatant and left to stand for 15 minutes. Thereafter, the mixture was centrifuged at 9000 x g for 10 minutes, and 800 μL of 0.2 M sodium phosphate buffer (pH8.0) and 100 μL of 0.02% DTNB in 1% sodium citrate solution were added to 200 μL of the supernatant. After 5 minutes, the absorbance at 412 nm was measured.

Comet assay

The comet assay was conducted as described by Ostling (9) with some modifications. Briefly, 48 hours after UV radiation, the capsule was separated from the lens and treated with trypsin at 37°C for 6 minutes. Epithelial cells were separated from the capsule and centrifuged at 1500 rpm for 5 minutes. The cells were counted and suspended with culture medium at a concentration of 1 x 10⁶ Cells/mL. Slides were prepared by creating a thin film of 0.75% agarose on the glass, after which the slides were dried. The cells were then mixed with 1% low melting point agarose [ultra PURE (Low Melting), GIBCO BRL] at a ratio of 1:1, put on the dried thin film, and covered with the cover.

After cooling, the cover glass was removed and the slides were immersed in mixing solution [2.5 M NaCl, 100 mM EDTA, 10 mM Tris (pH 10.0), 1% N-lauroyl-sarcosine, 0.01% TritonX-100, 1% DMSO] for 75 minutes. After the slides were immersed in electrophoresis buffer [300 mM NaOH, 1 mM EDTA (pH 13.0)] for 20 minutes, the slides and the resulting solution was electrophoresed at 20 V for 15 minutes. After being three times immersed in 0.4 M Tris (pH 7.6) for 5 minutes, the gel was stained with ethidium bromide (20 mg/mL). DNA-strand damage was detected by fluorescence microscopy and comet lengths were measured from microscopic photographs.

Statistical analysis

The degree of opacity, the amount of GSH, and the comet length in MCI-186-treated UV-irradiated lenses were compared to the same measurements made with untreated UV-irradiated lens by t-tests. P values of <0.05 were regarded as significant.

Results

1. Effect of MCI-186 on UV light-induced lens opacity

Rat lenses were incubated with or without varying concentrations of MCI-186 for 1 hour, exposed to 5 kJ/m² or 2 kJ/m² UVB, and then incubated for another 48 hours with or without the drug. The degree of
opacity in the cultured lenses was then scored by the system depicted in Figure 1 and Table 1. All of the 5 kJ/m² UVB unirradiated control lenses had an opacity score of 0 while almost all of the irradiated lenses had an opacity score of 1, regardless of whether the lenses were treated with MCI-186 or not (Fig.2). Thus, MCI-186 does not protect rat lenses from 5 kJ/m² UV radiation-induced opacity.

2. Effect of MCI-186 on GSH levels

The GSH levels in the lens served as indicators of the antioxidant effect of the drug. Rat lenses were irradiated with 5 kJ/m² or 2 kJ/m² UV and then cultured with or without varying concentrations of MCI-186 for 48 hours, after which the GSH levels were measured. The 5 kJ/m² irradiated lenses contained significantly less GSH than the unirradiated control lenses (Fig.3).

However, a t-test revealed that MCI-186 treatment had no effect on the GSH levels, although the 10 and 20 µM-treated lenses did show slightly higher GSH levels compared to those in the untreated irradiated lenses (Fig.3). Thus, MCI-186 does not elevate GSH levels in 5 kJ/m² nor 2 kJ/m² UV-irradiated rat lenses.

3. Effect of MCI-186 on UV light-induced DNA damage.

DNA damage was determined by the comet assay. When exposed to stresses such as UV light, DNA segments, moves, and forms a comet-like pattern. If the damage to the DNA is severe, the comet length increases. Therefore, the comet length can be used to determine whether MCI-186 prevents UV light-induced DNA segmentation.

The epithelial cells from the irradiated lenses were obtained and their comet lengths were measured from microscopic photographs. The comet lengths of the MCI-186-treated irradiated lenses are indicated as ratios relative to the average comet length in the untreated UV light-irradiated lenses. Little DNA damage was detected in the unirradiated control lenses but 5 kJ/m² UV radiation markedly increased the comet tail lengths (Fig.4b). A t-test revealed that treatment with 1, 10, 20 or 40 µM MCI-186 significantly decreased the comet tail lengths (Fig.4c and data not shown) compared to those in the untreated UV-irradiated lenses (Fig.4b). However, the differences in comet tail lengths were not particularly profound (data not shown).

Fig.3 Effect of MCI-186 on GSH levels after UV irradiation.

Lenses were irradiated with 5 kJ/m² UV and then cultured with DMEM only (b) or with 0.1 µM (c), 2 µM (d), 10 µM (e), 20 µM (f), or 40 µM MCI-186 (g). Unirradiated lenses are shown in (a) as a control. Twenty lenses were tested per group. The GSH levels in the lenses decreased significantly after UV irradiation but were not altered by MCI-168 treatment.

Fig.4 MCI-186 protects lens epithelial cells from UV light-induced DNA damage.

Shown are the DNA comet tails from lenses before irradiation (a) and 48 hours after the 5 kJ/m² UV-light irradiation (b, c), during which time the lenses were cultured with (b) or without (c) 10 µM MCI-186.
We also examined the effect of MCI-186 on DNA damage induced by a milder UV ray dose, 2 kJ/m². Little DNA damage was detected in the unirradiated control lenses (Fig.4a) but 2 kJ/m² UV radiation markedly increased the comet tail lengths (Fig.4b). Treatment with 10 μM MCI-186 decreased the comet tail length compared to it in the untreated UV-irradiated lenses (Fig.4c). A t-test revealed that treatment with 10, 20, 30 or 40 μM MCI-186 significantly decreased the comet tail lengths (Fig.5). These differences were strongly statistically significant, especially for the 10 μM MCI-186-treated lenses. Notably, when 2 kJ/m²-irradiated lenses were treated with 10 μM MCI-186, significantly smaller comet lengths were also observed. This suggests that even lower doses of MCI-186 may be effective in protecting lenses from irradiation-induced DNA damage Thus, MCI-186 does protect lenses from UV light-induced DNA damage, especially at lower levels of UV light exposure.

Discussion

In this study, the effect of the antioxidant MCI-186 on UV light-irradiated lens damage was examined. For this purpose, rat lenses were irradiated with 5 kJ/m² UVB rays, which results in opacity in the following 48 hours. Since human cataracts develop gradually, these experimental opacity-inducing conditions are relatively severe. Moreover, to generate the 5 kJ/m² exposure, the lenses were irradiated with 2 mW/cm² ultraviolet rays for 250 seconds. This is also rather unnatural as short periods of radiation with a strong UV source may inflict greater damage on the lens compared to when the lenses are irradiated over a longer time with a weaker UV source, even though the cumulative UV light dose may be the same. Thus, to experimentally generate lens opacity via a more natural process, it may be better to irradiate the lenses for a longer time period with a weaker UV light source.

In this study, we also examined the effect of irradiating the lenses with 333 mW/cm² UVB rays for 600 seconds to provide a total radiation dose of 2 kJ/m². Given that the UVB ray intensity in Tokyo is about 300 mW/cm² in summer (July – August) and about 100 μW/cm² in winter (January), the 333 mW/cm² UVB source used in our latter experiments may more closely reflect the natural conditions to which human eyes are exposed. A final point regarding the experimental procedure employed in this study is that the UVB zone (275~316; 375 nm max. 312 nm) that we used is known to directly damage the organism due to the formation of both DNA dimers and free radicals (10, 11, 12).

This study clearly revealed that MCI-186 can protect lenses from UV light-induced DNA damage. This suggests that free radicals generated by the UV radiation were eliminated by MCI-186, thus inhibiting their ability to damage the DNA. This was particularly apparent when the lenses were exposed to the lower, more physiological 2 kJ/m² UV light dose. Notably, an effect was detected with MCI-186 concentrations as low as 0.1 μM. Since plasma concentrations of MCI-186 at 6 μM are used in clinical settings to protect the brain from ischemia (13), it may thus be appropriate, in further experiments, to administer MCI-186 at concentrations below 0.1 μM. Moreover, the effect of MCI-186 on damage induced by using a longer wavelength zone should also be investigated.
References


