ABSTRACT—SPR-210 \{2-[4-(4,5,7-trifluorobenzothiazole-2-yl)methyl-3-oxo-3,4-dihydro-2H-1,4-benzothiazin-2-yl] acetic acid\}, a novel aldose reductase (AR) inhibitor, exhibited highly potent inhibition of partially purified AR from porcine lens (IC\(_{50}\)=9.5 \times 10^{-9} \text{ M}) and human placenta (IC\(_{50}\)=1.0 \times 10^{-8} \text{ M}). On the other hand, very weak inhibition by SPR-210 was observed against human placenta aldehyde reductase, which is the most closely related enzyme to AR, and against several adeninenucleotide-requiring enzymes. SPR-210 showed a noncompetitive mechanism with respect to DL-glyceraldehyde against porcine lens AR. Sorbitol accumulation in isolated human erythrocytes was effectively inhibited by SPR-210 during incubation with 50 mM glucose (IC\(_{50}\)=1.6 \times 10^{-8} \text{ M}). Oral administration of SPR-210 (1–30 mg/kg/day for 5 days) to streptozotocin-induced diabetic rats decreased the sorbitol contents in the sciatic nerve and lens (ED\(_{50}\)=1.9 and 6.8 mg/kg/day, respectively). SPR-210 had higher potency in the lens than other AR inhibitors. Moreover, the deterioration in motor nerve conduction velocity in diabetic rats was ameliorated by treatment with SPR-210 (1–30 mg/kg/day) accompanying the reduction in sorbitol content in the sciatic nerve. SPR-210 induced the recovery of the delayed peak latency of oscillatory potentials (O, 04) in the electroretinogram in diabetic rats (10 mg/kg/day). These results suggest that the specific AR inhibitor SPR-210 will be a useful therapeutic agent for preventing and improving some diabetic complications, especially diabetic neuropathy and retinopathy, and therefore, can be discriminated from other AR inhibitors.

Keywords: Aldose reductase inhibitor, Diabetic neuropathy, Motor nerve conduction velocity, Diabetic retinopathy, Electroretinogram

The polyol pathway consists of two enzymes, aldose reductase (AR) (E.C.1.1.1.21) and sorbitol dehydrogenase (SDH) (E.C.1.1.1.14). AR catalyzes the reduction of glucose to sorbitol, which is consecutively converted to fructose by SDH. Glucose has a high affinity for hexokinase, but its affinity for AR is quite low (1, 2). However, when there are elevated glucose concentrations, as observed in diabetes mellitus, increased AR activity (2, 3) and enhanced conversion of glucose to sorbitol and fructose occur, which have been implicated in the pathogenesis of diabetic complications; that is, cataracts (4, 5), retinopathy (6–8), neuropathy (2, 9) and nephropathy (10, 11). AR inhibitors can reduce the elevated tissue sorbitol contents through the inhibition of AR, the rate-limiting enzyme of the polyol pathway. To date, a number of AR inhibitors (12, 13) such as epalrestat (Ono Pharm., Osaka) (14, 15), tolrestat (Ayerst, Princeton, NJ, USA) (16, 17), zenarestat (FK-366; Fujisawa Pharma., Osaka) (18), zopolrestat (Pfizer, Groton, CT, USA) (19) and sorbinil (Pfizer) (20–22) have been found to improve some diabetic complications in animal experiments and have been developed in clinical stages of evaluation. Although these compounds are effective in the nerves, few compounds are effective in other tissues, such as the lens and retina, which are vulnerable to hyperglycemia in the diabetic state.

Recently, we have found a novel AR inhibitor, SPR-210 \{2-[4-(4,5,7-trifluorobenzothiazole-2-yl)methyl-3-oxo-3,4-dihydro-2H-1,4-benzothiazin-2-yl] acetic acid\} (Fig. 1), that possesses high AR inhibitory activity especially in the lens and retina as well as in nerves. The present paper describes its in vitro properties and its effects on diabetic neuropathy and retinopathy in STZ-induced diabetic rats. A part of the present studies was published elsewhere (23).
MATERIALS AND METHODS

Care of experimental animals

Male Sprague-Dawley rats (5 weeks of age) were purchased from Charles River Japan, Inc. (Tokyo). The animals were housed in plastic cages under conditions of controlled temperature, humidity and light. The rats were fed a normal laboratory diet (CRF-1; Oriental East Co., Tokyo) and water ad libitum. All procedures were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee.

In vitro studies

Preparation of AR: Porcine lens AR was prepared as follows (1): Lenses were homogenized in a Teflon homogenizer with 3 volumes of cold distilled water and then centrifuged at 10,000 × g for 15 min to remove insoluble materials. The supernatant was fractionated with 0-40% ammonium sulfate and centrifuged at 10,000 × g for 10 min, and then the precipitate was discarded. The supernatant was dialyzed overnight against 0.05 M sodium chloride. The dialyzed solution was stored at 4°C prior to use. When the unit of activity is defined as a change in absorbance of 0.001 unit per minute, its specific activity was 160.5 U/protein. Human placental AR was partially purified using a minor modification of the method of Clements and Winegrad (24). Its specific activity was 1454 U/protein. Both ARs did not contain aldehyde reductase (ALR).

Other enzymes: Bovine kidney ALR and human placenta ALR were prepared using a minor modification of the method of VanderJagt et al. (25). Sorbitol dehydrogenase (SDH) from sheep liver, lactate dehydrogenase (LDH) from chicken heart, and alcohol dehydrogenase (ADH) from bakers yeast and horse liver were purchased from Boehringer Mannheim (Mannheim, FRG), Wako Chem. (Osaka), and Sigma (St. Louis, MO, USA), respectively. All operations during the preparation of AR and ALR were performed at 4°C.

Determination of AR and ALR activities and effects of inhibitors: AR activity was assayed spectrophotometrically with an auto-analyzer Cobas Fara II (F. Hoffman-La Roche, Ltd., Basel, Switzerland) using a minor modification of the method of Hayman and Kinoshita (1). For this assay, the absorbance of NADPH at 340 nm was measured at 25°C for 2 min after starting the reaction by addition of the substrate (D,L-glyceraldehyde or D-glucose). All inhibitors were dissolved in dimethylsulfoxide (DMSO) to make 10 mM solutions and diluted with distilled water. The assay was carried out in 0.25 ml volumes of 40 mM sodium phosphate buffer (pH 6.2) containing 0.4 M lithium sulfate, 0.1 mM NADPH, the enzyme, various concentrations of inhibitor and 3 mM D,L-glyceraldehyde or 25 mM D-glucose as a substrate. The reference blank contained all of the above compounds except for the enzyme. The final concentration of DMSO in the reaction mixture never exceeded 1%, an upper limit of concentration which did not influence AR activity. The concentration of inhibitors required for 50% inhibition of enzyme activity (IC50) was estimated from the least-squares regression line of the log concentration-response curve.

ALR activity was assayed by the same method as that used for AR activity, except for the substrate (15 mM D-glucuronic acid instead of D,L-glyceraldehyde), the phosphate buffer (200 mM phosphate buffer (pH 7.0) instead of the 40 mM buffer (pH 6.2)) and the absence of lithium sulfate (26). Other enzyme activities were also measured relative to the change in the absorbance of NADH or NAD+ at 340 nm.

Sorbitol accumulation in human erythrocytes: The effects of inhibitors on sorbitol accumulation in human erythrocytes were assayed by the method of Terashima et al. (14). Erythrocytes from fresh human blood were washed twice with cold saline, and 0.5 ml of washed packed cells was incubated in 2.5 ml of Krebs-Ringer bicarbonate buffer with 50 mM glucose equilibrated with 95% O2 and 5% CO2 at 37°C for 3 hr. Inhibitors were then added to the incubation tubes to give final concentrations of 0 to 10-6 M. The sorbitol content was assayed enzymatically (27) using an F-kit (D-sorbitol/xylitol, Boehringer Mannheim).

In vivo studies

Rats, fasted overnight, were made diabetic by a single intravenous (i.v.) injection of 60 mg/kg body weight of streptozotocin (STZ, Sigma), which was dissolved in 0.9% saline immediately before the injection. Normal control rats were injected with the vehicle alone. The volume of the injection was set at 1 ml/kg body weight.
One week after STZ injection, blood samples of all rats were collected from the tail vein with heparinized capillary tubes. The glucose levels in the blood were measured by an enzymatic assay based on glucose oxidase and mutarotase (Glucose CII-Test, Wako Chem.). Rats with a plasma glucose level greater than 300 mg/dl were grouped at random, so that the mean body weights and blood glucose levels of each group were not significantly different.

Three in vivo experiments were designed as follows: Experiment 1 was performed to estimate the inhibitory effect of SPR-210 on the tissue sorbitol accumulation in vivo. One week after STZ injection, SPR-210 was orally administered once daily for 5 days. Rats were anesthetized with ether after final dosing of the inhibitor, and a blood sample was collected from the abdominal aorta into heparinized (10 U/ml of blood) test tubes. The whole blood was centrifuged at 2,000 x g for 10 min at 4°C. Erythrocytes were then separated from the platelets and leukocytes and washed twice with cold saline. At the same time, the sciatic nerves and lenses were weighed immediately and frozen at −80°C prior to the measurement of sorbitol and glucose contents.

To measure the sorbitol contents in various tissues, the assay was carried out by the method of Terashima et al. (14). Briefly, packed erythrocytes (0.5 ml) were deproteinized with 16% perchloric acid (PCA, 0.25 ml) and centrifuged at 5,000 x g for 10 min at 4°C. Sciatic nerve, lens and retina tissues were homogenized in 0.6 ml of 16% PCA and centrifuged. All the supernatants were neutralized with 2.5 M K2CO3 and centrifuged again. The supernatants were used to assay sorbitol and glucose contents.

Sorbitol was assayed enzymatically as described above. The sorbitol contents in various tissues were expressed as nmol per ml for the erythrocyte or μmol per g of tissue wet weight for the sciatic nerve and lens. The ED50 values for sorbitol accumulation were obtained by least-squares linear regression.

Experiments 2 and 3 were undertaken to evaluate the recovery effect of SPR-210 on motor nerve conduction velocity (MCV) and the electroretinogram (ERG), respectively. In Experiment 2, four weeks after STZ injection, SPR-210 was orally administered once daily for 2 weeks. The MCV of the rat tail was measured under ether anesthesia while the caudal temperature (about 37°C) was maintained by a heater with thermostat (TK-43; Asahi Electric, Tokyo) according to the method of Miyoshi and Goto (28) at three points: before STZ injection, at the starting point of treatment and after the final administration. Electrical stimulation was done by a rectangular pulse, 0.3 msec in duration with a voltage sufficient to evoke a supramaximal response. The evoked potentials were amplified and recorded on a Synax ER1100 (NEC San-ei, Tokyo). The MCV (m/sec) was determined from the distance between two stimulation points and from the difference between two latencies. Data were expressed as Recovery %, which was calculated as follows:

\[
\text{Recovery \%} = \frac{\lvert [N] - [T] \rvert}{\lvert [N] - [D] \rvert} \times 100,
\]

where [T] is the MCV of diabetic rats treated with drugs (after the final administration), and [N] and [D] are the MCV of age-matched normal and vehicle-treated diabetic rats, respectively.

In Experiment 3, four weeks after STZ injection, SPR-210 was orally administered once daily for 4 weeks. The reason why the duration of treatment with SPR-210 was longer than in Experiment 2 was that the administration of SPR-210 for 2 weeks did not ameliorate the ERG in the STZ-diabetic rats. The rats were fully dilated with instillations of 0.5% tropicamide and 0.5% phenylephrine hydrochloride. A 10-joule xenon discharge lamp was flashed 30 cm above the eye after a 1-hr dark adaptation. The potential between the corneal contact lens electrode (a gold ring embedded in a hard contact lens; Kyoto Contact Lens Laboratories, Kyoto) and the reference electrode on the median forehead was amplified and displayed with an a.c. amplifier-oscilloscope using the same instruments as in Experiment 2 at a time constant of 0.3 sec through a low-pass filter (0.5 Hz), before STZ injection, at the starting point of treatment and after the final administration.

The oscillatory potentials (OP) were denoted as O1, O2, O3 and O4 in the sequence of appearance (Fig. 2). The peak latency (msec) of the OPs were defined as the horizontal difference from the stimulus to the peak of OPs.

After measuring the MCV or the ERG, all the rats were sacrificed under ether anesthesia, and the sciatic nerves or the retinas were immediately removed, weighed, and frozen at −80°C prior to measurement of sorbitol contents.

**Drugs**

The following drugs were used: SPR-210, epalrestat, zopolrestat, zenarestat and tolrestat were synthesized in the Pharmaceutical Research Laboratories of Sapporo Breweries, Ltd. For the in vivo experiments, SPR-210 was suspended in 0.5% sodium carboxymethylcellulose (CMC-Na).

**Statistical analyses**

Results are expressed as the mean±S.E. The significance of the difference between the two groups was analyzed by Student's *t*-test for unpaired observations. The one-way analysis of variance and Tukey's multiple range test were used for a multigroup comparison.
RESULTS

Inhibition of aldose reductase and other enzymes

The inhibitory effects of SPR-210, epalrestat, zopolrestat, zenarestat and tolrestat on AR and ALR from various sources are summarized in Tables 1 and 2. The IC_{50} values of SPR-210 for porcine lens AR and human placenta AR were $9.5 \times 10^{-9}$ and $1.0 \times 10^{-8}$ M (with D- glyceraldehyde as the substrate), respectively. SPR-210 appeared to be a highly potent inhibitor of AR based on the sources employed. It is noteworthy that inhibition by SPR-210 against human placental AR is more intense than those by epalrestat and tolrestat. As for the bovine kidney ALR and the human placental ALR, which is the most closely related enzyme to AR, SPR-210 displayed

| Table 1. Inhibition of aldose reductase by aldose reductase inhibitors |
|---------------------|--------|--------|
| ARI                | IC_{50} (nM) |        |
|                    | D-glyeraldehyde/ |        |
|                    | DL-glyeraldehyde | D-glucose |
|                    | PL | HP | PL | HP |
| SPR-210            | 9.5 | 10 | 8.9 | 9.3 |
| Epalrestat         | 21  | 24 | 29 | 28 |
| Zopolrestat        | 13  | 10 | 8.9 | 9.8 |
| Zenarestat         | 12  | 9.4 | 7.6 | 8.0 |
| Tolrestat          | 17  | 21 | 15 | 15 |

ARI: aldose reductase inhibitors, PL: porcine lens, HP: human placenta.
much less inhibition against these enzymes than did the other inhibitors (Table 2). For the adeninenucleotide-requiring enzyme (sorbitol dehydrogenase, lactate dehydrogenase and alcohol dehydrogenase) activities, 10 pM SPR-210 exhibited no effect or slight inhibition, similar to the other inhibitors (Table 2).

Fig. 3. Lineweaver-Burk plot of porcine lens aldose reductase activity with glyceraldehyde as the substrate. The ordinate represents the reciprocal of initial velocity expressed as the change in optical density per 2 min. The abscissa represents the reciprocal of glyceraldehyde concentrations ranging between 0.025 and 1 mM. ○, 5 nM; △, 10 nM; and ◊, uninhibited control.

Table 3. Inhibitory effect of SPR-210 on sorbitol accumulation in intact human erythrocytes

| Conc. (M) | ARI | SPR-210 | zopolrestat | zenarestat |
|----------|-----|---------|-------------|------------|
| 3 × 10⁻⁸ | 57.9±3.8 ** * | 40.0±1.7 | 40.5±1.2 |
| 10⁻⁷     | 67.9±3.2 ** * | 44.0±2.7 | 45.4±1.2 |
| 3 × 10⁻⁷ | 82.4±4.2  | 53.0±14.2 | 75.8±4.2 |
| 10⁻⁶     | 83.7±2.5  | 86.8±1.6  | 80.6±2.2  |

Tukey’s multiple range test: **P<0.01 (vs. zopolrestat-treated group), *P<0.01 (vs. zenarestat-treated group). n=4-7.

Inhibition of sorbitol accumulation in various tissues of diabetic rats in vivo

To investigate the inhibitory effect of SPR-210 on sorbitol accumulation in various tissues of diabetic rats, we designed appropriate experiments as described in Materials and Methods. Blood glucose levels of STZ-diabetic rats (365–448 mg/dl) were certainly higher than those of normal rats (92–114 mg/dl). SPR-210 had no effect on body weight and blood glucose levels.

Figure 4 shows the time-course of tissue sorbitol contents in erythrocytes, sciatic nerves and lenses of rats after STZ injection. In the erythrocytes and sciatic nerve, the sorbitol contents reached maximal levels within one day after STZ injection. However in the lens, the sorbitol content linearly increased more than one day after STZ injection (Fig. 4).

The treatment with SPR-210 was started at 7 days after STZ injection. After the final administration of SPR-210, the variations in mean sorbitol contents of tissues in the vehicle-treated diabetic rats (two experiments) were 15.5–20.9 nmol per ml for the erythrocytes, 1.5–1.7 μmol per g of tissue wet wt. for the sciatic nerve and 37.8–41.9 μmol, respectively per g of tissue wet wt. for the lens. The treatment with SPR-210 (1–30 mg/kg/day for 5 days) inhibited the sorbitol accumulation in a dose-dependent manner (Table 4). The ED₅₀ values were 1.9 mg/kg/day for the sciatic nerve and 6.8 mg/kg/day for the lens.

Neither zopolrestat nor zenarestat had an effect on body weight and blood glucose level, but both compounds inhibited tissue sorbitol accumulation in a dose-dependent fashion (data not shown). The ED₅₀ values of zopolrestat were 3.8 mg/kg/day for the sciatic nerve and 29.3 mg/kg/day for the lens, and those of zenarestat were 8.5 mg/kg/day for the sciatic nerve and 45.4 mg/kg/day for the lens. These data indicate that SPR-210 was two-fold to sevenfold more potent compared with zopolrestat and zenarestat, especially in the lens.

Effect on MCV in diabetic rats

At the beginning of Experiment 2, just before STZ-injection, the mean MCVs of normal rats ranged from 20.9
to 21.7 m/sec. Four weeks after STZ injection, the mean MCVs were prolonged in normal rats (30.2–33.7 m/sec), while in STZ-diabetic rats, the mean MCVs were apparently smaller (27.3–28.8 m/sec). After the final administration of SPR-210 (six weeks after STZ injection), the differences in the mean MCVs between the normal rats and vehicle-treated diabetic rats enlarged further.

The treatment with SPR-210 (1–30 mg/kg/day), started 4 weeks after STZ-injection and continued for 2 weeks, dose-dependently ameliorated the mean MCV. Especially at 30 mg/kg/day, SPR-210 caused recovery of the mean MCV to a nearly normal control level (Table 5). The ED₅₀ values were 0.5 mg/kg/day for the recovery of MCV and 1.7 mg/kg day for the inhibitory effect on the sorbitol accumulation in the sciatic nerve. These improvements in MCV by SPR-210 were correlated with the sorbitol contents in the sciatic nerve (Fig. 5, r = −0.479, P < 0.01).

The treatments with zopolrestat and zenarestat did not fully recover the mean MCV in the STZ-diabetic rats even at a dose of 30 mg/kg/day, in spite of potent inhibition against the sorbitol accumulation in the sciatic nerve (data not shown). The ED₅₀ values of zopolrestat were 17.2 mg/kg/day for the recovery of MCV and 3.1 mg/kg/day for the inhibitory effect on sorbitol accumulation in the sciatic nerve. These data indicate that SPR-210

Table 4. Inhibitory effect of SPR-210 on sorbitol accumulation in the various tissues of diabetic rats

| Animal group (+SPR-210) | Dose (mg/kg/day) | Inhibition % sorbitol content (μmol/g) | Days after STZ injection |
|-------------------------|-----------------|----------------------------------------|-------------------------|
|                         |                 | erythrocytes                           | sciatic nerve           | lens                     |
| Diabetic                | 1               | 54.4 ± 9.6**                          | 21.2 ± 11.6             | 8.9 ± 6.5                |
| +SPR-210                | 3               | 69.7 ± 4.9**                          | 68.8 ± 3.0**            | 37.0 ± 7.4*              |
|                         | 10              | 77.6 ± 3.3**                          | 98.8 ± 0.5**            | 67.5 ± 6.8**             |
|                         | 30              | 84.1 ± 5.0**                          | 100.0**                 | 72.8 ± 7.8**             |

Tukey's multiple range test: *P < 0.05, **P < 0.01 (vs. vehicle-treated diabetic group). n = 4–5.

Table 5. Recovery effect of SPR-210 on motor nerve conduction velocity (MCV) and sciatic nerve sorbitol of diabetic rats

| Animal group (+SPR-210) | Dose (mg/kg/day) | MCV (m/sec) | Recovery % | Sciatic nerve sorbitol inhibition % |
|-------------------------|-----------------|-------------|------------|------------------------------------|
| Diabetic                | 1               | 56.3 ± 26.0 | 31.7 ± 7.3 |
| +SPR-210                | 3               | 72.2 ± 11.2*| 70.4 ± 5.6**|
|                         | 10              | 87.8 ± 7.0**| 100**      |
|                         | 30              | 95.7 ± 8.9**| 100**      |

Tukey's multiple range test: *P < 0.05, **P < 0.01 (vs. vehicle-treated diabetic group). n = 5–6.

Fig. 4. Time-course of tissue sorbitol contents in erythrocytes (left panel in the upper), sciatic nerve (left panel in the lower) and lens (right panel in the upper) after injection of streptozotocin (STZ) to rats. ○, normal control rats; ●, STZ-induced diabetic rats.
ameliorated the MCV in the STZ-diabetic rats much better than zopolrestat and zenarestat.

Effect on ERG in diabetic rats

The b-wave of the ERG was composed of four kinds of oscillatory potentials (O\textsubscript{1}–O\textsubscript{4}), which were not elucidated physiologically. Four weeks after STZ injection, the sum of the peak latencies (PLs) of the individual oscillatory potential in the diabetic rats was significantly prolonged compared to that of the normal control rats. Treatment of diabetic rats with SPR-210 (10 mg/kg/day for 4 weeks) caused significant recovery of the delayed sum of PLs to nearly normal control levels (Fig. 6). A statistically significant improvement in ERG was also observed at the PL of O\textsubscript{1}, O\textsubscript{3} and O\textsubscript{4} (Table 6). Zenarestat, however, did not ameliorate the delayed sum of PLs (data not shown).

The retinal sorbitol contents were significantly decreased to the normal control levels after the final administrations of SPR-210 and zenarestat (data not shown).

![Fig. 5. Correlation between motor nerve conduction velocity (MCV) and sciatic nerve sorbitol contents in normal control rats (○); vehicle-treated diabetic rats (●); and diabetic rats treated with SPR-210 at 1 mg/kg (■), 3 mg/kg (□), 10 mg/kg (▲) or 30 mg/kg (△). Total number of data points is 58.](image)

![Fig. 6. Recovery effect of SPR-210 on the sum of peak latencies (PLs) of oscillatory potentials (O\textsubscript{1}–O\textsubscript{4}) in the electroretinogram (ERG) in diabetic rats (described in Materials and Methods). ○, normal control rats; ●, vehicle-treated diabetic rats; and ▲, diabetic rats treated with SPR-210 at 10 mg/kg/day. *P<0.05 and **P<0.01, significantly different from the normal control rats, and #P<0.01, significantly different from the vehicle-treated diabetic rats by one-way analysis of variance and Tukey's multiple range test.](image)

| Table 6. Recovery effect of SPR-210 on the peak latency of oscillatory potential on electroretinogram (ERG) in diabetic rats |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Animal group                  | Dose (mg/kg/day) | O\textsubscript{1} | O\textsubscript{2} | O\textsubscript{3} | O\textsubscript{4} | SUM             |
| Normal                        |                 | 25.0±0.6         | 31.9±1.2         | 44.1±1.0         | 56.0±1.3         | 157.0±2.7       |
| Diabetic + vehicle            | 0               | 31.4±1.3**       | 40.3±1.7**       | 51.3±1.4**       | 64.8±2.2**       | 187.8±6.1**     |
| SPR-210                       | 10              | 27.1±0.5\textsuperscript{4} | 35.8±0.8         | 45.2±0.5\textsuperscript{4} | 55.8±0.6\textsuperscript{4} | 163.8±2.0\textsuperscript{4} |

Tukey's multiple range test: **P<0.01 (vs. normal group), \textsuperscript{4}P<0.05 and \textsuperscript{4}P<0.01 (vs. vehicle-treated diabetic group). n=6.
DISCUSSION

AR inhibitors including the hydantoin type, e.g., sorbinil, have been reported to show a high AR inhibition activity and a significant improvement in diabetic neuropathy (29), but show a lasting high concentration in the plasma; and adverse effects, such as skin rash, were also observed in clinical trials (30, 31). Based on these reports, we selected a 1,4-benzothiazin acetic acid compound from the various AR inhibitor prototypes (carboxylic acid derivatives) and finally chose SPR-210 as one of our candidate AR inhibitors among approximately 200 benzothiazin derivatives with an acetic acid moiety synthesized in our laboratories.

SPR-210 inhibited partially purified AR from porcine lens and human placenta with IC_{50} values 8.9 nM to 10 nM stronger than those of epalrestat. Although AR is known to display species differences, SPR-210 causes a potent inhibition of AR derived from not only animal sources but also human sources. Kinetic studies in vitro demonstrated its noncompetitive inhibition with di-glycereraldehyde as a substrate.

SPR-210 showed concentration-dependent inhibition of sorbitol accumulation in isolated human erythrocytes incubated in 50 mM glucose (IC_{50}=16.4 nM), apparently a more intense effect than those of zopolrestat and zenarestat, in spite of similar IC_{50} values for human placenta AR in vitro. This discrepancy may be due to the difference among these compounds with respect to membrane permeability, metabolic rate or concentration of each inhibitor in human erythrocyte cells (our unpublished data).

To evaluate the efficacy of SPR-210 against diabetic complications, we investigated the effect on the sorbitol accumulation in various tissues involved in diabetic complications in vivo. SPR-210 dose-dependently reduced the elevated sorbitol contents in the erythrocytes, sciatic nerve and lens (Table 4) without affecting the tissue glucose levels (data not shown). Thus the inhibitory effects of SPR-210 on the tissue sorbitol accumulations were approximately twofold to fourfold (sciatic nerve) and fourfold to sevenfold (lens) more potent than those of zopolrestat and zenarestat. SPR-210 showed a very potent inhibitory effect on the sorbitol accumulations, especially in the lens and retina. These data indicate that SPR-210 is expected to be more beneficial for the treatment of diabetic neuropathy and cataract than other AR inhibitors.

To further evaluate the therapeutic characteristics of SPR-210, we investigated the effects on the reduced MCV and the delayed peak latency of the ERG in the STZ-diabetic rats. Treatment of diabetic rats with SPR-210 for 2 weeks caused the amelioration of the MCV and inhibition of the sorbitol accumulation in the sciatic nerve in a dose-dependent manner. As reported previously (32), a significant correlation was also observed between the MCV and the sciatic nerve sorbitol contents in our experiments. Other AR inhibitors did not show any correlations between the two parameters. These results suggest that the inhibition of the tissue sorbitol accumulation may not be enough to ameliorate the MCV in the STZ-diabetic rats. Moreover, SPR-210 improved the delayed peak latency of the ERG accompanying a decrease in the sorbitol content. However, longer treatments were required to cause ERG recovery than MCV recovery. These results may be caused by the differences in the penetration of SPR-210 into the tissues.

Although other AR inhibitors decreased the sorbitol contents as SPR-210 did, they had no effects or weak effects on the MCV and the ERG. These results suggest that other new mechanisms, different from the 'osmotic theory', i.e., sorbitol accumulation (33), may be involved in diabetic neuropathy and retinopathy. The improvements in MCV and ERG by SPR-210 indicate that SPR-210 may restore the deteriorated microcirculation in these tissues participating in diabetic complications. Recently, it has been reported that prostaglandins are effective for the treatment of diabetic neuropathy (34) via vasodilation and antiplatelet action through the increase in cyclic adenosine 3',5'-monophosphate (cAMP) (35). Interestingly, the prostaglandins restored the cAMP content in sciatic nerves and the MCV without changing the sorbitol contents in the diabetic rats, and AR inhibitors were also reported to restore the cAMP levels (36). In addition, a positive correlation existed between the cAMP content in sciatic nerves and the MCV in diabetic rats. From these results, we speculated that some other mechanisms of SPR-210, such as the restoration of cAMP content in various tissues, may play a role in the improvement of physiological function in the diabetic rats.

In conclusion, the specific AR inhibitor SPR-210 shows potent AR inhibitory activity in vitro, the inhibition of tissue sorbitol accumulations especially in the lens and retina in vivo, and amelioration of the neuropathy and retinopathy in STZ-diabetic rats. These results suggest that SPR-210 can be expected to be useful for the clinical treatment of diabetic complications and can be discriminated from the other AR inhibitors.

Acknowledgments

We would like to thank Dr. N. Hotta (Nagoya University), Dr. S. Yagihashi (Hirosaki University) and Dr. K. Takahashi (Nippon Veterinary & Animal Science University) for their kind advice and thank Ms. T. Tsukamoto, Y. Aoshima and C. Hattori for their technical assistance.
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