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# A novel zwitterionic inhibitor of aldose reductase interferes with polyol pathway in *ex vivo* and *in vivo* models of diabetic complications

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Recently a zwitterionic principle has been suggested as an alternative to bioisosteric replacement for increasing low bioavailability of aldose reductase inhibitors bearing an acidic function. In the present work we studied the effect of a novel zwitterionic inhibitor of aldose reductase [(2-benzyl-2,3,4,5-tetrahydro-1*H*-pyrido[4,3-*b*]indole-8-yl)-acetic acid, compound **1**] on sorbitol accumulation in *ex vivo* and *in vivo* models of diabetic complications. The effect of **1** on sorbitol accumulation in isolated rat eye lenses incubated with high glucose and in selected organs of streptozotocin-induced diabetic rats was evaluated. Significantly increased sorbitol levels were recorded in the lenses incubated with 50 mM glucose in comparison with controls. Sorbitol production was inhibited by **1** at concentrations of 25 and 100 μM. Under *in vivo* conditions in diabetic rats, significant elevation of sorbitol levels in selected organs was recorded. Compound **1** administered *i.g.* for five consecutive days (twice a day 25 mg/kg) inhibited sorbitol accumulation in erythrocytes and the sciatic nerve, yet it was without effect in eye lenses. A similar picture of inhibition was observed after *i.p.* administration of **1.** To conclude, the results suggest that the zwitterionic principle may represent a practicable way of improving bioavailability of aldose reductase inhibitors bearing an acidic function.

## 1. Introduction

Although multiple biochemical pathways are likely to be responsible for the pathogenesis of diabetic complications, substantial evidence suggests a key role for the polyol pathway (Yabe-Nishimura 1998; Kador et al. 2000; Oates 2002, 2010; El-Kabbani et al. 2004; Alexiou et al. 2009; Obrosova et al. 2010; Obrosova and Kador 2011; Tang et al. 2012). Under hyperglycemia conditions in tissues that do not require insulin for glucose uptake, aldose reductase (ALR2, E.C.1.1.1.21), the first enzyme of the polyol pathway, reduces some of this excess glucose to the organic osmolyte sorbitol in an NADPH-dependent manner. Due to its poor membrane penetration and slow metabolism by sorbitol dehydrogenase, sorbitol accumulates intracellularly, resulting in disruption and eventually death of the cells. In this way, the polyol pathway is believed to contribute to the etiology of long-term diabetic complications such as cataract, retinopathy, nephropathy, neuropathy, micro-, and macroangiopathy.

Recently novel carboxymethylated pyridoindoles have been designed, synthesized and described as uncompetitive inhibitors of aldose reductase (Stefek et al. 2008). Of them, compound **1** (Fig. 1) was characterized by a corresponding $IC_{50}$ value in a low micromolar region. A reasonable degree of selectivity with respect to the closely related aldehyde reductase (Stefek et al. 2008) and the glycolytic pathway (Juskova et al. 2009) was recorded. In addition, antioxidant activity of **1** was proved (Juskova et al. 2010) pointing to its potential use as a multitarget agent in the pharmacological prevention of diabetic complications.

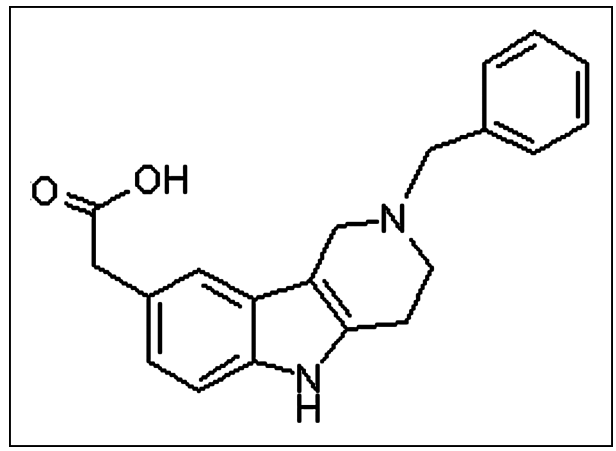


Fig. 1: Chemical structure of (2-benzyl-2,3,4,5-tetrahydro-1H-pyrido[4,3-b] indole-8-yl)-acetic acid (compound **1**).

The critical property for the efficacy of aldose reductase inhibitors (ARIs) is their ability to penetrate into target tissues. Owing to ALR2 pharmacophore requirements for an acidic proton (Lee et al. 1998; Schlitzer et al. 2001; DelCorso et al. 2008; Alexiou et al. 2009) the largest group of ARIs contains an acetic acid moiety. Carboxylic acids are ionized at physiological pH resulting in their poor biological availability. Substitution of the acetic acid chain with a hydantoin or succinimide moiety partially improved the yield in the target tissues (Costantino et al. 2000; Miyamoto 2002; Alexiou et al.

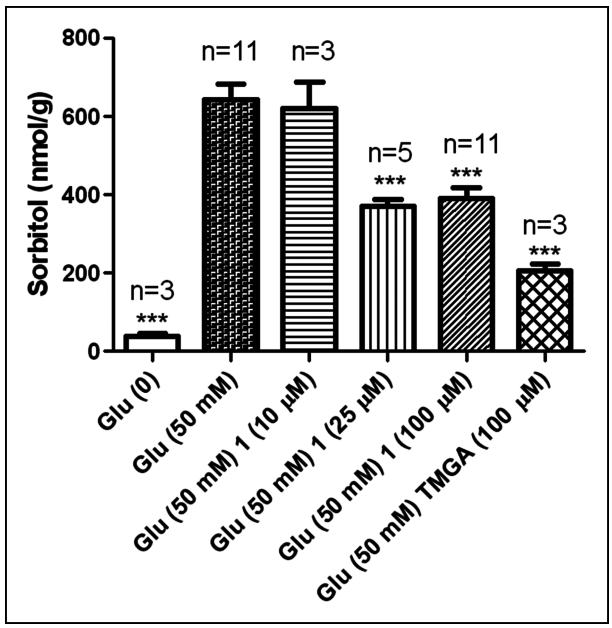


Fig. 2: Effect of compound **1** on sorbitol accumulation in isolated rat eye lenses incubated with high glucose in comparison with standard TMGA. Glucose, 50 mM; time of incubation, 3 hours; 37 °C. Results are mean values ± SEM from n independent incubations, *** $p < 0.001$ vs. Glu (50 mM).

2009). The bioisosteric principle may offer another way how to overcome unfavorable partitioning of acidic ARIs (Nicolaou et al. 2004; Rakowitz et al. 2005; Alexiou et al. 2008; Pekglidou et al. 2010; Maccari et al. 2010).

The presence of a basic center at the tertiary nitrogen of compound **1**, in addition to the acidic carboxylic function, predisposes the compound to form double-charged zwitterions. As a result of intramolecular interaction between the oppositely charged centers, such molecules usually exhibit a bell-shaped logD/pH profile with a maximum uptake around the isoelectric point, where zwitterionic species co-exist with the neutral form (Pagliara et al. 1997). Indeed, as previously reported, the distribution profile of **1** in a two-phase system of water/octanol was characterized by maximal distribution ratio lying near the physiological pH (Stefek et al. 2008). Physico-chemical proof of the zwitterionic nature of compound **1** was reported recently (Stefek et al. 2011).

The zwitterionic principle thus represents another potential alternative how to increase low bioavailability of aldose reductase inhibitors bearing an acidic function. In the present work we studied the effect of compound **1** on sorbitol accumulation in isolated rat eye lenses incubated in the presence of high glucose as well as in selected organs in streptozotocin-induced diabetic rats *in vivo*.

## 2. Investigations and results

As shown in Fig. 2, significantly increased sorbitol levels were recorded in the lenses incubated with glucose in comparison with control incubations without glucose. Sorbitol production was significantly inhibited by compound **1** at concentrations of 25 and 100 μM. No significant effect was observed at a concentration of 10 μM **l**. The efficacy was comparable to that of 100 μM tetramethylene glutaric acid (TMGA) used as a reference.

Under *in vivo* conditions in STZ-diabetic rats, significant elevation of sorbitol concentration in erythrocytes, sciatic nerve and eye lenses was recorded. Compound **1** administered *i.g.* (50 mg/kg/day) for five consecutive days significantly inhibited sorbitol accumulation in red blood cells and sciatic nerve, yet it was without effect in eye lenses (Fig. 3**A**). A very similar picture of inhibition was observed after *i.p.* administration of **1** at the same dosage regimen (Fig. 3**B**). In both experiments, the diabetic state was characterized by plasma glucose varying from 22.9 to 27.1 mM; no significant changes in body weight and blood glucose were recorded in the groups of diabetic rats treated with compound **1** in comparison with the untreated diabetic groups.

## 3. Discussion

Significantly increased sorbitol levels recorded in the lenses incubated with glucose, in comparison with control incubations without glucose, reflect an increased flux of the sugar through lens intracellular ALR2. Similarly, other authors (Terashima et al. 1984) observed more than 10-fold increases of sorbitol levels in the eye lenses incubated with glucose under comparable conditions (50 mM glucose, 4 h incubation). Sorbitol production was significantly inhibited by compound **1** present in the incubation media at 25 and 100 μM concentrations indicating ready uptake of **1** by lens tissue followed by inhibition of the cytosolic ALR2. Increase in the concentration of **1** from 25 to 100 μM did not result in any significant change of inhibition. This finding may point to some degree of saturation of the uptake process. Inhibition of sorbitol dehydrogenase, the second enzyme of the polyol pathway, would result in a similar effect, yet it may be excluded since, as we reported earlier (Juskova 2010), compound **1** did not affect the activity of sorbitol dehydrogenase at concentrations below 250 μM. Alternatively, decreased solubility of **1** at higher concentrations might be another reason for the observed irregularity of the concentration dependence. Yet this option was excluded by obtaining identical results from incubations performed in the presence of DMSO (1% final concentration, data not shown). Overall, the inhibitory efficacy of **1** was lower than that of the equimolar reference TMGA. The latter is a specific inhibitor of ALR2, used as a reference also by other authors (Malone et al. 1980; Das and Srivastava 1985; Schmidt and Michal 1989).

The result of the *in vivo* experiments showed efficient inhibitory effects of compound **1** on sorbitol accumulation in erythrocytes and sciatic nerves, regardless the way of drug administration. This result points to ready uptake of compound **1** into the central compartment both after its enteral and parenteral administration. The treatment of diabetic animals had no effect on sorbitol levels in the eye lens. The absence of any effect of compound **1** on sorbitol accumulation in lenses under *in vivo* conditions may be explained by the limited availability of the drug at this site. The lens is a bloodvessel-less organ supplied through the anterior eye chamber. The same order of organ sensitivity to therapy by an aldose reductase inhibitor was reported by Mylari et al. (1991) for ranirestat and Matsumoto et al. (2008) for zopolrestat. The latter authors recorded steady state levels of zopolrestat in rat blood plasma on the third day of repeated oral dosage of the drug. A fourteen-day period was required to reach steady state concentration of zopolrestat in the sciatic nerve. Obviously a longer period is needed for eye lenses, reported as even less sensitive to zopolrestat inhibition.

The above mentioned results are in compliance with the amphoteric behavior of compound **1**. Its isoelectric pH = 6.4 (Stefek et al. 2008), lying closely to the physiologically relevant pH 7.4, predisposes this compound for good bioavailability. As we reported earlier (Stefek et al. 2008), the distribution profile of compound **1** showed a maximal extraction yield around pH 7 in an 1-octanol/water system. This behavior can obviously be ascribed to the zwitterionic nature (Stefek et al. 2011) of the compound and by higher lipophilicity of the doubly charged zwitterionic form, compared to a singly charged species.

On balance then, the present results point to good systemic availability of compound **1**, after both enteral and parenteral

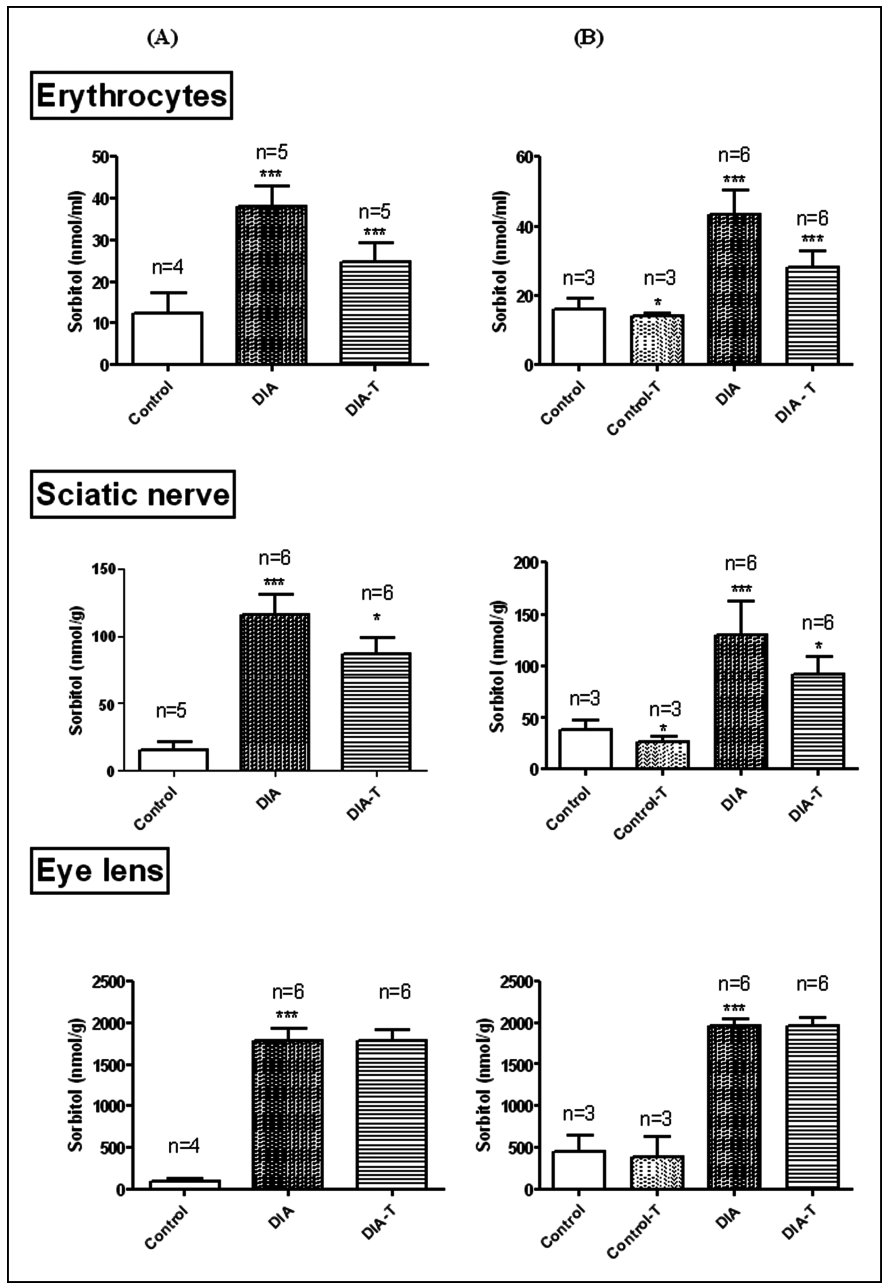


Fig. 3: Accumulation of sorbitol in erythrocytes, sciatic nerves and eye lenses of rats under conditions of STZ-induced experimental diabetes. Effect of compound **1** administered *i.g.* (**A**) or *i.p.* (**B**) for five consecutive days according to the following dosage schedule: 25 mg/kg twice daily (8:30 and 15:30) for the first four days and 25 mg/kg on the fifth day three hours before killing the animals. Results are mean values ± SD with n = number of animals in a group. +++ $p \leq 0.001$ for D vs. C and for D vs. CT; * $p \leq 0.05$ for CT vs. C and for DT vs. D; *** $p \leq 0.001$ for DT vs. D.

administration. The zwitterionic principle is thus proposed as a practicable way of improving bioavailability of aldose reductase inhibitors bearing an acidic function. Assessment of the overall disposition and pharmacokinetics of compound **1** may provide a definite answer, which however, was beyond the scope of the present study.

## 4. Experimental

### *4.1. Chemicals and instruments*

Compound **1**, (2-benzyl-2,3,4,5-tetrahydro-1*H*-pyrido[4,3-*b*]indole-8-yl)-acetic acid was synthesized at the Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences (Stefek et al. 2008) and was available as hydrochloride salt. Tetramethylene glutaric acid (TMGA), streptozotocin, sorbitol dehydrogenase, diaphorase, β-$NAD^+$, and resazurin were obtained from Sigma-Aldrich Chemie (Steinheim, Germany). BIO-LA-TEST Glucose GOD 1500 kit was from Pliva-Lachema Diagnostika Ltd. (Brno, Czech Republic). Other chemicals were purchased from local commercial sources and were of analytical grade quality. Spectrophotometric and fluorometric analysis was performed using an Infinite M 200 analyzer (Tecan Austria GmbH).

### *4.2. Animals*

Male Wistar rats 8 - 9 weeks old, weighing 230 - 250 g, were used as organ donors. The animals came from the Breeding Facility of the Institute of Experimental Pharmacology and Toxicology, Dobra Voda (Slovak Republic). 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).

### *4.3. Eye lens cultivation*

The compound studied dissolved in distilled water was added into the tubes containing freshly dissected eye lenses (1 lens per tube) in 10 mM isotonic phosphate buffered saline (pH 7.4, 1.9 mM $NaH_2PO_4$, 8.1 mM $Na_2HPO_4$ and 150 mM NaCl), bubbled at 37 °C with pneumoxide (5% $CO_2$, 95% $O_2$), to the final concentrations as reported, 30 min before adding glucose.

The incubation was initiated by adding glucose to the final concentration of 50 mM and then continued at 37 °C with occasional (in about 30-min intervals) bubbling of the mixture for approximately 30-s periods with pneumoxide. The incubations were terminated after a 3-h period by cooling the mixtures in an ice bath, followed by washing the lenses three times with ice-cold phosphate buffered saline (1 mL). The short term cultivations were preferred to avoid substantial permeability changes of the eye lenses. The washed lenses were kept deep-frozen for sorbitol determination.

### *4.4. Experimental diabetes*

Experimental diabetes was induced by triple i.p. doses of streptozotocin (STZ, 30 mg/kg) on three consecutive days. STZ was dissolved in citrate buffer (0.1 M, pH 4.5). The animals were fasted overnight prior to STZ administration. Control animals received the citrate buffer only. Water and food were available immediately after dosing. Two days after the last dose of STZ, all animals with plasma glucose level > 15 mM were considered diabetic and were included in the study.

Control and diabetic animals were randomly assigned to untreated and treated groups as indicated in the result section. Compound **1**, in the form of a hydrochloride salt, was dissolved in physiological solution. The treatment was initiated on the fifth day of the experiment and continued for the next four days either by *intragastric* (*i.g.*) administration *via* a gavage (experiment A) or *intraperitoneal (i.p.)* administration (experiment B). In both experiments the dosage schedule was as follows: 25 mg/kg dose of compound **1** was applied twice daily (8:30 and 15:30) for four days; on the fifth day, the treatment was applied in the morning, three hours before killing the animals. The animals were killed by cervical dislocation under ether anesthesia followed by exsanguination of the carotid artery. Washed erythrocytes, eye lenses and sciatic nerves were frozen and kept in deep-freeze for sorbitol assay.

### *4.5. Sorbitol assay*

#### *4.5.1. Erythrocytes*

The frozen erythrocytes (0.2 mL) were let to melt at ambient temperature. Thereafter, ice cold distilled water (0.4 mL) was added to hemolyze the red blood cells, followed by addition of $HClO_4$ (9%, 0.6 mL) to precipitate proteins. The suspension was then ultra-sounded for 5 min. The mixture was kept on ice for 30 min followed by centrifugation at 700 × g for 15 min at 4 °C. The supernatant was neutralized with $K_2CO_3$ (4 M).

#### *4.5.2. Sciatic nerve*

The frozen nerves were let to melt at ambient temperature, dried on filter paper and powdered under liquid nitrogen. Distilled water (0.4 mL) was added and the suspension was ultra-sounded for 5 min. Thereafter, ice cold $HClO_4$ (9%, 0.4 mL) was added, mixed thoroughly and ultra-sounded again for 5 min. The mixture was kept on ice for 30 min followed by centrifugation at 700 × g for 15 min at 4 °C. Aliquot (0.6 mL) was transferred to a clean tube and neutralized with $K_2CO_3$ (4 M).

#### *4.5.3. Eye lens*

The frozen lenses were let to melt at ambient temperature. Then distilled water (0.2 mL/1 lens) was added. The lenses were disrupted by a glass rod. The rod was washed twice with distilled water (0.1 mL) and the suspension was ultra-sounded for 5 min. Thereafter, icecold $HClO_4$ (9%, 0.4 mL) was added and mixed thoroughly. The mixture was ultra-sounded for further 5 min and then kept on ice for 30 min to let proteins precipitate. The precipitated protein was spun off (15 min at 3 000 rpm) at 4° C. The supernatant was neutralized with concentrated $K_2CO_3$ (4 M).

#### *4.5.4. Analytical procedure*

The neutralized supernatants, obtained as described above, were used for determination of sorbitol concentration by modified enzymatic analysis according to Mylari et al. (2003). In brief, sorbitol was oxidized to fructose by sorbitol dehydrogenase (SDH) with concomitant reduction of resazurin by diaphorase to the highly fluorescent resorufin. The final concentrations of the assay solutions were: diaphorase (11.5 U/25 mL triethanolamine buffer), $NAD^+$ (25 mg/25 mL triethanolamine buffer), resazurin (25 μL of 2 mmol/L resazurin solution in 25 mL of triethanolamine buffer), SDH (15.025 U/mL triethanolamine buffer). Reaction mixtures were incubated for 60 min at room temperature with an opaque cover. The sample fluorescence was determined at 544 nm excitation and 590 nm emission. After the appropriate blanks had been subtracted from each sample, the amount of sorbitol was determined in each sample by comparison with a linear regression of sorbitol standards.

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