Effects of Ophthalmic Formulations Containing Cilostazol Nanoparticles on Retinal Vasoconstriction in Rats Injected with Endothelin-1

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Abstract

Cilostazol (CLZ) is useful for the management of diabetic retinal vascular dysfunction and neuronal degeneration. However, drug delivery in a posterior segment, such as retina, is not possible using eye drops with traditional formulations. In this study, we designed new ophthalmic formulations containing CLZ solid nanoparticles, and investigated whether these ophthalmic formulations provide noninvasive delivery systems targeting the posterior segment of the eye. The new ophthalmic formulations containing 1% CLZ solid nanoparticles were prepared by adding various additives (0.005% benzalkonium chloride (BAC), 0.5% o-mannitol, 2-hydroxypropyl-β-cyclodextrin (HPβCD) and 1% methylcellulose) and subjecting the mixtures to mill methods (CLZβCD ophthalmic formulations; particle size 61 ± 43 nm, mean ± S.D.). The addition of HPβCD and mannitol enhanced the stability of the CLZ dispersion (CLZman), and no precipitation from the CLZβCD ophthalmic formulations was observed until 21 days after preparation. In addition, in the measurement of the antimicrobial activity against Escherichia coli (ATCC 8739), the CLZ nanoparticles in ophthalmic formulations didn’t affect the antimicrobial activity by preservative, such as BAC. In this study, retinal vasoconstriction was produced in rats by intravitreal injection of 1x10^-5 M endothelin-1 (15 μL, ET-1); retinal vasoconstriction in ET-1-injected rats returned to normal by 48 h after injection. On the other hand, the instillation of CLZman ophthalmic formulations suppressed the retinal vasoconstriction in ET-1-injected rats, and theretinal vascular caliber in rats instilled with CLZβCD was similar than that in non-treated rats 3 h after intravitreal injection. It is possible that dispersions containing CLZ nanoparticles provide new possibilities for an effective, non-invasive method to deliver therapeutic agents to intraocular tissues such as the retina, and that an ocular drug delivery system using drug nanoparticles may expand their usage as therapy in the ophthalmologic field.

Keywords: Nanoparticle; Cilostazol; Retina; Drug delivery system; Eye drop

Introduction

Most vision-threatening ocular diseases are associated with the intraocular structures, particularly the posterior segment-related diseases including age-related macular degeneration, diabetic macular edema and endophthalmitis. Recently, pharmaceutical approaches to these diseases have used steroids and oligonucleotides [1,2], and these drugs are generally administered via invasive methods, such as intravitreal injections and subtenon injections, because noninvasive methods to deliver these drugs are not available. However, repeated injections are associated with potential risks of complications, such as cataracts, vitreous hemorrhages and retinal detachment [3]. Moreover, patients may not comply with such regimens. Although, systemic administration has also been used to deliver therapeutic agents to the posterior segment of the eye, this route of administration requires large administration doses because of the inner and outer blood-retinal barriers that separate the retina and the vitreous humor from the systemic circulation [4]. Thus, there is a pressing need for noninvasive delivery systems targeting the posterior segment of the eye.

In treating the posterior segments, it is very important to increase the effectiveness of ocular drugs by enhancing their bioavailability [5]. In order to overcome side-effects and increase ocular drug bioavailability, several strategies, including the preparation of viscous solutions, micro/nanoparticles and hydrogels, have been developed and investigated [6-12]. In the case of viscous solutions, numerous studies have demonstrated that they do not possess sufficient mechanical strength to resist the ocular clearance mechanism, and offer only a transient improvement in ocular residence time [13]. On the other hand, it has been reported that the capability of drugs to penetrate across the cornea can be significantly improved by decreasing the particle size using nanoparticles [8-10,14-16]. Ophthalmic formulations containing drug nanoparticles present a possible solution to the limitations surrounding ocular drug penetration [17-19], and it is known that decreasing direct cellular stimulation and reducing the amount of a drug used by increasing its bioavailability are useful ways to circumvent the side effects related to drug delivery [5]. It is expected that ophthalmic drug systems using nanoparticles may provide an alternative strategy for increasing ocular drug penetration [17-19]. Our previous reports showed that dispersions containing tranilast and indomethacin nanoparticles prepared by a bead mill method cause less corneal damage to human corneal epithelium cells and greater corneal penetration than commercially available tranilast and indomethacin eye drops (RIZABEN® eye drops, INDOMELOL® ophthalmic solution) [20-24]. It is possible that ophthalmic drug delivery systems using nanoparticles will provide a noninvasive way to target drugs to the posterior segment of the eye.

Cilostazol(6-[4-(1-cyclohexyl-1H-tetrazol-5-yl)butoxy]-

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3,4-dihydrocarbostyril, CLZ) is well known to have anti-platelet aggregation and vasodilatory effects with minimal cardiac effects, and has been applied clinically to cerebrovascular diseases. Pharmacologically, CLZ has been found to increase intracellular cyclic Adenosine Monophosphate (AMP) levels by inhibiting its hydrolysis by type 3 phosphodiesterase, resulting in vasculoprotective effects [25,26]. In addition, it has been reported that CLZ treatment is useful for the management of diabetic retinal vascular dysfunction and neuronal degeneration [27], and intra-arterially administered CLZ induces vasodilation of the retinal arterioles of rats, which results in an increase in blood supply to the retina independent of changes in mean arterial pressure [28]. Therefore, if CLZ can be delivered to the retina by the instillation of an ophthalmic formulations containing CLZ, such formulation may beuseful for the therapeutic treatment of the retina.

In this study, we designed new ophthalmic formulations containing CLZ solid nanoparticles using the bead mill method, and investigated whether these formulations provide noninvasive delivery systems targeting the posterior segment of the eye. In addition, we also demonstrate the preventive effect of ophthalmic formulations containing CLZ nanoparticles on retinal vasocostriction.

Methods

Animals and materials

Male Wistar rats (72 rats), 7 weeks of age were housed under standard conditions (12 h/d fluorescent light (07:00-19:00), 25°C room temperature), and allowed free access to a commercial diet (CE-2, Clea Japan Inc., Tokyo, Japan) and water. All procedures were performed in accordance with the Kinki University Faculty of Pharmacy Committee Guidelines for the Care and Use of Laboratory Animals and the Association for Research in Vision and Ophthalmology resolution on the use of animals in research. CLZ microparticles (original CLZ) were kindly donated by Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan). 2-Hydroxypropyl-β-cyclodextrin (HPβCD, average molar substitution, 0.6; average MW, 1380) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Mannitol (n-mannitol) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals used were of the highest purity commercially available.

Preparation of ophthalmic dispersions containing CLZ nanoparticles

CLZ nanoparticles were prepared using zirconiaballs, Pulverisette 7 (a planetary ball mill, Fritsch Japan Co., Ltd., Tokyo, Japan) and Bead Smash 12 (a bead mill, Wakenyaku Co. Ltd., Kyoto, Japan). Zirconia balls (diameter: 10 mm) were added to a zirconia cup (diameter: 45 mm) containing CLZ microparticles (solid, original CLZ), BAC, mannitol or MC, and the mixture was crushed with the Bead Smash 12 for 24 h (400 rpm, room temperature). The mixture was dispersed in saline with or without 5% HPβCD, and crushed with the Bead Smash 12 (5,500 rpm, 30 sec × 30 times, 4°C) using zirconia beads (diameter: 0.1 mm). The compositions of the dispersions containing CLZ are shown in Table 1. One percent CLZ is equivalent to 27.1mM CLZ; the particle sizes and images were obtained using a nanoparticle size analyzer SALD-7100 (Shimadzu Corp., Kyoto, Japan; refractive index 1.60-0.10i) and scanning probe microscope SPM-9700 (Shimadzu Corp., Kyoto, Japan), respectively. The image of dispersions containing CLZ nanoparticles (CLZnano) as described in Table 1 was created by combining a phase and height image using image analysis software connected to the SPM-9700. The solubility of CLZ in saline containing BAC, mannitol, MC and 5% HPβCD was 0.037% (the solubility of CLZ in saline was 0.0005%). In the penetration, the solvent containing additive was filtered through a Minisart CE (pore size of 0.20 μm, Costar, Cambridge, MA, USA), and was performed in aseptic technique.

Stability of ophthalmic dispersions containing CLZ

Three milliliters of ophthalmic dispersions containing CLZ as described in Table 1 were incubated in 5 ml test tubes in the dark at 20°C for 7 day, after which 50μl of sample solution was withdrawn from 5 mm under the surface at the indicated time intervals (total height of liquid, 4 cm). The CLZ concentrations in the samples were determined by the following HPLC method. Fifty microliters of filtrate was added to 100 µl methanol containing 0.3μg benzophenone (internal standard), and the mixture was filtered through a Chromatodisk 4A (pore size 0.45 μm, Kurabo Industries Ltd., Osaka, Japan). The solution (10 µl) was injected into an Inertsil ODS-3 (3 μm, column size: 2.1 mm × 50 mm) column (GL Science Co., Inc., Tokyo, Japan) on a Shimadzu LC-20AT system equipped with a column oven CTO-20A (Shimadzu Corp., Kyoto, Japan). The mobile phase consisted of acetonitrile/methanol/water (35/15/50, v/v) at a flow rate of 0.25 ml/min; the column temperature was 35°C, and the wavelength for detection was 254nm. The retention times of CLZ was 3.7 min, and internal standard used was benzophenone.

Antimicrobial activity of dispersions containing CLZ nanoparticles

CLZnano as described in Table 1 was tested for antimicrobial activity against E. coli (ATCC 8739). The organism was selected based on Japanese Pharmacopoeia (JP) test protocols [29]. According to the standard methodology, the bulk dilution was split into 10 mL aliquots, each of which was inoculated with between 10^7 and 10^9 CFU/mL of E. coli(ATCC 8739) (1 organism per aliquot) and incubated in the presence of Colony-Forming Units vehicle (solution containing 0.001% BAC, 0.5% mannitol, 5% HPβCD and 0.5% MC) or CLZ-containing dispersions at 20°C to 25°C. The inoculated samples were sampled and counted on days 2, 7, 14 and 28. One milliliter aliquots were serially diluted in phosphate buffer, plated induplicate on soybean-casein digest agar (casein soya bean digest agar for JP general test, Wako, Osaka, Japan), and incubated at 31°C for 3 days. Raw data counts were converted to log (CFU) values. Since the samples were diluted at least 1:10 at the time of testing, 10 CFU reduction is the lowest sensitivity allowed by the test.

Assay of CLZ concentration in blood, cornea, lens, vitreous body, sclera, choroid, retina, anterior and posterior part

Thirty microliters of dispersions containing CLZ micro- or nanoparticles (CLZnano or CLZmicro) as described in Table 1 was instilled into the right eye of rats, and the eyes were kept open for about 1 min to prevent the CLZnano or CLZmicro from overloading. After that, the rats were killed under deep isoflurane anesthesia, and the blood was collected from the vena cava10, 30 and 60 min after instillation of CLZ (n=6). The cornea, lens, vitreous body, sclera, choroid, retina, anterior and posterior part as described in Fig. 1 were excised (the portion other than the retina is defined asclera and choroid because the tissues were not further separable), homogenized in methanol on ice, and
centrifuged at 10,000 rpm for 15 min at 4°C. CLZ in the supernatant was analyzed by the HPLC method described above. The area under the curve ($AUC_{CLZ}$) of the CLZ concentration versus time (minutes) (the area under the CLZ concentration-time curve), under the first moment curve ($AUMC_{CLZ}$) and mean residence time ($MRT_{CLZ}$) were calculated according to the following equations (Eqs. 1-3):

$$AUC_{CLZ} = \int_{0}^{60} C_{CLZ} \, dt$$  \hspace{1cm} (1)

$$AUMC_{CLZ} = \int_{0}^{60} C_{CLZ} \, dt$$  \hspace{1cm} (2)

$$MRT_{CLZ} = \frac{AUMC_{CLZ}}{AUC_{CLZ}}$$  \hspace{1cm} (3)

Where $t$ is the time after instillation of eye drops, $C_{CLZ}$ is the CLZ concentration at time $t$. $AUC$ was determined according to the trapezoidal rule up to the last CLZ concentration measurement point.

**Measurement of retinal vascular caliber**

Thirty microliters of CLZ micro or CLZ nano, as described in Table 1 was instilled into the right eye of rabbits, and the eyes were kept open for about 1 min to prevent the CLZ micro or CLZ nano from overflowing. After 5 min, the eyes were dilated by the instillation of 0.1% pivalephrine (Santen Pharmaceutical Co., Osaka, Japan) under anesthesia (isoflurane), and 1 x 10$^{-4}$ M endothelin-1 (15 µL, ET-1) was injected (intravitreal injection). Changes in retinal vascular caliber (RVC) were monitored using a Digital Microscope (Bio Medical Science Inc., Tokyo, Japan) 0, 10, 30, 60, 120, 180 and 240 min after injection of ET-1 (n=6); RVC was analyzed with image analyzing software Image J. Retinal vasoconstriction (%) was calculated according to the following Eq. 4:

$$\text{Ratio of retinal vasoconstriction} \% = \frac{\text{RVC without injection} - \text{RVC with injection}}{\text{RVC without injection}} \times 100$$  \hspace{1cm} (4)

$\Delta$RVC (%) was analyzed as the difference in the ratio of retinal vasoconstriction in rats instilled with saline or eye drops (the enhancement of $\Delta$RVC shows a high protective effect against the retinal vasoconstriction). The area under the curve ($AUC_{\Delta\text{RVC}}$) of $\Delta$RVC versus time (minutes) (area under $\Delta$RVC-time curve) area under the first moment curve ($AUMC_{\Delta\text{RVC}}$) and mean residence time ($MRT_{\Delta\text{RVC}}$) were calculated according to the following equations (Eqs. 5-7):

$$AUC_{\Delta\text{RVC}} = \int_{0}^{240} \Delta\text{RVC} \, dt$$  \hspace{1cm} (5)

$$AUMC_{\Delta\text{RVC}} = \int_{0}^{240} \Delta\text{RVC} \cdot t dt$$  \hspace{1cm} (6)

$$MRT_{\Delta\text{RVC}} = \frac{AUMC_{\Delta\text{RVC}}}{AUC_{\Delta\text{RVC}}}$$  \hspace{1cm} (7)

Where $t$ is a time after ET-1 injection. AUC was determined according to the trapezoidal rule up to the last RVC value measurement point.

**Statistical analysis**

All values are presented as mean ± Standard Deviation (S.D.) or Standard Error of the mean (S.E.). Unpaired Student’s-t test was used to evaluate statistical differences, and multiple groups were evaluated by one-way analysis of variance followed by Dunnett’s multiple comparison. P values less than 0.05 were considered significant.

**Results**

**Establishment of ophthalmic dispersions containing CLZ nanoparticles by bead mill methods**

Figure 2 and Table 2 show the particle size distributions (Figure 2) and mean particle diameters (Table 2) of dispersions containing 1% CLZ as described in Table 1. CLZ microparticles (18.8 ± 14.0 µm) containing BAC, mannitol, HPβCD and MC were milled by the mill method to a mean particle size of 61 ± 43 nm (mean ± S.D., CLZ nano, Fig. 2E and G). On the other hand, CLZ reached a meringue state by the mill method using CLZ microparticles containing BAC, mannitol and HPβCD (Milled-CLZ nano, Fig 2F). The CLZ particles obtained by the addition of BAC, mannitol and MC had a mean particle size of 610 ± 101 nm (Milled-CLZ nano, HPβCD means ± S.D.), and the mean particle size of Milled-CLZ nano, HPβCD was larger than that of CLZ nano. There was no difference in particle size among Milled-CLZ nano, Milled-CLZ nano, and CLZ nano. Figure 3 shows the stability of dispersions containing 1% CLZ as described in Table 1. The CLZ nano preparation precipitated 12 days after preparation. The stability of dispersions containing CLZ was increased by the combination of additive (BAC, mannitol or HPβCD) and the bead mill method. The addition of HPβCD and mannitol enhanced the stability of the CLZ dispersion (CLZ nano). The Milled-CLZ nano, HPβCD and Milled-CLZ nano, preparations precipitated 4 h after preparation. The stability of dispersions containing CLZ was observed up to 21 days after preparation. Although, the antimicrobial activity of Milled-CLZ nano, was observed, the CLZ nano preparation showed high antimicrobial activity approximately equal to that of the 0.001% BAC solution (Figure 4).

**Effect of ophthalmic dispersions containing CLZ nanoparticles on theretinatmPT-1-injected rats**

Figure 5 shows the CLZ concentrations in blood and cornea, lens,
vitreous body, sclera, choroid, retina, anterior, posterior part of the right eyes of rats after eye drop instillation, and Table 3 summarizes the pharmacokinetic parameters calculated from the data in Fig. 5. No significant difference in plasma CLZ concentration was observed. On the other hand, in the right eye field, including the cornea, lens, vitreous body, sclera, choroid, retina, anterior and posterior part (Fig. 5B-H), the CLZ concentration in rats instilled with CLZ nanoparticles was significantly higher than those in rats instilled with CLZ microparticles. The AUC values in the cornea, lens, vitreous body, sclera containing choroid, retina, anterior and posterior part were approximately 1.9, 1.8, 8.0, 6.2, 8.2, 24.1 and 16.0-fold higher in rats instilled with CLZ nanoparticles than those instilled with CLZ microparticles. In contrast to the results in CLZ concentration in the right retina of rat instilled with CLZ nanoparticles, the CLZ in the left retinas of rats, which did not receive instillations, was undetectable. Figure 6 shows the preventive effects of the instillation of CLZ nanoparticles on retinal vasoconstriction in ET-1-injected rats, and Table 4 summarizes the pharmacokinetic parameters calculated from the data in Fig. 6. Retinal vasoconstriction was induced by the
intravitreal injection of ET-1, and the RVC in ET-1-injected rats was 49.6% that of non-treated rats (normal rats) at 30 min after intravitreal injection. The retinal vasoconstriction in ET-1-injected rats recovered by 48 h after intravitreal injection. Although, retinal vasoconstriction have been seen as an effective preservative and to be indispensable in the preparation of eye drops. However, BAC has been shown to be highly toxic both in vitro and in vivo due to a stimulatory effect on epithelial cell death [30-32]. Clinically, these iatrogenic effects are found most frequently for eye drops used to treat long-term pathologies and inflammation. The side effects of BAC seem to be both dose- and time-dependent, increasing with larger amounts used for longer periods. On the other hand, we previously reported that the addition of mannitol prevents the corneal stimulation caused by BAC [33]. Moreover, Mori et al. [34] reported that adsorption to the surface of cyclodextrin decreases the cohesion of nanoparticulate solids, and we previously reported that the addition of HPβCD is suitable for the preparation of nanoparticles using mill methods [22-24,35]. Jansen et al. [36] have reported no observable irritation of the eye membrane by solutions containing HPβCD at levels less than 12.5%. Taken together, in this study we attempted to prepare a CLZ dispersion containing BAC, mannitol and 5% HPβCD using the bead mill method. However, the CLZ became meringue-like when subjected to the bead mill method. Therefore, BAC has been shown as an effective preservative and to be indispensable in the preparation of eye drops. However, BAC has been shown to be highly toxic both in vitro and in vivo due to a stimulatory effect on epithelial cell death [30-32]. Clinically, these iatrogenic effects are found most frequently for eye drops used to treat long-term pathologies and inflammation. The side effects of BAC seem to be both dose- and time-dependent, increasing with larger amounts used for longer periods. On the other hand, we previously reported that the addition of mannitol prevents the corneal stimulation caused by BAC [33]. Moreover, Mori et al. [34] reported that adsorption to the surface of cyclodextrin decreases the cohesion of nanoparticulate solids, and we previously reported that the addition of HPβCD is suitable for the preparation of nanoparticles using mill methods [22-24,35]. Jansen et al. [36] have reported no observable irritation of the eye membrane by solutions containing HPβCD at levels less than 12.5%. Taken together, in this study we attempted to prepare a CLZ dispersion containing BAC, mannitol and 5% HPβCD using the bead mill method. However, the CLZ became meringue-like when subjected to the bead mill method. When CLZ microparticles containing BAC, mannitol and HPβCD (Milled-CLZ (β- Figure 2F) were used. Therefore, a new innovation for the preparation of CLZdispersions was required.

Discussion

We designed new ophthalmic formulations containing CLZ solid nanoparticles using the bead mill method [22-24], and investigated the possibility of using these formulations as noninvasive delivery systems targeting the posterior segment of the eye. In addition, we also demonstrated the preventive effect of these ophthalmic formulations containing CLZ nanoparticles on retinal vasoconstriction.

In the design of ophthalmic formulations containing CLZ nanoparticles, the selection of additives is important. BAC is known to have a strong preservative effect, and its surface-active effects increase the corneal penetration of the main component. Therefore, BAC has been shown as an effective preservative and to be indispensable in the preparation of eye drops. However, BAC has been shown to be highly toxic both in vitro and in vivo due to a stimulatory effect on epithelial cell death [30-32]. Clinically, these iatrogenic effects are found most frequently for eye drops used to treat long-term pathologies and inflammation. The side effects of BAC seem to be both dose- and time-dependent, increasing with larger amounts used for longer periods. On the other hand, we previously reported that the addition of mannitol prevents the corneal stimulation caused by BAC [33]. Moreover, Mori et al. [34] reported that adsorption to the surface of cyclodextrin decreases the cohesion of nanoparticulate solids, and we previously reported that the addition of HPβCD is suitable for the preparation of nanoparticles using mill methods [22-24,35]. Jansen et al. [36] have reported no observable irritation of the eye membrane by solutions containing HPβCD at levels less than 12.5%. Taken together, in this study we attempted to prepare a CLZ dispersion containing BAC, mannitol and 5% HPβCD using the bead mill method. However, the CLZ became meringue-like when subjected to the bead mill method. When CLZ microparticles containing BAC, mannitol and HPβCD (Milled-CLZ (β- Figure 2F) were used. Therefore, a new innovation for the preparation of CLZ dispersions was required.

MC, a derivative of cellulose, is a water-soluble substance with a high degree of purity, uniformity and transparency. The MC is highly biocompatible [37-39] and is used in the preparation of ophthalmic

### Table 2: Changes in CLZ particle size in CLZ dispersions with or without BAC, mannitol, HPβCD and MC.

<table>
<thead>
<tr>
<th>Blood</th>
<th>AUC&lt;sub&gt;CLZ&lt;/sub&gt; (nmol•min/ ml)</th>
<th>AUMC&lt;sub&gt;CLZ&lt;/sub&gt; (nmol•min/g or ml)</th>
<th>MRT&lt;sub&gt;CLZ&lt;/sub&gt; (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLZ</td>
<td>8.0 ± 2.6</td>
<td>237.5 ± 21.9</td>
<td>29.6 ± 1.9</td>
</tr>
<tr>
<td>CLZα</td>
<td>11.2 ± 3.4</td>
<td>335.3 ± 30.8</td>
<td>30.0 ± 2.2</td>
</tr>
<tr>
<td>Cornea</td>
<td>80.8 ± 5.6</td>
<td>1582.5 ± 105.7</td>
<td>19.5 ± 1.1</td>
</tr>
<tr>
<td>Lens</td>
<td>165.4 ± 10.7</td>
<td>3397.5 ± 195.8</td>
<td>20.5 ± 1.5</td>
</tr>
<tr>
<td>Vitreous</td>
<td>10.4 ± 1.1</td>
<td>264.3 ± 11.9</td>
<td>25.3 ± 1.1</td>
</tr>
<tr>
<td>Sclera and Choroid</td>
<td>1284.4 ± 97.1</td>
<td>23625.6 ± 896.8</td>
<td>18.4 ± 0.7</td>
</tr>
<tr>
<td>Retina</td>
<td>6756.1 ± 592.7</td>
<td>141975.0 ± 4517.7</td>
<td>20.9 ± 1.1</td>
</tr>
<tr>
<td>Anterior part</td>
<td>16.8 ± 1.6</td>
<td>446.1 ± 16.4</td>
<td>23.7 ± 0.9</td>
</tr>
<tr>
<td>Posterior part</td>
<td>4.3 ± 0.1</td>
<td>103.8 ± 5.4</td>
<td>23.9 ± 1.2</td>
</tr>
</tbody>
</table>

### Table 3: Pharmacokinetic parameters for the suppression of retinal vasoconstriction by CLZ<sub>α</sub> in rats injected intra vitreally with ET-1.

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>AUC&lt;sub&gt;α&lt;/sub&gt; (%)• min</th>
<th>AUMC&lt;sub&gt;α&lt;/sub&gt; (%)• min</th>
<th>MRT&lt;sub&gt;α&lt;/sub&gt; (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>181.3 ± 7.9</td>
<td>7623.0 ± 321.5</td>
<td>42.0 ± 0.8</td>
</tr>
<tr>
<td>CLZ</td>
<td>904.1 ± 31.3&lt;sup&gt;*&lt;/sup&gt;</td>
<td>61593.7 ± 2286.4&lt;sup&gt;*&lt;/sup&gt;</td>
<td>68.1 ± 1.1&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>CLZ&lt;sub&gt;α&lt;/sub&gt;</td>
<td>3909.4 ± 117.5&lt;sup&gt;**&lt;/sup&gt;</td>
<td>423018.2 ± 14105.3&lt;sup&gt;**&lt;/sup&gt;</td>
<td>108.2 ± 1.7&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

### Table 4: Pharmacokinetic parameters for the suppression of retinal vasoconstriction by CLZ<sub>α</sub> in rats injected intra vitreally with ET-1.

Retinal vasoconstriction in the right eye was caused by intravitreal injection of ET-1. Parameters were calculated according to Eqs. 4-7 (see Materials and methods). *P< 0.05, vs. CLZ<sub>α</sub>-instilled rats; CLZ<sub>α</sub>-instilled rats. The data are presented as means ± S.E. of 6 independent rats. 

References


vasoconstriction of the right eye. CLZ to Eq. 4 (see Materials and methods). The data are presented as the means ± S.E. of 6 independent experiments. *P < 0.05, vs. CLZ

Figure 5: Changes in CLZ concentration in the blood (A), cornea (B), lens (C), vitreous body (D), sclera with choroid (E), retina (F), anterior (G) and posterior part (H) of rats instilled with dispersions containing CLZ micro- or nanoparticles. Rat eyes were instilled with 30 μl of dispersions containing CLZ microparticles (CLZmic) or nanoparticles (CLZnano). The data are presented as the means ± S.E. of 6 independent experiments. *P < 0.05, vs. CLZnano.

Next, we evaluated CLZ concentrations in blood and cornea, lens, vitreous body, sclera, choroid, retina, anterior and posterior part of the right eye of rats after the instillation of CLZnano. The determination of the concentration in ophthalmic formulation is important. We previously reported that the instillation of 0.05% CLZ ophthalmic solution decrease the enhanced Intraocular Pressure (IOP), and was useful for the therapeutic treatment of the glaucoma [35]. However, it is difficult to deliver the drug to retina by the ophthalmic formulation containing low drug concentration. Therefore, we attempted to prepare the CLZ ophthalmic formulation containing high drug concentration, which would be changed by labeling. Therefore, we measured changes in the CLZ concentration in ocular tissues. In general, topically administered drugs are absorbed either through the corneal route (cornea → aqueous humor → intraocular tissues) or non-corneal route (conjunctiva → sclera → choroid/retinalpigment epithelium)[50]. In addition, drugs absorbed into the conjunctiva can enter the aqueous humor as well as the sclera, showing good access to the trabecular meshwork, iris root and pars plana [51]. In the CLZ-instilled right eye field, such as the cornea, lens, vitreous body, sclera, choroid, retina, anterior and posterior part (Fig. 5B-H), the CLZ concentrations in rats instilled with CLZnano were significantly higher than in rats instilled with CLZmic in this study. In contrast to the results for AUCCLZ and MRT CLZ in the right eye field of rats instilled with CLZnano, CLZ concentrations in the left retina, which received no drug instillation, were undetectable.

formulations. Mueller and Deardorff et al. [40] stated that MC does not cause eye irritation or damage, and they used 1% MC in the development of ophthalmic formulations. The gel strength depends on the degree of substitution and the molecular weight [41,42]. In addition, our previously reports showed that the addition of MC enhanced the crushing efficiency of bead mill method [22,24]. The CLZ particle size was decreased using a combination of MC and the bead mill method, and the CLZ particle size reached the nano order by the bead mill method using CLZ nanoparticles containing BAC, mannitol, HPβCD and MC (CLZnano, Figure 2E and G). The size of a particle influences its functionality in terms of uptake, residence time in circulation, adherence, degradation, as well as clearance [43-47]. The fate of particles inside the body has been reported as follows: ≥ 2 μm, trapped inside liver cells; ≥ 300 - 400 nm, captured by macrophages and excreted; ≥ 200 nm, filtered in the spleen; ≤ 100 nm, escape from blood vessels through the endothelial lining. Thus, size governs the movement of nanoparticles inside tissues. In the ophthalmic field, nanoparticles in sizes ranging from 10 to 1000 nm allow for improved topical passage of large, water insoluble molecules through the barriers of the ocular system [48]. In this study, the particles size of CLZnano is 61 nm, and it is expected that CLZnano may provide an alternative strategy for increasing ocular drug penetration. In addition, the stability of dispersions containing CLZnanoparticles increased by the addition of 5% HPβCD (CLZnano, Fig. 3). Furthermore, we examined whether the preservative effect and stability of CLZ in the CLZnano formulation are changed, or not. Although, no antimicrobial activity of CLZ itself was observed, the CLZnano preparation showed high antimicrobial activity approximately equal to that of a 0.001% BAC solution (Fig. 4), and no degradation or reduction in the amount of CLZ in CLZ formulations with or without BAC was detected by HPLC methods.

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addition, no significant difference in plasma CLZ concentration was observed. Taken together, although further investigation is required, the delivery of CLZ nano to the posterior segment of the eye might occur via both corneal and non-corneal pathways.

In order to study accurately the effects of ophthalmic formulations containing CLZ nanoparticles on the retina and posterior segment, the selection of the experimental model is very important. ET-1, which is thought to be a highly relevant factor for ocular blood flow, is known to be a very potent and long-lasting vasoconstrictor peptide originating in endothelial cells [52]. In the retina, the ET-1 is a potent vasoactive peptide that causes vasoconstriction of retinal vessels. ET-1 and its receptors have been found in ocular tissues where it appears to have a regulatory function [53,54]. ET-1 is found in both the aqueous and vitreous humors and its concentration is elevated in glaucoma patients [55-58] and in animal models of glaucoma [59-61]. ET-1 is an important contributing factor in retinal injury of retina and optic neuropathy. Therefore, we evaluated the effects of CLZ nano using ET-1-induced retinal injury in rats. Retinal vasoconstriction was observed following the intravitreal injection of ET-1, and this retinal vasoconstriction was suppressed by the instillation of CLZ nano. In addition, no suppression of retinal vasoconstriction was observed in the left eye (non-instillation) of ET-1-injected rats. These results suggest that the instillation of CLZ nano can prevent retinal vasoconstriction via an ocular route.

Further studies are needed to elucidate the usefulness and the route of CLZ after the instillation of dispersions containing CLZ nanoparticles. Therefore, we are now investigating the route of CLZ after the instillation of dispersions containing CLZ nanoparticles using rabbits. In addition, we are demonstrating the effect by the ophthalmic formulation containing the different CLZ concentration.

In the present study, we attempted to establish a new method for preparing drug solid nanoparticles, and succeeded in preparing a high quality dispersion containing CLZ nanoparticles. The state of the dispersions containing CLZ nanoparticles does not affect the antimicrobial activity of BAC against E. coli, and the instillation of the ophthalmic dispersions containing CLZ nanoparticles suppresses retinal vasoconstriction in ET-1-injected rats. It is possible that dispersions containing CLZ nanoparticles provide new possibilities for the effective delivery of therapeutic agents to intraocular tissues such as the retina using non-invasive delivery methods, and that an ocular drug delivery system using drug nanoparticles may expand their usage for therapy in the ophthalmologic field.

References


