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(2-Benzyl-2,3,4,5-tetrahydro-1*H*-pyrido[4,3-*b*]indol-8-yl)-acetic acid: An aldose reductase inhibitor and antioxidant of zwitterionic nature

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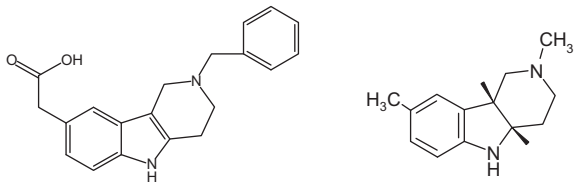
ABSTRACT

Novel carboxymethylated pyridoindoles, characterized by antioxidant activity combined with the ability to inhibit aldose reductase, represent an example of a multitarget approach to the treatment of diabetic complications – severe diabetes-related health disorders of multifunctional nature. One of the novel carboxymethylated pyridoindoles, (2-benzyl-2,3,4,5-tetrahydro-1*H*-pyrido[4,3-*b*]indol-8-yl)-acetic acid (compound **1**), was found to inhibit aldose reductase with the IC_{50} value 18.2 ± 1.2 μM. Owing to aldose reductase pharmacophore requirements for an acidic proton, most aldose reductase inhibitors contain an acetic acid moiety, ionized at physiological pH, resulting in poor bioavailability of the drugs. The presence of a basicity center at the tertiary nitrogen of the carboxymethylated pyridoindoles, in addition to the acidic carboxylic function, predisposes these compounds to form double-charged zwitterionic species. The zwitterionic nature of compound **1** may remarkably affect its pH-lipophilicity profile allowing for increased membrane penetration in the pH region around its isoelectric point, which lies close to the physiological pH 7.4. In the first part of this study, the presence of zwitterionic species was experimentally proved by the concentration-dependent effect of sodium 1-hexanesulphonate on the distribution profile of compound **1**. Then a series of experiments was performed in the cellular system of isolated erythrocytes in vitro. Isolated rat erythrocytes exposed to peroxyl radicals, generated in the solution by decomposition of the hydrophilic azoinitiator AAPH or intracellularly by decay of lipophilic *t*-BuOOH, underwent progressive hemolysis. The onset of the hemolysis was shifted from the starting zero point by the time interval assigned as a lag period. In the presence of compound **1** the lag period was significantly prolonged. Finally, under conditions of a short-term experiment in STZ-diabetic rats in vivo, increase in sorbitol levels in erythrocytes was recorded. Compound **1** administered in the dose 50 mg/kg/day (i.g.) significantly decreased the sorbitol level in the erythrocytes. To conclude, the physico-chemical proof of the zwitterionic nature of compound **1** was established and the results obtained in isolated red blood cells indicated good cellular availability of the compound. In addition, in diabetic rats, sorbitol accumulation in red blood cells was significantly inhibited by compound **1** administered intra-gastrically, suggesting its ready uptake into the central compartment. The zwitterionic principle thus may have significant consequences for increased bioavailability of drugs bearing an acidic function.

1. Introduction

Recently novel carboxymethylated congeners of stobadine (Fig. 1), an efficient pyridoindole antioxidant,[1] were designed, synthesized and characterized as uncompetitive inhibitors of aldose reductase, the first enzyme of the polyol pathway, involved in the etiology of diabetic complications.[2] 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 was recorded. The



(**1**) Stobadine (**2**)

Figure 1. Chemical structure of (2-benzyl-2,3,4,5-tetrahydro-1*H*-pyrido[4,3-]indole-8-yl)-acetic acid (**1**) and stobadine [(−)-*cis*-2,8-dimethyl-2,3,4,4a,5,9b-hexahydro-1*H*-pyrido[4,3,-*b*]indole] (**2**).

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inhibitory mode, efficacy and selectivity were preserved even under conditions of prolonged experimental diabetes of rats.[3] Antioxidant action of **1** was documented in a DPPH test, and in liposomal and erythrocyte membrane models oxidatively stressed by peroxyl radicals.[2,4] In the system of isolated erythrocytes, compound **1** was readily taken up by the cells without affecting significantly their osmotic fragility. Under euglycemic conditions, compound **1** did not interfere with the glycolytic pathway of glucose elimination from isolated red blood cells.[5]

The presence of a basicity center at the tertiary nitrogen, in addition to the acidic carboxylic function, predisposes these compounds to form double-charged zwitterions, which are partially neutralized molecular species, as the result of intermolecular interaction between the oppositely charged centers. Such molecules usually exhibit a bell-shaped log*D*/pH profile with a maximum around the isoelectric point, where zwitterionic species co-exist with the neutral form.[6] 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.[2] The zwitterionic principle thus offers an interesting alternative how to increase low bioavailability of aldose reductase inhibitors bearing an acidic function.

In the first part of the present study, physico-chemical evidence of zwitterionic nature of compound **1** is presented, based on the effect of a hydrophobic counter ion on the distribution profile.[7] Secondly, the antioxidant action of **1** was studied in the cellular system of isolated erythrocytes in vitro under oxidative stress induced by peroxyl radicals generated by decay of both hydrophilic AAPH and lipophilic *t*-BuOOH. Finally, the ability of compound **1** to interfere with the polyol pathway in the whole animal in vivo was evaluated.

2. Results

The effect of 1-hexanesulphonate anion on octanol/water log*D* of compound **1** is depicted in Figure 2. At low concentration of sodium 1-hexanesulphonate, a small increase in the distribution ratio is observed (see the high resolution insertion), which is followed by a decrease with increasing concentration, reaching a low-level plateau at high concentrations of the counter ion.

In parallel experiments performed with piroxicam, a well known zwitterionic drug[8] used as an auxiliary compound, an analogous effect of hexanesulphonate anion on log*D* values was

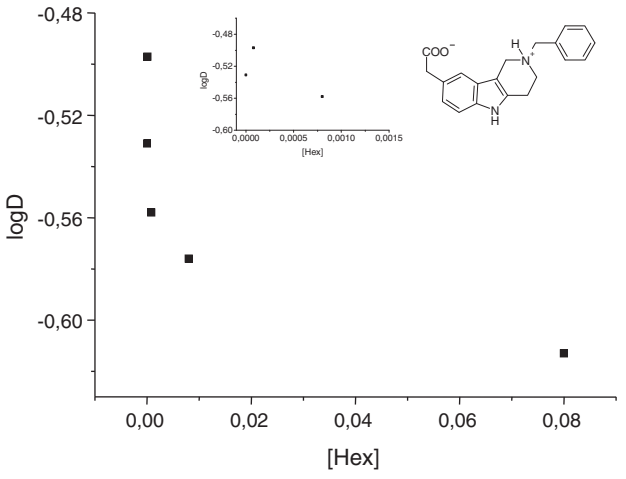

Figure 2. Effect of 1-hexanesulphonate on log*D* of compound **1** in the octanol-water system at pH 7.4. Insertions: left, log*D* versus [1-hexanesulphonate] in a low concentration range; right, zwitterions derived from compound **1**.

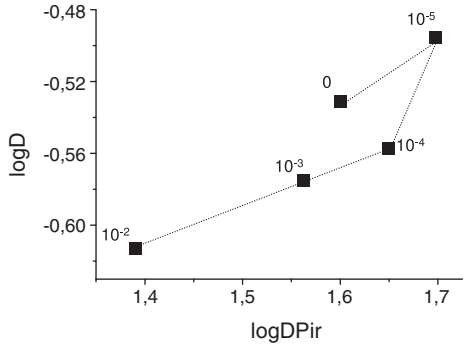

Figure 3. Plot of the log*D* values of compound **1** versus log*D* values of piroxicam (log*D*pir) at equal concentrations of 1-hexanesulphonate. Initial increase is followed by decrease at higher hexanesulphonate concentration.

determined at its isoelectric point (data not shown). After initial increase upon addition of sodium hexanesulphonate, a systematic decrease in log*D* follows at higher counter ion concentration in both cases. This behavior is illustrated in Figure 3.

Isolated rat erythrocytes exposed to AAPH or *t*-BuOOH underwent progressive hemolysis, determined by measuring the release of hemoglobin. The onset of hemolysis was shifted from the starting zero point by the time interval assigned as a lag period. In the presence of compound **1** (100 μM), the lag period increased significantly (Fig. 4).

Figure 5 summarizes the antioxidant effect of compound **1** in comparison with reference antioxidants in isolated rat erythrocytes oxidatively stressed by AAPH- or *t*-BuOOH-derived peroxyl radicals. Compound **1** (100 μM), trolox (100 μM, Fig. 5a) and carnosine (10 mM, Fig. 5b) significantly prolonged the lag time compared to the control value. Melatonin (Fig. 5b) at 100 μM concentration had no effect. In the case of compound **1**, a slight nonsignificant decrease of the lag period was observed when pre-incubation was omitted and the compound was added 30 min after hemolysis was started by *t*-BuOOH (Fig. 5b, −30 min).

Under in vivo conditions in STZ-diabetic rats, significant elevation of sorbitol concentration in erythrocytes was recorded. Compound **1** administered intra-gastrically for five consecutive days significantly inhibited sorbitol accumulation in red blood cells (Fig. 6). The diabetic state was characterized by plasma glucose varied from 22.9 to 27.1 mM. No significant change of blood glucose was recorded in the group of diabetic rats treated with compound **1** in comparison with the untreated diabetic group.

3. Discussion

The presence of a basicity center at the tertiary nitrogen of compound **1**, in addition to the acidic carboxylic function, predisposes the compound to form double charged zwitterionic species shown in the insertion of Figure 2. As reported before,[2] the dissociation constants of **1** were determined as follows: $pK_a^{Acidic} = 4.34 \pm 0.07$ and $pK_a^{Basic} = 8.65 \pm 0.07$. Since $pK_a^{Acidic} < pK_a^{Basic}$, the acidic and basic functionalities can be ionized at the same time resulting in double charged zwitterionic species.[6] Zwitterionic ampholytes often exhibit incompletely understood effects of intramolecular neutralization that may render the zwitterionic species markedly more lipophilic than the single charged cations and anions, giving typically a lipophilicity profile of a characteristic bell-shape with the maximum around the isoelectric point,[6] which indeed was the case of compound **1**.[2]

In the present study, the presence of zwitterions was experimentally proved by the concentration-dependent effect of the counter-anion sodium 1-hexanesulphonate on the distribution

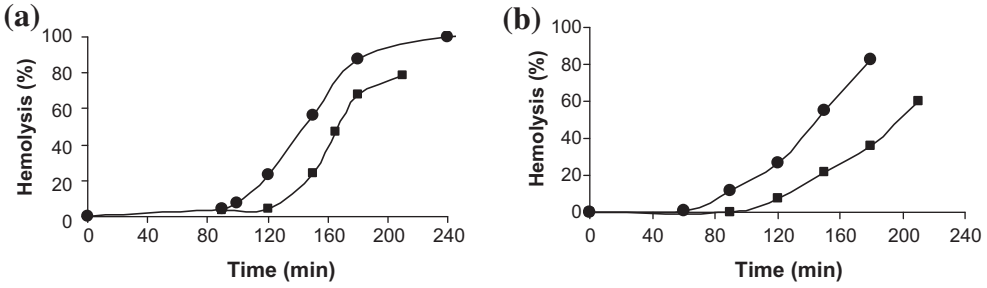

Figure 4. Time course of hemolysis of rat erythrocytes induced by AAPH (a) or *t*-BuOOH (b). Erythrocyte suspensions (1.5%) were incubated (a) with 30 mM AAPH alone (circles) or in the presence of 100 μM of compound **1** (cubes), or (b) with 250 μM *t*-BuOOH alone (circles) or in the presence of 2.5 μM of compound **1** (cubes). Results of two typical experiments.

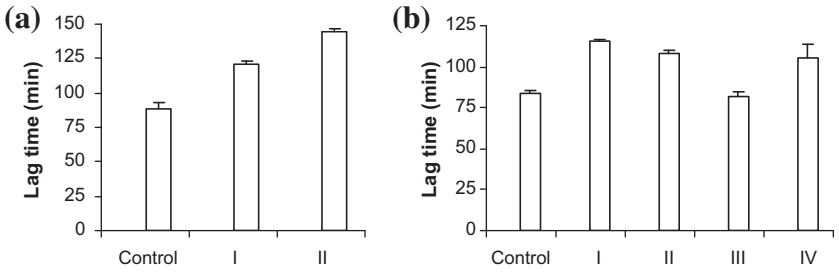

Figure 5. Compound **1** protected rat erythrocytes against (a) AAPH- or (b) *t*-BuOOH – induced hemolysis. Trolox, melatonin and carnosine were used as references. (a) I, compound **1** (100 μM, +30 min); II, trolox (100 μM, +30 min); (b) I, compound **1** (100 μM, +30 min); II, compound **1** (100 μM, −30 min); III, melatonin (100 μM, +30 min); IV, carnosine (10 mM, +30 min); +30 min, erythrocytes were pre-incubated with the compound studied for 30 min, then AAPH or *t*-BuOOH was added; −30 min, pre-incubation period was omitted and compound **1** was added 30 min after addition of *t*-BuOOH. Results are presented as means ± SD from at least three measurements.

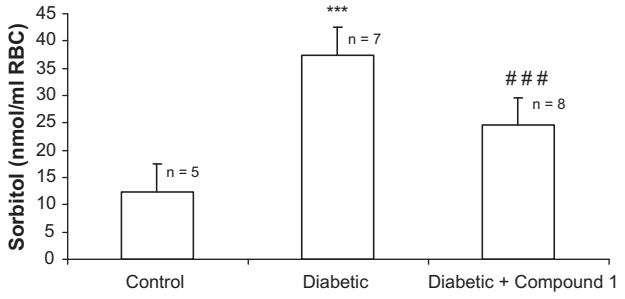

Figure 6. Accumulation of sorbitol in red blood cells of rats under conditions of experimental diabetes in vivo. Effect of compound **1**. Results are presented as means ± SD, where *n* is the number of animals in each group. $^{***}p$ <0.001 diabetic versus control; $^{\#\#\#}p$ <0.001 diabetic + compound **1** versus diabetic.

profile of **1** in a two-phase octanol-water model system. Experiments were performed at pH 7.4. In presence of a protonated basic center, normally an increase in log*D* values is expected with increase in the concentration of sodium 1-hexanesulphonate reaching an upper-level plateau, as a result of ion pair formation with the protonated basic group.[7] On the contrary, in the case of compound **1**, an opposite effect was observed, namely a decrease in log*D* with increase of 1-hexanesulphonate concentration resulting in a low-level plateau, although a small increase was noticed at low concentration of the counter ion. This behavior provides evidence of the zwitterionic nature of compound **1** with charge compensation around its isoelectric point. This intramolecular interaction is affected in the presence of the hydrophobic counter-anion. Low concentrations of 1-hexanesulphonate disrupt the zwitterionic structure only to a small extent so that the log*D* value increases slightly, since the effect of the release of carboxylate anion is counterbalanced by ion pair formation. As the concentration of 1-hexanesulphonate increases more anions are exposed to the solvent and log*D* decreases reaching a low level plateau at a high concentration of 1-hexanesulphonate at which the zwitterionic structure is totally disrupted and there is no interaction between the carboxylate anion and the protonated amine function.

In order to further support our assumption, analogous experiments with comparable results were performed with piroxicam at its isoelectric point (data not shown). The zwitterionic nature of piroxicam has been extensively studied with direct determination of its tautomeric constant Kz.[8] It should be noted that such a direct method could not be applied in the case of compound **1** since there is no difference in the spectra of the zwitterionic species and the neutral form. The correspondence of the effect of 1-hexanesulphonate on both compound **1** and piroxicam is illustrated in Figure 3. After initial increase at low 1-hexanesulphonate concentration, a systematic decrease in log*D* follows in both cases.

In our further experiments we used isolated erythrocytes as a cellular model. Peroxidation of plasma membrane eventually resulting in hemolysis was induced both by the hydrophilic AAPH,[9] which simulates an attack by free peroxyl radicals from the outside aqueous region, and by lipophilic *t*-BuOOH, which generates peroxyl radical intracellularly.[10,11]

At 100 μM concentration, compound **1** gave distinct prolongation of the initial inhibition period (lag phase) in the hemolysis kinetic curve, indicating its antioxidant action characterized by a much higher reaction rate of **1** with peroxyl radicals compared to the rate of chain propagation of cell membrane peroxidation (Fig. 4). In the first case, when red blood cells were treated with hydrophilic AAPH azoinitiator, 100 μM trolox was found to be more protective than equimolar compound **1**. On the other hand, when lipophilic *t*-BuOOH was applied, compound **1** (100 μM) protected red blood cells more efficiently than carnosine, present at 100 times higher concentration. Compound **1** was found to block efficiently the hemolysis even when added to already pre-peroxidized erythrocytes. The results suggest a ready uptake of **1** into the cells followed by direct inhibition of chain propagation inside

the cellular membrane and they are in accord with our previous findings pointing to a rapid passive transport of **1** through the plasmatic membrane of red blood cells.[5] Trolox, carnosine and melatonin were used for comparison as physiologically relevant reference antioxidants. Kang et al.[12] found carnosine efficient in protecting ceruloplasmin against oxidative damage induced by AAPH derived peroxyl radicals, most likely through a mechanism of peroxyl radical-scavenging. Absence of any protective effect of melatonin is in accord with the notion that melatonin is rather ineffective for scavenging less reactive peroxyl radicals.[13]

The result of the in vivo experiment showed efficient inhibitory effect of compound **1** on sorbitol accumulation in erythrocytes. No significant change of blood glucose was recorded in the group of diabetic rats treated with compound **1** in comparison with the untreated group. As we reported before,[14] compound **1** in pharmacologically relevant concentrations did not affect the activity of sorbitol dehydrogenase, the second enzyme of polyol pathway. The only explanation for the observed reduction of sorbitol level in erythrocytes of diabetic animals after i.g. administration of compound **1** appears to be the inhibitory effect of the compound on intracellular aldose reductase. On balance then, we believe that this finding serves as an indirect evidence for penetration of compound **1** into the central compartment after its intra-gastric administration and eventually into red blood cells.

4. Conclusions

In conclusion, the present results offer physico-chemical evidence of the zwitterionic nature of compound **1**. As hypothesized at the beginning of this study, the isoelectric pH lying closely to the physiologically relevant pH 7.4 predisposes this compound for good bioavailability. Indeed, the results obtained in isolated red blood cells indicated good cellular availability of compound **1**. In addition, in diabetic rats, sorbitol accumulation in red blood cells was significantly inhibited by the compound administered intra-gastrically, suggesting its uptake into the central compartment. 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, this however was outside the scope of the present study.

5. Experimental

5.1. Chemicals and instruments

Compound **1**, (2-benzyl-2,3,4,5-tetrahydro-1*H*-pyrido[4,3-*b*]indol-8-yl)-acetic acid, and stobadine (**2**), (−)-*cis*-2,8-dimethyl-2,3,4,4a,5,9b-hexahydro-1*H*-pyrido-[4,3-*b*]indole (Fig. 1) were synthesized at the Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences and were available as hydrochlorides. 2,2′-Azobis(2-amidinopropane) hydrochloride (AAPH) was obtained from FLUKA Chemie GmbH (Steinheim, Germany). *t*-BuOOH and trolox were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Melatonin, carnosine, were obtained from Sigma Chemical Co. (St. Louis, MO, USA). *N*-Octanol (extra pure) and sodium 1-hexanesulphonate were purchased from Lab-Scan Analytical Sciences Ltd, Ireland. Piroxicam was of pharmaceutical grade and was kindly donated by the National Organization of Drugs, Greece. Other chemicals were purchased from local commercial sources and were of analytical grade quality. Plasma glucose was determined by BIO-LA-TEST Glucose GOD 1500 kit (Pliva-Lachema Diagnostica, Brno, Czech Republic). Spectrophotometric analysis was performed using a Hewlett–Packard Diode Array Spectrophotometer 8452A.

5.2. Distribution experiments

Octanol/water partition experiments were performed by the shaking flask method in thermostated vials according to standard procedure[15] using the following experimental conditions. The aqueous phase consisted of phosphate buffer, pH 7.4 in the case of compound **1** and 3.65 in the case of piroxicam. Sodium 1-hexanesulphonate was added at concentrations varying from 8.0×10^{-5} to 8.0×10^{-2} M. The aqueous phase was mutually saturated with *n*-octanol before the experiment. Compounds were dissolved in the aqueous phase at concentration 1.5×10^{-5} M. The phase volume ratio Vaq/Voct was 2:1 in the case of compound 1 and 40/1 in the case of piroxicam. Equilibration was performed for approximately 3 h. Centrifugation followed for 15 min at 700 g. The aqueous phase, before and after equilibration, was analyzed spectrophotometrically at 272 nm and 353 nm, respectively. Distribution coefficients D were calculated according to Eq. 1 and converted to $\log D$:

$$D = \frac{(A_0 - A_1)}{A_1} \times \frac{V_{aq}}{V_{oct}} \quad (1)$$

A_0 and A_1 being absorbance before and after equilibration and V_{aq}, V_{oct} the volumes of aqueous phase and octanol, respectively.

5.3. Animals

Male Wistar rats, 8–9 weeks old, weighing 200–250 g, were used. The animals came from the Breeding Facility of the Institute of Experimental Pharmacology, 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).

5.4. Preparation of packed erythrocytes

The animals in light ether anesthesia were killed by exsanguination by the carotid artery. The blood was collected in 3.8% sodium citrate (1 vol. of sodium citrate: 9 vol. of blood) and centrifuged at 500 × g for 15 min at 4 °C. Plasma and white blood cells were removed by aspiration. The retrieved erythrocytes were washed three times with 6 vol. of ice-cold phosphate buffered saline (PBS, pH 7.4, 1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4 and 150 mM NaCl). The entire procedure was conducted at 2–6 °C. After the last washing, the red blood cells were used for further studies or sorbitol determination.

5.5. Hemolysis measurements

The hemolysis studies were performed in rat erythrocyte suspensions in PBS with the hematocrit of 1.5%. Compound **1**, stobadine, melatonin, carnosine, AAPH and *t*-BuOOH were dissolved in PBS directly. Trolox was dissolved in distilled water and titrated with 5 mM KOH. Further dilutions were made with PBS. The compounds studied were added from stock solutions in PBS to the erythrocyte suspensions to the final concentrations as reported in the result section. Controls received an equivalent volume of PBS alone. Samples were then pre-incubated for 30 min at 37 °C. AAPH or *t*-BuOOH solutions were added to samples to the final concentrations of 30 mM or 250 μM, respectively, and incubation continued at 37 °C up to 5 h. In one series of experiments, the pre-incubation period was omitted and compound **1** was added 30 min after addition of *t*-BuOOH, as indicated in the result section. Aliquots were withdrawn after different time periods. The incubations were terminated by cooling the suspensions in an ice bath followed by centrifugation at 700 × g for 10 min. The degree of

hemolysis was determined by spectrophotometry of the hemoglobin released into the supernatant fraction, as described by Winterbourn.[16] The results were calculated as percentage of hemolysis. Total hemolysis (100%) was obtained by incubation of control erythrocytes in 10 mM hypotonic phosphate buffer, pH 7.4 (1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4) at 37 °C for 1 h.

5.6. Animal study in vivo

Experimental diabetes was induced in rats by triple i.p. doses (30 mg/kg) of streptozotocin (STZ) administered on three consecutive days.[3,17] STZ was dissolved in 0.1 M citrate buffer, pH 4.5. The animals were fasted overnight prior to STZ administration. Control animals received an equal volume of 0.1 M citrate buffer, pH 4.5. Water and food were available immediately after dosing. On day 7 after beginning of the experiment, all animals with plasma glucose level >15 mM were considered diabetic. The diabetic animals were randomly assigned to two groups: untreated diabetic rats kept on a standard diet and diabetic rats treated with compound **1** (25 mg/kg, i.g., twice a day). The treatment continued for four consecutive days. On the fifth day the animals received the morning dose of compound **1** (25 mg/kg, i.g.). Three hours after the last dose, the animals were sacrificed in light ether anesthesia and blood was collected. 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 °C and relative humidity of 40–70%.

5.7. Sorbitol determination

To determine sorbitol, the pellet of packed erythrocytes was washed three times with isotonic PBS. Thereafter, ice cold $HClO_4$ (9%, 0.6 ml) was added to an aliquot (0.2 ml) of packed erythrocytes to precipitate proteins. 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). The neutralized supernatant was used for determination of sorbitol concentration by modified enzymatic analysis.[18] 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 2 mM resazurin solution in 25 ml of triethanolamine buffer), SDH (15.025 U/1 ml triethanolamine buffer). Reaction mixtures were incubated for 60 min at room temperature with an opaque cover. The sample fluorescence was determined at excitation 544 nm, emission 590 nm. After the appropriate blanks were subtracted from each sample, nanomole of sorbitol per milliliter of packed RBCs in each sample was determined by comparison with a linear regression of sorbitol standards.

Acknowledgments

To the memory of Dr. Vladimir Snirc PhD, who suddenly passed away in the middle of this study. This work was supported by VEGA Grant No. 2/0067/11 and COST-CM1001.

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