In vitro Inhibition of Lens Aldose Reductase by (2-Benzyl-2,3,4,5-Tetrahydro-1H-Pyrido[4,3-b]Indole-8-yl)-Acetic Acid in Enzyme Preparations Isolated from Diabetic Rats

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Abstract. (2-benzyl-2,3,4,5-tetrahydro-1H-pyrido[4,3-b]indole-8-yl)-acetic acid (compound 1), a novel aldose reductase inhibitor, was assayed for efficacy and selectivity to inhibit rat lens aldose reductase under *in vitro* conditions by using enzyme preparations obtained from diabetic animals. The inhibitory efficiency was characterized by IC_{50} in micromolar region. Enzyme kinetics analysis revealed uncompetitive type of inhibition, both in relation to the D,L-glyceraldehyde substrate and to the NADPH cofactor. In testing for selectivity, comparisons to rat kidney aldehyde reductase, an enzyme with the highest homology to aldose reductase, was used. The inhibition selectivity of the compound tested was characterized by selectivity factor around 20 and was even slightly improved under conditions of prolonged experimental diabetes. These findings were identical with those in the control rats. To conclude, the inhibitory mode, efficacy and selectivity of compound 1, a novel aldose reductase inhibitor, was preserved even under the conditions of prolonged STZ-induced experimental diabetes of rats.

Key words: Polyol pathway — Aldose reductase inhibition — Diabetic complications — Experimental diabetes

Introduction

An important role for aldose reductase (ALR2, E.C.1.1.1.21), the first enzyme of the polyol pathway, has been suggested in the pathogenesis of diabetic complications (Yabe-Nishimura 1998). ALR2 reduces glucose to sorbitol with concomitant consumption of NADPH. This alternative route of glucose metabolism is accelerated

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during hyperglycemia eliciting osmotic imbalance, impaired ion transport, altered redox status, and persisting exposure to production of free radicals. In this way, the polyol pathway in cells is believed to contribute to the etiology of long-term diabetic complications such as cataract, retinopathy, nephropathy, neuropathy, micro- and macroangiopathy. Under physiological conditions, ALR2 serves as an extrahepatic detoxifying enzyme against endogenous and xenobiotic aldehydes (Yabe-Nishimura 1998).

Being potentially harmful, aldose reductase became an ideal target of drug action. Search for inhibitors of the enzyme has become an important pharmacological goal (Costantino et al. 2000; Miyamoto 2002; El-Kabbani et al. 2004). Inhibition of aldose reductase can prevent abnormal intracellular accumulation of sorbitol and the accompanying depletion of glutathione, so that normal function of cells can be preserved even under hyperglycemia conditions in diabetic patients (Hotta 1995).

Recently, a series of new carboxymethylated tetrahydropyridoindoles was synthesized and characterized as aldose reductase inhibitors, exerting also antioxidant activity (Stefek et al. 2005). Under *in vitro* conditions, (2-benzyl-2,3,4,5-tetrahydro-1H-pyrido[4,3-b]indole-8-yl)-acetic acid (compound 1), a representative compound of the series, inhibited ALR2 isolated from healthy rat eye lenses with IC_{50} (the concentration of the inhibitor required to produce 50 % inhibition of the enzyme reaction) in micromolar region.

Since several studies have shown that ALR2 isolated from diabetic or hyperglycemic tissues is kinetically different from the enzyme purified from normal tissues and less susceptible to inhibition by aldose reductase inhibitors such as sorbinil or tolrestat (Das and Srivastava 1985; Srivastava et al. 1985, 1986a,b; Chandra et al. 2002), in the present work we studied the inhibitory efficiency and selectivity of compound 1 for enzyme preparations obtained from diabetic rats. The experimental data of the diabetic animals were compared with those of control healthy rats.

Materials and Methods

Experimental diabetes

The study was approved by the Ethics Committee of the Institute and performed in accordance with the Principles of Laboratory Animal Care (NIH publication 83–25, revised 1985) and the Slovak law regulating animal experiments (Decree 289, Part 139, July 9th 2003). Wistar rats (sex specified in result section), 8–9 weeks old, weighing 200–230 g, were used. The animals came from the Breeding Facility of the Institute of Experimental Pharmacology Dobrá Voda (Slovakia). Experimental diabetes was induced in rats by either single i.p. or triple i.v. or i.p. doses of streptozotocin (STZ) administered on three consecutive days (the mode of application and STZ dose are specified in the section Results). STZ was dissolved in 0.1 mol/l citrate buffer, pH 4.5. The animals were fasted overnight prior to STZ administration. Control animals received 0.1 mol/l citrate buffer. Water and food

were available immediately after dosing. Ten days after STZ administration, all animals with plasma glucose level >10 mmol/l were considered diabetic. For three animals randomly chosen in each diabetic group, an oral glucose tolerance test was performed at the end of the experiment. Fasting and 2-h plasma glucose values after 1 g/kg glucose dose were >10 mmol/l and >20 mmol/l, respectively. During the experiment, the animals were housed in groups of two in cages of type T4 Velaz (Prague, Czech Republic) with bedding composed of wood shaving (changed daily). Tap water and pelleted standard diet KKZ-P-M (Dobrá Voda, Slovakia) were available ad libitum. The animal room was air-conditioned and the environment was continuously maintained at a temperature of 23 ± 1 ℃ and relative humidity of 40–70 %.

Preparation of ALR2

Rat lens ALR2 was partially purified using a procedure adapted from Hayman and Kinoshita (1965) as follows: lenses were quickly removed from rats following euthanasia and homogenized in a glass homogenizer with a teflon pestle in 5 vol. of ice cold distilled water. The homogenate was centrifuged at $10,000 \times g$ at 0–4 ℃ for 20 min. The supernatant was precipitated with saturated ammonium sulfate at 40, 50 % and then at 75 % salt saturation. The supernatant was retained after the first two precipitations. The pellet from the last step, possessing ALR2 activity, was dispersed in 75 % ammonium sulfate and stored in smaller aliquots in liquid nitrogen container.

Preparation of ALR1

Rat kidney aldehyde reductase (ALR1) was partially purified according to the reported procedure of Costantino et al. (1999) as follows: kidneys were quickly removed from rats following euthanasia and homogenized in a knife homogenizer followed by processing in a glass homogenizer with a teflon pestle in 3 vol. of 10 mmol/l sodium phosphate buffer, pH 7.2, containing 0.25 mol/l sucrose, 2.0 mmol/l EDTA dipotassium salt and 2.5 mmol/l β-mercaptoethanol. The homogenate was centrifuged at $16,000 \times g$ at 0–4 ℃ for 30 min and the supernatant was subjected to ammonium sulfate fractional precipitation at 40, 50 and 75 % salt saturation. The pellet obtained from the last step, possessing ALR1 activity, was redissolved in 10 mmol/l sodium phosphate buffer, pH 7.2, containing 2.0 mmol/l EDTA dipotassium salt and 2.0 mmol/l β-mercaptoethanol to achieve total protein concentration of approximately 20 mg/ml. In the final purification step, diethylaminoethyl DE 52 (DEAE-DE 52) resin was added to the solution (33 mg/ml) and after gentle mixing for 15 min removed by centrifugation. The supernatant containing ALR1 was then stored in smaller aliquots in liquid nitrogen. No appreciable contamination by ALR2 in ALR1 preparations was detected since no activity in terms of NADPH consumption was observed in the presence of glucose substrate up to 150 mmol/l.

Enzyme assays

ALR1 and ALR2 activities were assayed spectrophotometrically by determining NADPH consumption at 340 nm and were expressed as decrease of the optical density$\cdot$s$^{-1}\cdot$protein^{-1}.

To determine ALR2 activity (Da Settimo et al. 2005), the reaction mixture contained 4.67 mmol/l D,L-glyceraldehyde as a substrate, 0.11 mmol/l NADPH, 0.067 mol/l phosphate buffer, pH 6.2 and 0.05 ml of the enzyme preparation in a total volume of 1.5 ml. The reference blank contained all the above reagents except the substrate D,L-glyceraldehyde to correct for the oxidation of NADPH not associated with reduction of the substrate. The enzyme reaction was initiated by addition of D,L-glyceraldehyde and was monitored for 4 min after an initial period of 1 min at 30°C.

ALR1 activity was assayed analogically (Costantino et al. 1999) using 20 mmol/l D-glucuronate as a substrate in the presence of 0.12 mmol/l NADPH in 0.1 mol/l phosphate buffer, pH 7.2 at 37°C.

Enzyme activities were adjusted by diluting the enzyme preparations with distilled water so that 0.05 ml of the preparation gave an average reaction rate for the control sample of 0.020 ± 0.005 absorbance units/min. The effect of an inhibitor on the enzyme activity was determined in reaction mixtures with required inhibitor concentrations. The inhibitor at the same concentration was included in the reference blank. IC$_{50}$ values were determined from the least-square analysis of the linear portion of the semilogarithmic inhibition curves. Each curve was generated using at least four concentrations of inhibitor causing an inhibition in the range from at least 25 to 75 %.

Total protein in enzyme preparations was determined according to Geiger and Bessman (1972).

Blood measurement

Heparinized blood samples were taken from the rat tails after overnight fasting of the animals. Plasma glucose levels were measured using the commercial glucose kit, GLU 250 E (Bio-La test, Lachema, Brno, Czech Republic).

Chemicals

Compound 1, (2-benzyl-2,3,4,5-tetrahydro-1H-pyrido[4,3-b]indole-8-yl)-acetic acid (Scheme 1), was synthesized at the Institute of Experimental Pharmacology, Slovak Academy of Sciences (Stefek et al. 2005) and was available as a potassium carboxylate salt. STZ, NADPH, β-mercaptoethanol, D,L-glyceraldehyde, D-glucuronate were obtained from Sigma Chemical Co. (St. Louis, MO, USA). DEAE-DE 52 cellulose was from Whatman International Ltd. (Maidstone, England). Other chemicals were purchased from local commercial sources and were of analytical grade quality.
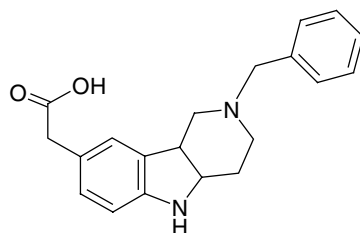
Scheme 1. Chemical structure of 2-benzyl-2,3,4,5-tetrahydro-1H-pyrido[4,3-b]indole-8-yl)-acetic acid (compound 1).

Results

The enzyme kinetics for compound 1 was analyzed in ALR2 preparations isolated from diabetic rats. Uncompetitive type of inhibition was observed either in relation to the substrate D,L-glyceraldehyde or the cofactor NADPH, as shown in Figures 1 and 2, respectively. Table 1 shows values of the corresponding kinetic parameters in comparison with control data.

As summarized in Table 2, extension of experimental diabetes in male rats from 1 up to 5 months did not significantly affect the sensitivity of the lens ALR2 to the inhibition by compound 1, expressed by corresponding IC_{50} values. In female
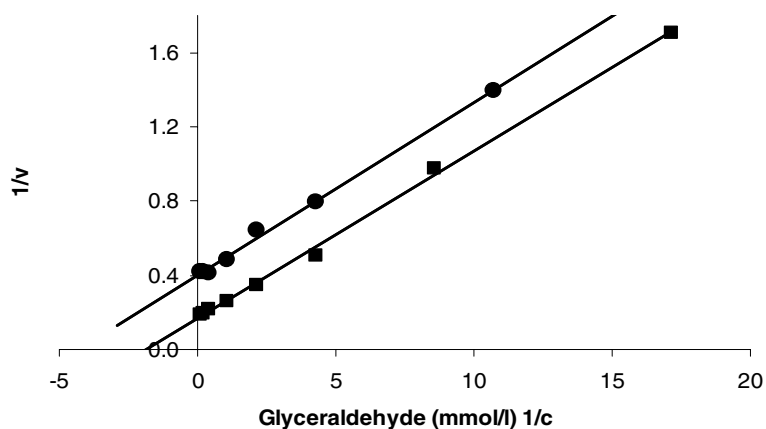
Figure 1. Inhibitory effect of compound 1 on diabetic male rat lens aldose reductase (1-month experimental diabetes, induced in male rats by three i.v. doses of STZ, 25 mg/kg, on three consecutive days, was characterized by glycemia >10 mmol/l for the duration of the whole experiment). Typical double reciprocal plot of initial enzyme velocity (v) *versus* concentration (c) of substrate (D,L-glyceraldehyde) in the presence or absence of compound 1: ■ no inhibitor; ● 20 μmol/l of compound 1 (uncompetitive type of inhibition).
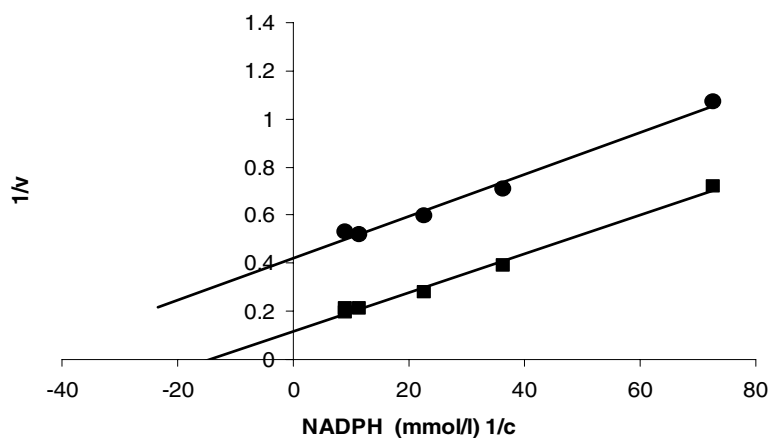
Figure 2. Inhibitory effect of compound 1 on diabetic male rat lens aldose reductase (1-month experimental diabetes, induced in male rats by three i.v. doses of STZ, 25 mg/kg, on three consecutive days, was characterized by glycemia >10 mmol/l for the duration of the whole experiment). Typical double reciprocal plot of initial enzyme velocity (v) *versus* concentration (c) of cofactor (NADPH) in the presence or absence of compound 1: ■ no inhibitor; ● 20 μmol/l of compound 1 (uncompetitive type of inhibition).

Table 1. Kinetic parameters of ALR2 in partially purified male rat eye lens preparations and the inhibitory effect of compound 1 under control and diabetic conditions

Varied substrate/ cofactor	State	K_m (μmol/l)	V_{max} (10,000 O.D./s/mg)	K_i compound 1[a] (μmol/l)
Glyceraldehyde	Control	585.0 ± 90.0	4.3 ± 1.3	17.9 ± 3.0
	Diabetes[b]	478.0 ± 110.0	4.5 ± 0.8	16.1 ± 3.0
NADPH	Control	54.0 ± 12.0	6.8 ± 1.4	11.0 ± 1.9
	Diabetes[b]	69.0 ± 26.0	8.7 ± 2.9	10.6 ± 1.6

[a] Uncompetitive type of inhibition. Results are mean values $\pm$ SD from at least three experiments. [b] 1-month experimental diabetes, induced in rats by three i.v. doses of STZ (25 mg/kg) on three consecutive days, was characterized by glycemia >10 mmol/l for the duration of the whole experiment. O.D., optical density.

rats, similarly the inhibitory efficacy of compound 1 was not affected by two-month experimental diabetes when compared with age-matched controls. Notable is the sex difference indicating a higher sensitivity of female ALR2 to compound 1 in comparison with the enzyme preparations isolated from lenses of male animals.

To test the selectivity of compound 1 in inhibition of ALR2, the latter was compared with the effect achieved when inhibiting ALR1, an enzyme with high-

Table 2. Inhibtion of ALR2 by compound 1 in partially purified rat eye lens preparations. Effect of diabetic state

Experimental diabetes	Control group IC$_{50}$ (μmol/l)	Diabetic group IC$_{50}$ (μmol/l)
1-month[a]	18.2 $\pm$ 1.2 ($n = 8$)	16.6 $\pm$ 1.2 ($n = 7$)
2.5-month[b]	n.d.	16.4 $\pm$ 0.7 ($n = 8$)
5-month[c]	n.d.	14.6 $\pm$ 0.1 ($n = 8$)
2-month[d]	13.9 $\pm$ 0.9 ($n = 6$)	12.0 $\pm$ 1.6 ($n = 6$)

[a] Experimental diabetes, induced in male rats by three i.v. doses of STZ (25 mg/kg) on three consecutive days, was characterized by glycemia >10 mmol/l for the duration of the whole experiment. [b] Experimental diabetes, induced in male rats by three i.p. doses of streptozotocin (30 mg/kg) on three consecutive days, was characterized by glycemia >10 mmol/l for the duration of the whole experiment. [c] Experimental diabetes, induced in male rats by three i.p. doses of STZ (30 mg/kg) on three consecutive days, was characterized by glycemia >10 mmol/l for the duration of the whole experiment. [d] Experimental diabetes, induced in female rats by single i.p. dose of STZ (65 mg/kg), was characterized by glycemia >15 mmol/l for the duration of the whole experiment. Results are mean values $\pm$ SD from at least three parallel measurements by using eye lens preparations pooled from n animals; IC$_{50}$, the concentration required to produce 50 % inhibition; n.d., not determined.

Table 3. Inhibtion of ALR1 by compound 1 in partially purified male rat kidney preparations. Effect of diabetic state

Experimental diabetes	Control group IC$_{50}$ (μmol/l)	Diabetic group IC$_{50}$ (μmol/l)
1-month[a]	328.5 $\pm$ 21.9 ($n = 8$)	349.2 $\pm$ 39.3 ($n = 7$)
2.5-month[b]	n.d.	433.8 $\pm$ 17.0 ($n = 8$)
5-month[c]	n.d.	387.2 ($n = 8$)

[a] Experimental diabetes, induced in rats by three i.v. doses of STZ (25 mg/kg) on three consecutive days, was characterized by glycemia >10 mmol/l for the duration of the whole experiment. [b] Experimental diabetes, induced in rats by three i.p. doses of STZ (30 mg/kg) on three consecutive days, was characterized by glycemia >10 mmol/l for the duration of the whole experiment. [c] Experimental diabetes, induced in rats by three i.p. doses of STZ (30 mg/kg) on three consecutive days, was characterized by glycemia >10 mmol/l for the duration of the whole experiment. Results are mean values from two measurements or mean values $\pm$ SD from at least three parallel measurements by using eye lens preparations pooled from n animals; IC$_{50}$, the concentration required to produce 50 % inhibition; n.d., not determined.

est homology to ALR2. Table 3 summarizes the IC$_{50}$ values of compound 1 for inhibition of the reduction of glucuronate substrate by ALR1 obtained from kidneys of rats exposed to different periods of experimental diabetes. The correspond-

Table 4. Selectivity factors under control and diabetic conditions

Experimental diabetes	Control group $(IC_{50})^{ALR1}/(IC_{50})^{ALR2}$	Diabetic group $(IC_{50})^{ALR1}/(IC_{50})^{ALR2}$
1-month[a]	~18	~21
2.5-month[b]	n.d.	~26
5-month[c]	n.d.	~27

[a] Experimental diabetes, induced in male rats by three i.v. doses of STZ (25 mg/kg) on three consecutive days, was characterized by glycemia >10 mmol/l for the duration of the whole experiment. [b] Experimental diabetes, induced in male rats by three i.p. doses of STZ (30 mg/kg) on three consecutive days, was characterized by glycemia >10 mmol/l for the duration of the whole experiment. [c] Experimental diabetes, induced in male rats by three i.p. doses of STZ (30 mg/kg) on three consecutive days, was characterized by glycemia >10 mmol/l for the duration of the whole experiment. $(IC_{50})^{ALR1}$ and $(IC_{50})^{ALR2}$ represent IC_{50} values for inhibition of ALR1 and ALR2, respectively; n.d., not determined.

ing selectivity factors for different experimental conditions, defined as the ratio of $(IC_{50})^{ALR1}/(IC_{50})^{ALR2}$, are summarized in Table 4.

Discussion

Numerous structurally diverse compounds have been identified as potent *in vitro* aldose reductase inhibitors, nevertheless, with few exceptions, these compounds have produced little clinical benefit (Costantino et al. 2000; Miyamoto 2002). The limited long-term efficacy of these drugs may be linked, at least in part, to diabetes-induced changes in properties of ALR2, which may alter its ligand binding characteristics and efficacy of catalysis. Indeed, under hyperglycemic conditions, both elevated expression (Vinores et al. 1988; Ghahary et al. 1989, 1991; Nishigami 1990; Srivastava et al. 2005) and post-translational modifications (Bhatnagar et al. 1989; Del Corso et al. 1989; Liu et al. 1989; Capiello et al. 1995; Grimshaw and Lai 1996) of ALR2 were reported in the target organs of diabetic complications as well as in cells cultured under hyperglycemic conditions (Tawata et al. 1992; Spycher et al. 1997; Nakamura et al. 2000; Yabe-Nishimura et al. 2003; Srivastava et al. 2005). As compared to ALR2 from control tissues, the enzyme isolated from diabetic tissues was found either to be qualitatively identical (Ghahary et al. 1989, 1991) or displayed altered kinetic properties and became relatively insensitive to hydantoin inhibitors such as sorbinil or tolrestat (Das and Srivastava 1985; Srivastava et al. 1985, 1986a,b; Chandra et al. 2002). Similar changes in inhibitor sensitivity and kinetic properties have been reported in oxidatively modified forms of ALR2 (Bhatnagar et al. 1989; Del Corso et al. 1989; Liu et al. 1989; Capiello et al. 1995; Grimshaw and Lai 1996).

In the present study we compared the inhibitory potency and selectivity of compound 1 in control and diabetic states. ALR2 obtained from diabetic lenses dis-

played uncompetitive type of inhibition by compound 1. This finding was identical with that in control rats (Stefek et al. 2005). The uncompetitive type of inhibition, recorded either in relation to D,L-glyceraldehyde as the substrate or to NADPH as the cofactor, indicates that under physiological conditions glucose would not compete with the inhibitor for the binding site on the enzyme molecule. Thus, under hyperglycemic conditions in diabetics, the excessive free glucose would not decrease the inhibitory efficacy of the drug. The Michaelis constant values K_m for the control rat aldose reductase, in relation to glyceraldehyde substrate $(K_m)^{glyceraldhyde}$ = 0.585 mmol/l and in relation to NADPH co-factor $(K_m)^{NADPH}$ = 0.054 mmol/l, were within the range of values determined by other authors in partially purified rat lens ALR2 (DeRuiter et al. 1989; DeRuiter and Mayfield 1990; Haraguchi 2003).

An important feature of pharmacologically applicable aldose reductase inhibitors is the selectivity of their action. A co-inhibition of structurally related physiological oxidoreductases might have unwanted side effects. For testing the inhibition selectivity of compound 1, we compared its inhibitory effect on ALR2 with that on ALR1, an enzyme exhibiting the highest homology with ALR2 (Barski et al. 1995). The selectivity for compound 1 was characterized by a selectivity factor close to 20. Under diabetic conditions, a slight increase in the selectivity was recorded.

To conclude, the inhibitory mode, efficacy and selectivity of compound 1, a novel aldose reductase inhibitor, was preserved even under conditions of prolonged STZ-induced experimental diabetes of rats.

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