Full Paper

Ranirestat (AS-3201), a Potent Aldose Reductase Inhibitor, Reduces Sorbitol Levels and Improves Motor Nerve Conduction Velocity in Streptozotocin-Diabetic Rats

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Abstract. Ranirestat (AS-3201) is a novel aldose reductase (AR) inhibitor with potentially beneficial effects on diabetic sensorimotor polyneuropathy. In this study, we performed a kinetic analysis to determine the mode of inhibition of ranirestat on AR and investigated the effects of ranirestat on sorbitol levels in the sciatic nerves and lens of streptozotocin (STZ)-diabetic rats. We also evaluated the effects on motor nerve conduction velocity (MNCV) in STZ-diabetic rats. Kinetic analyses revealed that the ranirestat inhibition of AR is uncompetitive and reversible. In the sciatic nerve and lens of STZ-diabetic rats, single oral administration of ranirestat slightly reduced sorbitol levels. However, repeated oral administration of ranirestat for 5, 21, or 60 days enhanced the reducing effect of the ranirestat on sorbitol levels in the sciatic nerves and lens of STZ-diabetic rats with maximum effects after 21 days of treatment. Finally, repeated oral administration of ranirestat for 21 or 42 days dose-dependently improved the STZ-induced decrease in MNCV in STZ-diabetic rats. These findings demonstrate that repeated oral administration of ranirestat reduces sorbitol accumulation and improves MNCV in STZ-diabetic rats, indicating that ranirestat is an agent for the management of diabetic sensorimotor polyneuropathy.

Keywords: aldose reductase inhibitor, ranirestat (AS-3201), polyol pathway, motor nerve conduction velocity, streptozotocin-diabetic rat

Introduction

Aldose reductase (AR, alcohol: NADP+ oxidoreductase, EC 1.1.1.21) is an enzyme that catalyzes the reduction of various carbonyl compounds (aldehyde substrates) to alcohols in the presence of nicotinamide adenine dinucleotide phosphate (NADPH). In vivo, the reduction of glucose to sorbitol by AR is the first rate-determining step in the polyol pathway, one of the glucose metabolic pathways (Fig. 1). AR is widely distributed throughout the body, including the target organs that are affected by diabetes mellitus such as the lens, retina, kidney (1), and peripheral nerves (2). Activation of the polyol pathway during hyperglycemia results in the accumulation of sorbitol in various tissues so it is believed to be a factor in the development of the complications associated with diabetes (3). During hyperglycaemia the polyol pathway can account for the reduction of more than 30% of the glucose. However, the exact physiological role of this pathway remains
unclear (4).

AR inhibitors (ARI) capable of preventing sorbitol accumulation typical of hyperglycemia may be useful in the management of the complications of diabetes such as retinopathy (5), nephropathy (6), and neuropathy (7). Thus, a number of ARIs have been developed, and some have been found to prevent or reverse diabetes-induced biochemical, functional, and histological changes in the lens, retina, and peripheral nerves (8 – 10).

Ranirestat (AS-3201) (Fig. 2), a novel, new chemical entity ARI developed in our laboratories, has been shown to have a potentially beneficial effect on diabetic sensorimotor polyneuropathy in humans (8). Although the pharmacodynamics of ranirestat in humans was predicted from preclinical studies in diabetic rats, the pharmacological effects of ranirestat on AR in diabetic animal models have not been reported. In the present studies, we performed a kinetic analysis to determine the mode of inhibition of ranirestat on AR and investigated the effects of ranirestat, given orally as single or repeated doses, on sorbitol levels in the sciatic nerve, lens, and erythrocytes of streptozotocin (STZ)-diabetic rats. We also evaluated the effects of repeated oral administration of ranirestat on nerve function in STZ-diabetic rats.

**Materials and Methods**

**Animals**

Male STD-Wistar rats (260 – 290 g, 12-week-old) were purchased from Japan SLC Co., Ltd. (Hamamatsu). The animals were maintained in a temperature- and humidity-controlled animal room (20°C – 26°C, 40% – 70%) under a 12-h light/dark cycle (light on 0600 to 1800 h) with free access to food (pellet chow CE-2; Clea Japan, Tokyo) and water. The rats were made diabetic by the intravenous injection of STZ (SIGMA SO130; St. Louis, MO, USA) (either 30 mg/kg for measurement of tissue sorbitol levels or 40 mg/kg for measurement of motor nerve conduction velocity, MNCV) into the tail vein. STZ was dissolved in physiological saline containing 0.75 mM citrate buffer (pH 4.5) immediately before dosing. One day after STZ injection, plasma glucose levels was measured, and all STZ-treated rats were confirmed to be hyperglycemic (plasma glucose level: >300 mg/dl). All experimental procedures were approved by the Institutional Animal Care and Use Committee of Dainippon Pharmaceutical Co., Ltd.

**Preparation of AR**

Recombinant human aldose reductase (rhAR) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka), and lens aldose reductase (lAR) was collected from rats under anesthesia and purified according Hayman’s method (11, 12).

**Evaluation of the inhibitory effects of ranirestat on rhAR and lAR**

AR activity was determined following Hayman’s method (11). In brief, a reaction mixture containing 100 mM phosphate buffer (pH 6.5), 400 mM lithium sulfate, 0.05 mM 2-mercaptoethanol, 0.2 mM NADPH, and AR (rhAR or lAR, 0.007 unit/ml) was incubated at
37°C for 5 min in the presence or absence of ranirestat (1 – 300 nM in DMSO). Then d, l-glyceraldehyde was added to this reaction mixture as a substrate (1.5 mM), and AR activity was determined from the decrease in NADPH absorbance at 340 nm in a HITACHI U-3210 spectrophotometer (Hitachi Ltd., Tokyo). One unit of AR activity was defined as the amount of enzyme that catalyzes oxidation of 1 mmol NADPH/min.

**Kinetic analysis of ranirestat inhibitory activity on rhAR**

The mode of rhAR inhibition induced by ranirestat was determined and compared to that induced by sorbinil and ponalrestat, two other ARIs. Kinetic constants were determined by fitting AR activity at various d-glucose concentrations to either the Michaelis-Menten equation or the general equation for substrate inhibition (13). Reaction mixtures free of AR or glucose were used as controls.

**Determination of sorbitol levels in the sciatic nerve, lens, and erythrocytes following ranirestat administration**

Ranirestat was suspended in 0.5% tragacanth solution and administrated orally to STZ-diabetic rats as a single dose or repeated doses once daily for 5, 21, or 60 days starting 7 days after STZ injection. In the single dose experiment, ranirestat was administrated at doses of 0.01 – 10 mg/kg; and in the repeated dose experiments, ranirestat was administrated at doses of 0.03 – 1 mg/kg per day for 5 days, 0.003 – 0.3 mg/kg per day for 21 days, or 0.002 – 0.2 mg/kg per day for 60 days. Four hours after the final dose of ranirestat in each experiment, the sciatic nerve and lens were removed and blood was collected under anesthesia. Sorbitol contents in the sciatic nerve and lens tissues were determined by the method of Clements (14). Sorbitol levels in non-diabetic rats (N) and in untreated STZ-diabetic rats (D) were used as controls. The isolated sciatic nerves were treated promptly with distilled water (1 ml/40 mg wet weight) at 100°C for 2 min, homogenized in 6% perchloric acid (1 ml/10 mg wet weight), and centrifuged at 3,000 rpm at 4°C for 10 min. The supernatant was neutralized with 2 M potassium carbonate and centrifuged again (3,000 rpm, 4°C, 10 min). The new supernatant was diluted with distilled water and used for sorbitol assay. Finally, the erythrocytes were washed with saline, thoroughly mixed with 6% perchloric acid, and centrifuged at 3,000 rpm (4°C) for 10 min. The supernatant was neutralized with 2 M potassium hydroxide and centrifuged again (3,000 rpm, 4°C, 10 min). The new supernatant was then applied to a cation exchange column (AG50W-X4, bed volume 1.5 ml; Bio-Rad, Hercules, CA, USA), and the column eluate was neutralized with 2 M potassium carbonate and centrifuged (3,000 rpm, 4°C, 10 min). The resultant supernatant was used for the sorbitol assay. After the addition of 2 ml of 0.05 M glycine buffer (pH 9.4) containing 2 mM nicotinamide adenine dinucleotide (NAD) and 0.05 ml sorbitol dehydrogenase (SDH) (25.6 U/ml) to 1.0 ml of tissue extract, the mixture was incubated at room temperature for 60 min. The fluorescence generated by nicotinamide adenine dinucleotide reduced form (NADH) was measured at an excitation wavelength of 366 nm and an emission wavelength of 452 nm using a spectrofluorometer (F-3000, Hitachi). Based on fluorescence intensity in the presence or absence of SDH, the sorbitol concentration in each extract was determined from the calibration curve of reference d-sorbitol. Sorbitol contents were expressed in µmol/g wet weight (WW) for the sciatic nerve and lens or in nmol/gram hemoglobin (Hb) for the erythrocytes. Hb content in the erythrocytes was quantitated by the Hemoglobin B-TEST WAKO (Wako Pure Chemical Industries) based on the sodium lauryl sulfate-hemoglobin method. Assuming that the mean sorbitol contents in the non-diabetic control rats (N) and in STZ-diabetic control rats (D) were 0 and 100%, respectively, the % reduction in sorbitol content was calculated based on the sorbitol content in ranirestat-treated STZ-diabetic rats (DI) using the following equation:

\[
\% \text{ Reduction in sorbitol content} = \frac{(D – DI)\text{sorbitol}}{(D – N) \times 100}
\]

The dose of ranirestat that produced a 50% reduction in sorbitol content was taken as the ED50.

**Measurement of nerve function (MNCV)**

Rats were made diabetic by the intravenous injection of STZ (40 mg/kg) on Day 0. Starting on Day 21, ranirestat (0.03 – 1 mg/kg) was administrated orally once daily for 42 days (Day 21 to Day 63). MNCV was measured in the sciatic nerve just before the injection of STZ (Day 0), just before the start of ranirestat administration (Day 21), and then 3 and 6 weeks after the start of ranirestat administration (Day 42 and Day 63). The MNCV in the rat sciatic nerve was determined by the method of Sharma et al. (15). Briefly, the muscle action potential of the rat haunches was recorded under Fluothane inhalation anesthesia. A constant rectal temperature of 37.5°C – 38.5°C was maintained using an animal body temperature controller (ATB-1100; Nihon Kohden, Tokyo). Using an electric
stimulator (SEN-3301, Nihon Kohden), the rat sciatic nerves were stimulated at two points, S1 and S2, by applying single rectangular pulses (duration: 0.1 ms, voltage: supramaximal), and the evoked action potentials from the recording electrode were displayed on an oscilloscope (VC-11, Nihon Kohden) and recorded on an XY recorder (RW-21S; Rikadenki Kogyo, Tokyo). The proximal and distal latencies were measured as the time from stimulation at S1 and S2 to the rise in action potential; t1 and t2 (ms), respectively. The distance, d (mm), between S1 and S2 was measured and the MNCV was calculated with the following formula: MNCV (m/s) = d / (t1 – t2)

Statistical analyses

Experimental data are each expressed as a mean ± S.E.M. Statistical analyses were performed by the SAS statistical processing software (Statistical Analysis System, Ver. 6.11.; SAS Institute, Cary, NC, USA). Student’s t-test was used for comparison between the diabetic control groups and the non-diabetic control groups. The F-test was used to confirm homogeneity of variance. For multiple comparisons between the diabetic control groups and ranirestat-treated diabetic groups, Dunnett’s multiple comparison test was used and the homogeneity of variance was confirmed using Bartlett’s test. Regression analysis was used for the dose-response relationship. In all cases, a P value less than 0.05 was considered statistically significant.

Results

Inhibitory effects of ranirestat on rhAR and lAR

The inhibitory effects of ranirestat on the rhAR and lAR were evaluated using D,L-glyceraldehyde as a substrate. Ranirestat potently inhibited rhAR and lAR with IC50 values of 15 and 11 nM, respectively.

Mode of inhibition of ranirestat on rhAR

The mode of rhAR enzyme activity inhibition by ranirestat was investigated by an assessment of enzyme reaction kinetics. Table 1 and Fig. 3 show these results. Ranirestat, sorbinil, and ponalrestat inhibit rhAR in an uncompetitive, reversible manner that increased with increasing glucose concentrations (Fig. 3: a, b, c). However, the affinity of ranirestat for rhAR was greater than that of sorbinil or ponalrestat as indicated by an inhibition constant (Kii = 0.38 nM) 640 or 240 times lower than that of sorbinil (Kii = 242 nM) or ponalrestat (Kii = 90.1 nM), respectively (Table 1).

<table>
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<tr>
<th>AR inhibitor</th>
<th>Inhibition mode</th>
<th>Inhibition constants Kii (nM)</th>
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<tr>
<td>Ranirestat</td>
<td>Uncompetitive</td>
<td>0.38 ± 0.03</td>
</tr>
<tr>
<td>Sorbinil</td>
<td>Uncompetitive</td>
<td>242 ± 2</td>
</tr>
<tr>
<td>Ponalrestat</td>
<td>Uncompetitive</td>
<td>90.1 ± 0.8</td>
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The uncompetitive inhibition constant Kii of ranirestat, sorbinil, and ponalrestat was calculated by fitting the apparent inhibition constant (Kii') obtained at different glucose concentration to the following equation: Kii' = Kii ([S] + Kii) / [S], where [S] is the glucose concentration and Kii is the Michaelis constant of glucose.

Effects of ranirestat on sorbitol levels in STZ-diabetic rats

A single oral administration of ranirestat 0.01 – 10 mg/kg reduced sorbitol in the sciatic nerve and lens of STZ-diabetic rats. However, repeated daily oral doses of ranirestat of 0.03 – 1 mg/kg for 5 days, 0.003 – 0.3 mg/kg for 21 days, or 0.002 – 0.2 mg/kg for 60 days decreased sorbitol levels in the sciatic nerve and lens of STZ-diabetic rats. Optimal effects were generally observed following 21 days of ranirestat administration (Table 2, Fig. 4). However, the reduction in sorbitol in erythrocytes induced by the single oral administration of ranirestat (0.01 – 10 mg/kg) was similar to that observed following repeated oral dosing with 0.03 – 1 mg/kg for 5 days, 0.003 – 0.3 mg/kg for 21 days, or 0.002 – 0.2 mg/kg for 60 days (Table 2, Fig. 4).

Effects of ranirestat on MNCV in STZ-diabetic rats

The MNCV in STZ-diabetic control rats 21, 42, and 63 days after administration of STZ (Day 0) was significantly (P<0.01, Student’s t-test) slower than in the non-diabetic control rats (Fig. 4). Repeated daily administration of 0.1 – 1 mg/kg per day ranirestat from 21 to 63 days after STZ injection dose-dependently improved the decrease in MNCV induced by STZ from 42 to 63 days, P<0.001 for the 0.1 mg/dg per day and higher dose groups. Although the change in MNCV with ranirestat administration on Day 63 was greater than observed on Day 42, the degree of improvement in MNCV from Day 21 to Day 42 was greater than the change from Day 42 to Day 62.

Discussion

Our aims in the present studies were to determine the mode of inhibition of ranirestat on AR; investigate its effects on sorbitol levels in the sciatic nerves, lenses, and erythrocytes of STZ-diabetic rats; and evaluate its beneficial effects on MNCV in STZ-diabetic rats. Our results show that ranirestat inhibits rhAR and rat lAR.
Ranirestat is a potent AR inhibitor with almost the same potency (IC\textsubscript{50} = 15 nM for rhAR and 11 nM for lAR) and that its inhibition of rhAR is of the uncompetitive reversible type (K\textsubscript{ii} = 0.38 ± 0.03 nM). As shown in Fig. 3a, the inhibitory activity of ranirestat increased with increasing glucose concentration, indicating that ranirestat inhibition of rhAR is hardly affected by high glucose concentrations. Therefore, ranirestat potently inhibits AR derived from different species and that it has high affinity for rhAR. Although sorbinil and ponalrestat, two classical ARIs, also showed uncompetitive reversible inhibition of rhAR in this study, their affinity for rhAR was much lower than that of ranirestat (Fig. 3: b and c, Table 1). Our previous study showed two reasons for the high affinity of ranirestat. One reason is that the 4-bromo-2-fluorobenzyl group is essential for high-affinity binding because this group is in a close stacking orientation with a nearby aromatic ring and its 2-fluorine and 4-bromine atoms make a hydrogen bond in the enzyme active site. The other reason is that the structural rigidity with almost the same potency (IC\textsubscript{50} = 15 nM for rhAR and 11 nM for lAR) and that its inhibition of rhAR is of

**Table 2.** Relationship between the effects of ranirestat on sorbitol levels in the sciatic nerve, lens, and erythrocytes of STZ-diabetic rats and ranirestat administration

<table>
<thead>
<tr>
<th>Treatment period</th>
<th>ED\textsubscript{50} (mg/kg per day)</th>
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<tbody>
<tr>
<td></td>
<td>Sciatic nerve</td>
</tr>
<tr>
<td>1 day</td>
<td>3.3</td>
</tr>
<tr>
<td>5 day</td>
<td>0.19</td>
</tr>
<tr>
<td>21 day</td>
<td>0.034</td>
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<tr>
<td>60 day</td>
<td>0.023</td>
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The reduction in sorbitol level in each tissue was determined by assuming that mean values of sorbitol content in each tissue in the non-diabetic and STZ-diabetic control rats were 0 and 100%, respectively. The dose of ranirestat that reduced the relative value of sorbitol content by 50% (50% effective dose) was designated as the ED\textsubscript{50}.
of the succinimide ring in the pyrrolopyrazine framework may easily orient its three heteroatoms for hydrogen-bonding interactions towards the active site residue and significantly contributes to energetic stabilization in the enzyme-ranirestat complex (16). These findings may account for the different results in clinical trials of diabetic sensorimotor polyneuropathy with these 3 compounds (8). Interestingly, the 12-week clinical study in patients with sensorimotor polyneuropathy showed greater changes in sensory nerve conduction velocity than in motor nerve function, so perhaps longer treatment would show changes corresponding to those in the STZ-rat (8). Repeated oral administration of ranirestat (0.1–1 mg/kg) for 21 days improved the decrease in MNCV induced by STZ in diabetic rats (Fig. 5). In fact, ranirestat concentration in rat sciatic nerve following repeated oral administration reached a steady state level after at least 14 days (unpublished data). In addition, the concentration of ranirestat was confirmed to vary dose-dependently in patients sural nerve (8). These results suggest that ranirestat penetrates the sciatic nerve in a dose-dependent manner. The time-dependent increase of MNCV suggests that part of the improvement of nerve function depends on spontaneous recovery. Consequently, it is postulated that strict inhibition of the flux polyol pathway is important. The ranirestat effective dose for decreased MNCV was much lower than that of sorbinil or ponalrestat (17, 18). These findings suggest that AR inhibition potency is important in the improvement of MNCV. Another important results in this study are that the ranirestat dose required to improve nerve function was much higher than the ED<sub>50</sub> confirms previous experience (7) indicating that a high degree of polyol inhibition is required for this effect (80% in this study with ranirestat). STZ, given intravenously to rats at a dose of 30 mg/kg, increased blood glucose levels within 24 h to over 300 mg/dl in all treated rats. This increase in glucose level resulted in an increase in sorbitol levels in different tissues including the sciatic

Fig. 4. Effects of ranirestat dose on sorbitol levels in the sciatic nerve (a), lens (b), and erythrocytes (c) of STZ-diabetic rats. Ranirestat was orally administered to STZ-diabetic rats as a single dose (0.01–10 mg/kg) or repeated doses of 0.03–1 mg/kg per day for 5 days, 0.003–0.3 mg/kg per day for 21 days, or 0.002–0.2 mg/kg per day for 60 days starting 7 days after STZ administration. Sorbitol levels in the sciatic nerve, lens, and erythrocytes of STZ diabetic rats were measured 4 h after the last dose of ranirestat.

Fig. 5. Effects of ranirestat on motor nerve conduction velocity (MNCV) in the sciatic nerve of STZ-diabetic rats. Ranirestat 0.03–1.0 mg/kg per day was orally administered to STZ diabetic rats for 42 days starting 21 days after STZ administration. MNCV was measured in the sciatic nerve just before injection of STZ, just before the start of ranirestat administration, and then 21 and 42 days after the start of ranirestat administration. ***: Differences between the ranirestat-treated STZ-diabetic rats and the diabetic control rats were examined by Dunnett’s multiple comparison test (P<0.001). ###: Differences between STZ-diabetic control rats and non-diabetic control rats were examined by Student’s t-test (P<0.001).
nerve, lens, and erythrocytes. Both single and repeated oral administration of ranirestat to STZ diabetic rats reduced sorbitol levels in the sciatic nerve, lens, and erythrocytes. However, in the sciatic nerve and lens the effects of single oral administration of ranirestat on sorbitol levels were much weaker than those of its repeated oral administration (Fig. 4, Table 2), while in the erythrocytes the reduction in sorbitol level was independent of ranirestat regimen of administration. Although the reduction in sorbitol levels induced by single oral administration of ranirestat varied among the tested tissues, the normalization in sorbitol levels produced by ranirestat repeated oral administration for 21 days was almost the same in all tested tissues (Table 2). Ranirestat concentration in rat plasma following repeated oral administration reached a steady state level within 3 days. However, the administration period for ranirestat to reach a steady state level in the sciatic nerve is at least 14 days (unpublished data). These findings indicate that repeated oral administration of ranirestat potently reduces and normalizes sorbitol level in STZ diabetic rats without organ specificity. Otherwise, the finding of a similar decrease in sorbitol levels in nerve and lens with ranirestat administration for 21 days (Table 2) indicates potent AR inhibition without organ specificity. Others have reported that AR mRNA is widely distributed in organs that are affected by high levels of hyperglycemia, such as the lens, retina, and peripheral nerve (1). Consequently, to the extent that the polyol pathway contributes to the underlying pathophysiological cascade leading to the microvascular complications of diabetes, it may be predicted that ranirestat might prove effective in treating those microvascular complications, that is, retinopathy, nephropathy, and polyneuropathy.

In conclusion, the results of this study indicate that ranirestat is a potent aldose reductase inhibitor. These preclinical studies are consistent with the preliminary clinical results and indicate that ranirestat may be useful for the treatment of the complications associated with diabetes such as neuropathy.

References


