Effect of Instillation of Aldose Reductase Inhibitor FR74366 on Diabetic Cataract

Shizuo Ao, Chie Kikuchi, Takaharu Ono, and Yoshitodo Notsu

The authors investigate the effect of aldose reductase inhibitor FR74366 on diabetic cataract. Streptozocin (STZ)-induced diabetic rats were treated with eye drops of FR74366 (0.03%, 0.1%, and 0.3%) for 16 weeks. Lenses were examined using a slit lamp, and the score of lens opacity was determined on a scale of from 0 (normal lens) to 4 (matured nuclear cataract). Diabetic placebo control rats developed lens opacity linearly, beginning at 3 weeks and reaching a maximum at 8 weeks after STZ injection. Instillation of FR74366 to diabetic rats delayed the cataract formation and inhibited lens sorbitol accumulation in a dose-dependent manner. At 16 weeks after STZ injection, the score of lens opacity was more than 3 (diffuse central opacities) in diabetic placebo control rats, whereas it was less than 2 (peripheral vesicles and cortical opacities) and the lenses remained clear in animals treated with 0.3% of FR74366. Measurement of tissue drug concentrations indicated that FR74366 penetrated into the lens, where its levels were increased in a dose- and time-dependent manner. These three parameters (score of lens opacity and sorbitol and FR74366 levels) were well correlated with each other. Instillation of FR74366 also reduced the sorbitol levels in the retina. However, the sorbitol levels in the sciatic nerve and renal cortex were not changed by instillation of FR74366. Instillation or oral administration of FR74366 has not shown serious side effects in animal toxicity studies. These results suggested that instillation of FR74366 may be a useful therapeutic agent against diabetic cataract and retinopathy.

Several mechanisms have been proposed to explain cataract formation in diabetic rats. One hypothesis proposes that changes in the metabolism of phosphoinositides1 induce structural damage to the lens and cause lens opacification. An alternative theory suggests that ion imbalance, such as may involve Na+, K+, and Ca2+ ions, is associated with cataract formation.2-4 Recently, a polyol osmotic theory has been proposed. It suggests that a high glucose concentration stimulates aldose reductase (AR) (systematic name, alditol: NADP+ 1-oxidoreductase, EC number 1.1.1.21) activity,5-7 producing a high concentration of sorbitol in the tissues, which leads to changes in membrane permeability, membrane damage, tissue swelling, and cataract of the lens.8,9

Recently, several AR inhibitors have been shown to inhibit sorbitol accumulation in various tissues10-14 (lens, retina, sciatic nerve, and kidney) of diabetic rats and to be effective against diabetic complications in experimental14-18 animals and clinical trials.19-21 We found a chemically novel and potent AR inhibitor, FR74366 (FK366; [3-(4-bromo-2-fluorobenzyl)-7-chloro-2,4-dioxo-1,2,3,4-tetrahydro-quinazolin-1-yl] acetic acid), from various derivatives of quinazoline.22 We previously have shown that oral administration of FR74366 is effective in diabetic cataract and neuropathy.23 Topical treatment of ocular disease is interesting in clinical therapy because it may reduce side effects and unwanted effects of the drug on other tissues. The purpose of this paper is to investigate the effect of topical treatment of FR74366 on cataract formation in STZ-induced diabetic rats.

Materials and Methods

Animals

Male Wistar rats (CLEA Japan Inc., Tokyo, Japan) without lens opacity were given unlimited access to water and laboratory rat diet (CLEA, CE-2) and were maintained under a 12-hr-on/12-hr-off light cycle. Animal care and treatment conformed to the ARVO Resolution on the Use of Animals in Research. The rats (7 weeks old, 165–185 g of body weight) fasted overnight, were made diabetic by single intravenous (IV) injection of 60 mg/kg body weight of STZ (Sigma Chemical Co., St. Louis, MO) that had been freshly
dissolved in 2 millimoles per liter (mM) citric acid buffer pH 4.5. Normal control rats were injected with the vehicle only. One week and 16 weeks after induction of diabetes, plasma glucose levels were analyzed by enzymatic assay based on glucose oxidase and peroxidase (Glucose B-Test Wako, Osaka, Japan). Rats with a plasma glucose level greater than 450 mg/dl were used as diabetic animals in this study.

**Instillation of FR74366**

Each FR74366 drop contained glycerol 2.83%; p-oxy-methyl benzoate 0.026%; p-oxy-propyl benzoate 0.014%; ethylenediaminetetraacetic acid (EDTA) disodium salt 0.01%; FR74366 0.03%, 0.1%, 0.3% (dissolved in a minimum of 0.1-N NaOH) and adjusted to pH 7.0 with 0.1-N HCl.

Rats were treated with 2 eye drops of FR74366 (at 15-20-min intervals) on both eyes at 9:00 AM and at 5:00 PM for 16 weeks after induction of diabetes. Normal rats were treated with placebo drops according to the same protocol.

**Evaluation of Cataract Development**

Lenses were examined once a week using a slit lamp (Neitz, Tokyo, Japan) after dilating the pupils by Miodorin P (tropicamide 0.5% and phenylephrine HCl 0.5%; Santen Pharmaceutical, Osaka, Japan). Score of lens opacity is determined as follows according to the classification of Kador et al and Chylack et al:

- 0, clear normal lens
- 1, peripheral vesicles
- 2, peripheral vesicles and cortical opacities
- 3, diffuse central opacities
- 4, matured nuclear cataract

**Determination of Sorbitol and FR74366 Levels in Various Tissues**

Two and five weeks after induction of diabetes, three rats in each group were anesthetized with ether 1 hr after final instillation of FR74366; whole blood was taken from the abdominal aorta, and lenses were dissected. Sixteen weeks after induction of diabetes, rats were perfused from the left ventricle to the right atrium with saline 1 hr after final instillation of FR74366. Lens, retina, sciatic nerve, and kidney were dissected and weighed.

The sorbitol levels of sciatic nerve and renal cortex were measured by enzymatic assay using sorbitol dehydrogenase as described previously. Lens and retina were homogenized in 1 ml of H_2O containing 100 ng/ml of statil (ICI-128436) as internal standard for measurement of FR74366 levels by gas-liquid chromatography (GLC). Perchloric acid (2-N, 0.5 ml) was added to half of each homogenate (0.5 ml), and the resulting precipitates were centrifuged to deproteinize. The supernatants were neutralized with 5 M K_2CO_3 and centrifuged. The neutralized supernatants were used to assay sorbitol levels enzymatically as described previously.

FR74366 was extracted with benzene under acidic conditions with 0.1-N HCl from the remaining homogenates. The benzene was removed, and diazomethane in ether was added to the residue to esterify the carboxylic acid of FR74366. The ether was evaporated, and the residue was dissolved in toluene. The toluene fractions of esterified FR74366 were injected into a silicon-coated fused silica capillary column (25 m × 0.31 mm internal diameter) of a GLC (Hewlett Packard 5890) equipped with an electron capture detector. The column was maintained at 290°C and He was used as carrier gas (2 ml/min).

FR74366 levels in plasma were measured by high performance liquid chromatography (HPLC) using an octadodethyl silicone column as described previously.

**Statistical Methods**

Results are expressed as mean ± SD. The significance of differences was calculated by Student’s t-test.
except for the score of lens opacity, for which Mann-Whitney's U-test was used.

Results

General Features of Diabetic Rats

The increase of body weight was inhibited by induction of diabetes, and the blood glucose levels were increased to more than 450 mg/dl and were not significantly different under feeding conditions at 1, 2, 5, and 16 weeks after STZ injection. There were no effects of instillation of FR74366 on body weight and blood glucose (Table 1). One of the diabetic rats treated with 0.03% eye drops of FR74366 died at 8 weeks, but the cause of death was not known.

The lens sorbitol levels of diabetic placebo control rats were decreased gradually with duration of diabetes. The sorbitol levels were 31.1 ± 1.40 μmol/g at 2 weeks, 22.8 ± 2.61 μmol/g at 5 weeks, and 12.1 ± 4.11 μmol/g at 16 weeks after induction of diabetes. Instillation of FR74366 inhibited lens sorbitol accumulation in a dose-dependent manner after 2, 5, and 16 weeks of treatment (Table 1). The ED₅₀ values of the inhibitory effect on sorbitol accumulation in the lens were >0.3% at 2 weeks, 0.13% at 5 weeks, and 0.25% at 16 weeks of instillation.

Instillation of FR74366 for 16 weeks inhibited retinal sorbitol accumulation in a dose-dependent manner (ED₅₀ = 0.27%), whereas the sorbitol levels in the sciatic nerve and renal cortex were not changed (Table 1).

Prevention of Cataract Formation

By monitoring the progression of opaque area with slit-lamp microscopy, lens opacity initially was observed at 3 weeks, linearly developed from 4 to 8 weeks, and reached a plateau (diffuse central opacities or mature nuclear cataract) from 9 to 16 weeks after STZ injection in placebo control rats (Fig. 2).

Instillation of FR74366 to diabetic rats delayed cataract formation in a dose-dependent manner. Although the score of lens opacity in diabetic control rats rapidly progressed 4-8 weeks after STZ injection, lens opacification clearly was inhibited in diabetic rats treated with FR74366. When the rats were treated with 0.1% and 0.3% of FR74366, the score of lens opacity of the animals' right and left eyes was significantly (α = 0.05, Mann-Whitney's U-test) lower than that of the diabetic placebo control rats at 7-16 weeks.

Figure 2 shows that in diabetic placebo control rats the score of lens opacity was more than 3, and 7 of 20 lenses had a mature nuclear cataract (score 4) after 16 weeks. However, when the rats were treated with 0.3% of FR74366, the score of lens opacity was less than 2, and 3 of 20 lenses had shown no cataract formation (score 0), which was similar to normal lenses.

FR74366 Levels in Lens, Retina, and Plasma

Lenses, retina, and plasma FR74366 levels are shown in Table 2. FR74366 levels were increased in a dose-dependent manner in these tissues at 16 weeks. Lens FR74366 levels were increased in a dose- and time-dependent manner when treated with eye drops of FR74366 for 16 weeks. Sufficient lens FR74366 levels (1.35 ± 0.17 μg/g wet weight) to inhibit sorbitol accumulation were detected at 16 weeks when treated with 0.3% of FR74366.

FR74366 could penetrate into the retina, and FR74366 levels were 0.23 ± 0.08 μg/g wet weight in the retina after treatment with 0.3% eye drops for 16 weeks. The increase of body weight was inhibited by induction of diabetes, and the blood glucose levels were increased to more than 450 mg/dl and were not significantly different under feeding conditions at 1, 2, 5, and 16 weeks after STZ injection. There were no effects of instillation of FR74366 on body weight and blood glucose (Table 1). One of the diabetic rats treated with 0.03% eye drops of FR74366 died at 8 weeks, but the cause of death was not known.

Sorbitol levels were increased to more than 450 mg/dl and were not significantly different under feeding conditions at 1, 2, 5, and 16 weeks after induction of diabetes. Instillation of FR74366 inhibited lens sorbitol accumulation in a dose-dependent manner after 2, 5, and 16 weeks of treatment (Table 1). The ED₅₀ values of the inhibitory effect on sorbitol accumulation in the lens were >0.3% at 2 weeks, 0.13% at 5 weeks, and 0.25% at 16 weeks of instillation.

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FR74366 could penetrate into the retina, and FR74366 levels were 0.23 ± 0.08 μg/g wet weight in the retina after treatment with 0.3% eye drops for 16 weeks.

Table 1. General features and inhibitory effect of instillation of FR74366 on sorbitol accumulation in various tissues of diabetic rats

<table>
<thead>
<tr>
<th>Items</th>
<th>N</th>
<th>Normal placebo</th>
<th>STZ-diabetic placebo</th>
<th>STZ-diabetic + eye drop of FR74366</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.03%*</td>
</tr>
<tr>
<td>Body weight (g) 16 weeks</td>
<td>10</td>
<td>388 ± 20.9†</td>
<td>235 ± 37.6</td>
<td>217 ± 27.5</td>
</tr>
<tr>
<td>Plasma glucose (mg/dl)</td>
<td>10</td>
<td>166 ± 11.1†</td>
<td>556 ± 48.4</td>
<td>612 ± 50.9†</td>
</tr>
<tr>
<td>Sorbitol levels</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lens (μmol/g) 2 weeks</td>
<td>3</td>
<td>0.42 ± 0.01†</td>
<td>31.1 ± 1.40</td>
<td>30.8 ± 1.26</td>
</tr>
<tr>
<td>Lens (μmol/g) 5 weeks</td>
<td>3</td>
<td>0.87 ± 0.65†</td>
<td>22.8 ± 2.61</td>
<td>14.9 ± 4.36</td>
</tr>
<tr>
<td>Lens (μmol/g) 16 weeks</td>
<td>10</td>
<td>0.16 ± 0.13†</td>
<td>12.1 ± 4.11</td>
<td>10.4 ± 3.18</td>
</tr>
<tr>
<td>Retina (nmol/g)</td>
<td>10</td>
<td>94.8 ± 32.9†</td>
<td>994 ± 367</td>
<td>843 ± 282</td>
</tr>
<tr>
<td>Sciatic nerve (nmol/g)</td>
<td>10</td>
<td>116 ± 23.1†</td>
<td>1181 ± 103</td>
<td>1233 ± 246</td>
</tr>
<tr>
<td>Renal cortex (nmol/g)</td>
<td>10</td>
<td>102 ± 27.7†</td>
<td>259 ± 46.0</td>
<td>274 ± 71.7</td>
</tr>
</tbody>
</table>

Rats were injected with STZ (60 mg/kg, IV) and treated with two eye drops of FR74366 (0.03%, 0.1%, and 0.3%) at 9 AM and at 5 PM for 16 weeks. Tissues were dissected at 16 weeks and lenses were dissected at 2, 5, and 16 weeks after STZ injection. Tissue sorbitol levels were measured by enzymatic assay and expressed as nanomoles or micromoles per gram wet weight. Values are mean ± SD.

* N = 9 at 16 weeks.
† P < 0.001 vs STZ-diabetic placebo control.
‡ P < 0.05 vs STZ-diabetic placebo control.
§ P < 0.01 vs STZ-diabetic placebo control.
Time After STZ Injection (weeks)

**Fig. 2.** Effect of instillation of FR74366 on cataract formation in diabetic rats. Rats were injected with STZ (60 mg/kg, IV) and treated with two eye drops of FR74366 (0.03%, 0.1%, and 0.3%) at 9 AM and at 5 PM for 16 weeks. Values represent number of score of lens opacity. Score 0, open area; Score 1, dotted area; Score 2, hatched area; Score 3, stippled area; Score 4, closed area. *One of the diabetic rats treated with 0.03% of FR74366 died at 8 weeks. Cataract formation in normal control rats was not observed.

weeks. This value was approximately 17% of that of lens.

FR74366 was detected in the plasma although the levels were lower than lens levels (30–90% of lens levels).

**Discussion**

Several mechanisms have been proposed to explain diabetic cataract formation. Recently, a number of AR inhibitors have been developed to ameliorate diabetic complications. Both hydantoin type and carboxylic acid type AR inhibitors were reported to be effective on diabetic and galactosemic cataracts. The first demonstration that matured cataract can be markedly delayed was shown in rats fed a diet containing 30% galactose and 0.7% alrestatin.26 The oral administration of sorbinil27 and statil14 was shown to be effective in the prevention of cataract formation in diabetic rats. In addition, eye drops of sorbinil28 and M7917529 (hydantoin-type AR inhibitor) inhibited cataract formation in diabetic rats and rats fed galactose. Moreover, eye drops of CT-11230 showed remarkable beneficial effects on human corneal epitheliopathy.

We have shown previously23 that FR74366 exhibits a highly potent reversible and mixed-type inhibition of AR from rat lens (IC$_{50}$ = $4.4 \times 10^{-9}$ M) and rabbit lens (IC$_{50}$ = $5.7 \times 10^{-9}$ M). FR74366 inhibited sorbitol accumulation in the isolated rat lens (IC$_{50}$ = $3.5 \times 10^{-5}$ M) incubated with high glucose concentrations. The oral administration of FR74366 to STZ-induced diabetic rats for 2 weeks decreased sorbitol levels in various tissues (ED$_{50}$ = 3.7 mg/kg for sciatic nerve and 23 mg/kg for lens). The oral administration of 32 mg/kg FR74366 to diabetic rats for 17 weeks markedly and completely delayed cataract formation, whereas the ED$_{50}$ value for inhibition of sorbitol accumulation in the lens was 12 mg/kg.

The effective treatment of an ocular disease (cataract, retinopathy, and keratopathy) with topical application is of clinical interest because this may reduce any possible side effects and unwanted effects of the drug on other tissues. For this reason, we have studied the effectiveness of topical applied FR74366 in the development of cataracts in diabetic rats.

In this paper, we demonstrate that eye drops of FR74366, a carboxylic acid-type AR inhibitor, effectively delayed the initiation of cataract and prevented the progression of cataract (Fig. 2) as a result of the inhibition of sorbitol accumulation in the lens of dia-

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Duration (wk)</th>
<th>N</th>
<th>0.03%</th>
<th>0.1%</th>
<th>0.3%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lens</td>
<td>2</td>
<td>3</td>
<td>0.02 ± 0.01</td>
<td>0.21 ± 0.06</td>
<td>0.49 ± 0.08</td>
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<tr>
<td></td>
<td>5</td>
<td>3</td>
<td>0.11 ± 0.03</td>
<td>0.51 ± 0.18</td>
<td>0.90 ± 0.31</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>10</td>
<td>0.30 ± 0.02*</td>
<td>0.75 ± 0.05</td>
<td>1.35 ± 0.04</td>
</tr>
<tr>
<td>Retina</td>
<td>16</td>
<td>10</td>
<td>ND*</td>
<td>0.14 ± 0.07</td>
<td>0.23 ± 0.03</td>
</tr>
<tr>
<td>Plasma</td>
<td>16</td>
<td>10</td>
<td>0.09 ± 0.04*</td>
<td>0.36 ± 0.06</td>
<td>1.24 ± 0.11</td>
</tr>
</tbody>
</table>

FR74366 levels were measured by GLC in the lens and retina and measured by HPLC in the plasma. Values are mean ± SD. FR74366 levels are expressed as μg/g wet weight in the lens and retina and μg/ml in the plasma; ND, not detected. Tissue FR74366 levels were lower than detectable level (0.1 μg/g). *N = 9.
abetic rats (Table 1). FR74366 levels in the lens were increased in a dose- and time-dependent manner (Table 2). These three parameters (score of lens opacity and sorbitol and FR74366 levels) were well correlated with each other.

The lens sorbitol levels in the diabetic placebo controls after 2 weeks showed higher values compared with those observed 5 or 16 weeks after STZ-injection, showing that the increased sorbitol levels decreased with duration of diabetes (Table 1). This probably is attributable to the disruption and disintegration of lenticular fibers that occur with the formation of intense cataracts in the diabetic control rats; this action is accompanied by leakage and loss of metabolites. Although the 74 days of oral treatment of statil delayed cataract formation, lens sorbitol levels treated with 10 and 25 mg/kg of statil were higher than those of diabetic rats who received no treatment. In our studies, oral or eye drop (Table 1) treatment of FR74366 always reduced the lens sorbitol levels compared with those of untreated diabetic controls, even though a gradual decrease of sorbitol levels in diabetic control rats was observed. FR74366 showed highly potent activity in inhibition of polyol production in the lens, and consequently FR74366 inhibited lens swelling and prevented the progression of cataract.

We examined the various conditions of instillation of FR74366 (concentration and instillation times). In this study, we chose an experimental protocol in which the maximal concentration was 0.3% (because FR74366 has low water solubility). The diabetic rats were treated with 2 eye drops of FR74366 each morning and evening (4 drops a day). We thought this protocol would be useful for clinical trials. In the sorbinil study, rats were pretreated with 1% sorbinil drops on the right eye four times daily for 3 days and placed on a 50% galactose diet at day 4 while sorbinil drop administration was continued. The lenses of both eyes remained grossly clear, as judged ophthalmoscopically for up to 4 weeks; untreated galactosemic rats developed mature cataract by 3 weeks. Instillation of sorbinil prevented cataract formation, but the lens of the untreated eye was found to have the same level of dulcitol as that of the treated eye. Dulcitol levels in the sciatic nerve were substantially lower than in the tissues of untreated galactosemic rats. This was suggested to be attributable to a crossover effect as topically applied sorbinil entered the circulation system; however sorbinil levels were not determined.

In addition, sciatic nerve or renal cortex sorbitol levels were not changed, but retina sorbitol levels were inhibited in a dose-dependent manner (ED$_{50}$ = 0.27%) with topical treatment of FR74366. Instillation of FR74366 will be effective in treating diabetic retinopathy in addition to treating diabetic cataract.

We detected no deleterious side effects of chronic (1 yr) oral administration of FR74366 (56 mg/kg as non-effective dose) for rats and dogs. In addition, instillation of FR74366 (0.3%) to the diabetic rats (16 weeks) and the normal rabbits (13 weeks toxicity studies) showed no serious side effects. These results suggest that instillation of FR74366 will be a useful therapeutic agent against diabetic cataract and retinopathy.

Key words: aldose reductase inhibitor, eye drop, streptozocin-induced diabetic rat, cataract, FR74366.
Acknowledgments

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