A Novel Carboxymethylated Mercaptotriazinoindole Inhibitor of Aldose Reductase Interferes With the Polyol Pathway in Streptozotocin-Induced Diabetic Rats

M. SOLTESOVA PRNOVA[1], J. BALLEKOVA[1], A. GAJDOSIKOVA[1], A. GAJDOSIK[1], M. STEFEK[1]

[1]Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences, Bratislava, Slovak Republic

Summary

The aim of the present work was to study the effect of 3-mercapto-5H-1,2,4-triazino[5,6-b]indole-5-acetic acid (CMTI), an efficient aldose reductase inhibitor, on sorbitol accumulation in selected organs of streptozotocin-induced diabetic rats *in vivo*. In addition, the effect of CMTI on aldose reductase back reaction and on sorbitol dehydrogenase was determined. The model of experimental diabetes in male Wistar rats induced by streptozotocin was used. Experimental diabetes was induced by triple *intraperitoneal* doses of streptozotocin on three consecutive days. In diabetic rats, significant elevation of sorbitol concentration in the sciatic nerve and eye lenses was recorded. CMTI administered *intragastrically* (50 mg/kg/day) for five consecutive days significantly inhibited sorbitol accumulation in the sciatic nerve, yet it was without effect in eye lenses of diabetic animals. For aldose reductase back reaction, the substrate affinity of glycerol to aldose reductase was one order lower than that of glyceraldehyde in forward reaction. In addition, the back reaction was much slower, characterized by V_{max} value of about 30 times lower than that of the forward reaction. Inhibition of aldose reductase by CMTI was characterized by closely related IC_{50} values in submicromolar range for both forward and back reactions. No significant inhibition of the second enzyme of the polyol pathway, sorbitol dehydrogenase, by 100 µM CMTI was recorded (I=0.9±2.7 %, n=3). To conclude, the presented results showed the ability of CMTI to affect the polyol pathway in diabetic rats *in vivo* and represent thus a further step in a complex preclinical evaluation of CMTI as a potential agent for treatment of chronic diabetic complications.

Corresponding author

M. Stefek, Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences, Dubravska cesta 9, 841 04 Bratislava, Slovakia. E-mail: Milan.Stefek@savba.sk

Substantial evidence suggests a key role for the polyol pathway in the etiology of diabetic complications (Hotta 1995, Del Corso *et al.* 2008, Oates 2008, Alexiou *et al.* 2009, Obrosova *et al.* 2010, Tang *et al.* 2012). Under diabetic 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 a fraction of the excessive glucose to the osmotically active sorbitol in an NADPH-dependent way. Sorbitol dehydrogenase, the second enzyme of the polyol pathway, partially consumes sorbitol, yet under hyperglycemia sorbitol accumulates intracellularly, with ensuing cell disruption. Aldose reductase thus represents a promising therapeutic target in prevention of diabetic complications (Yabe-Nishimura 1998, Costantino *et al.* 2000, Miyamoto 2002, Obrosova and Kador 2011, Chatzopoulou *et al.* 2012, Maccari and Ottana 2015).

Recently novel carboxymethylated mercaptotriazinoindoles have been identified as efficient inhibitors of aldose reductase (Stefek *et al.* 2015). Of them, 3-mercapto-5H-1,2,4-triazino[5,6-b]indole-5-acetic

acid (CMTI) was characterized by a corresponding IC_{50} value in a submicromolar region. A reasonable degree of selectivity with respect to the closely related aldehyde reductase and AKR1B10 oxidoreductase (Stefek *et al.* 2015) was recorded.

Experimental diabetes in male Wistar rats (8-9 weeks, 230-250 g) was induced by triple *intraperitoneal* (*i.p.*) doses of streptozotocin (STZ, 30 mg/kg) on three consecutive days as described previously (Juskova-Karasova *et al.* 2014). 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 of six rats each. CMTI was dissolved in physiological solution. The treatment was initiated on the fifth day of the experiment and continued for the next four days by *intragastric* (*i.g.*) administration *via* gavage. The dosage schedule was as follows: 12.5 or 25 mg/kg dose of CMTI was applied twice daily (8:30 and 15:30) for four days; the last dose was applied on the fifth day in the morning, three hours before killing of the animals. The animals were killed by cervical dislocation in ether anesthesia followed by exsanguination of the carotid artery. Eye lenses and sciatic nerves were frozen and kept in deep-freeze for sorbitol assay. 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). The CMTI substance was provided by Matrix Scientific (Columbia, SC, USA) in 99.5 % purity, determined by the LC-MS technique (Stefek *et al.* 2015).

Diabetic rats with *ad libitum* access to food overnight were in the morning characterized by the levels of plasma glucose ranging from 24.6 to 27.7 mM. Morning plasma levels of glucose of STZ-treated rats fasting overnight were not significantly increased in comparison with control animals. Treatment of the animals with CMTI did not alter plasma glucose and body weight significantly either in control or diabetic animals.

In untreated diabetic rats, significant elevation of sorbitol concentration in the sciatic nerve and eye lenses was recorded. Sorbitol was determined by enzymatic analysis according to Mylari *et al.* (2003) modified by Juskova-Karasova *et al.* (2014). CMTI administered *intragastrically* (*i.g.*, 50 mg/kg/day) for five consecutive days significantly inhibited sorbitol accumulation in the

sciatic nerve, yet it was without effect in eye lenses of diabetic animals (Fig. 1 a,b). No significant inhibition of sorbitol accumulation either in the sciatic nerve or the eye lens was recorded in diabetic animals after administration of CMTI in the dosage regimen of 25 mg/kg/day. In control rats, administration of CMTI (50 mg/kg/day, *i.g.*) for five consecutive days did not affect significantly sorbitol levels in the sciatic nerve and the eye lens.
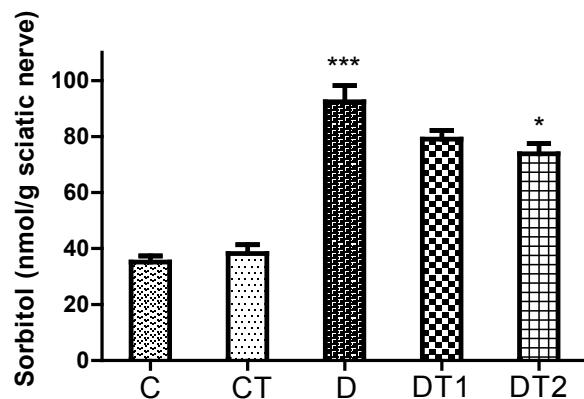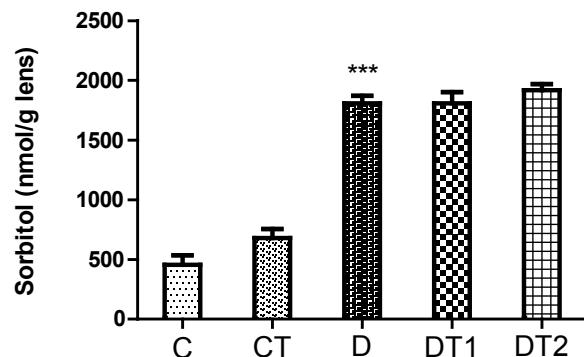

Fig. 1. Accumulation of sorbitol in **(a)** sciatic nerves and **(b)** eye lenses of rats under conditions of STZ-induced experimental diabetes. Effect of CMTI administered *intragastrically* for five consecutive days according to the following dosage schedule: Group DT1: 12.5 mg/kg twice daily (8:30 and 15:30) for the first four days and 12.5 mg/kg on the fifth day, three hours before killing of the animals. Group DT2: 25 mg/kg twice daily in the same schedule as group DT1. Group D, untreated diabetic rats; group C, untreated control animals; group CT, control rats treated by CMTI 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 of animals. Results are mean values ± SD with 6 animals in a group. * $p \leq 0.05$ for DT2 vs. D, *** $p \leq 0.001$ for D vs. C

This result points to a ready uptake of CMTI after its *i.g.* administration into the central compartment, its supply to the peripheral nerves and inhibition of

aldose-reductase-mediated sorbitol accumulation. Yet at the dosage regimen used, the sorbitol levels in nerves were not normalized to control values. Optimization of CMTI dosage regimen may improve the therapeutic outcome.

Table 1. Parameters of Michaelis-Menten kinetics for aldose reductase forward and back reaction with glyceraldehyde and glycerol as substrates.[a]

	K_m (mM)	V_{max} 10,000 x OD/s/mg
Forward reaction	0.26 ± 0.02	2.45 ± 0.38
Back reaction	2.50 ± 0.75	0.081 ± 0.029

[a] Activity of the forward reaction was assayed spectrophotometrically as described before (Stefek *et al.* 2015) by determining NADPH consumption at 340 nm and was expressed as decrease of the optical density (O.D.)/s/mg protein. The reaction mixture contained 4.67 mM D,L-glyceraldehyde as a substrate, 0.11 mM NADPH, 0.067 M phosphate buffer, pH 6.2 and 0.05 ml of the enzyme preparation in a total volume of 1.5 ml. 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. Activity of the back reaction was assayed under the same conditions as described for the forward reaction by using glycerol as a substrate and $NADP^+$ as a cofactor. A time dependent increase of absorbance at 340 nm was recorded for 4 min. Results are mean values ± SD from at least three experiments.

The treatment of diabetic animals with CMTI had no effect on sorbitol levels in the eye lens. The absence of any effect of CMTI on sorbitol accumulation in lenses under *in vivo* conditions may be explained by the limited availability of the drug at this site. The lens, an organ without any blood vessels, is supplied through the anterior eye chamber. The same absence of lens response to therapy by an aldose reductase inhibitor was reported by Matsumoto *et al.* (2008) for ranirestat and Mylari *et al.* (1991) 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 a steady state concentration of zopolrestat in the sciatic nerve. Obviously, a longer period is needed for eye lenses. Assessment of the overall disposition and pharmacokinetics of CMTI may provide a definite answer, which was however beyond the scope of the present study.

As we reported recently (Stefek *et al.* 2015), in isolated rat eye lenses incubated in the presence of high glucose, CMTI inhibited sorbitol accumulation in a concentration-dependent manner. For CMTI concentrations ranging from 0.1 to 100 µM, the degree of sorbitol inhibition spanned from about 14 % to 50 %. This finding obviously points to accessibility of isolated lenses to the inhibitor.

Under *in vivo* conditions, in addition to pharmacokinetic behavior, a potential effect of CMTI on aldose reductase back reaction and/or on sorbitol dehydrogenase, the second enzyme of the polyol pathway, should be taken into account. We studied Michaelis-Menten kinetics of aldose reductase in forward and back reactions using glyceraldehyde and glycerol, respectively, as substrates. The kinetic parameters, summarized in Table 1, indicated that the substrate affinity of glycerol to aldose reductase in back reaction is by one order lower than that of glyceraldehyde in forward reaction. In addition, the back reaction is much slower, characterized by V_{max} value about 30 times lower than that of the forward reaction.

For aldose reductase back reaction with 7.5 mM glycerol substrate, CMTI concentration dependence gave $IC_{50}=0.070\pm0.013$ µM, which is closely related to the value of $IC_{50}=0.097\pm0.019$ µM for the forward reaction (Stefek *et al.* 2015). This finding indicates that the affinity of CMTI to the ternary complex „ALR2-NADPH-glyceraldehyde" in forward reaction is similar to that of the ternary complex „ALR2-NADP+-glycerol" in back reaction. Sluggishness of the back reaction resulted into poor reproducibility of experimental kinetic data, especially for lower substrate concentration, which prevented us to determine the type of inhibition of the back reaction by CMTI. The above findings indicated that the back reaction contributed to sorbitol consumption only marginally, thus even its effective inhibition would not affect the sorbitol level profoundly.

Inhibition of sorbitol dehydrogenase, the second enzyme of the polyol pathway, concurrently with inhibition of aldose reductase, should, at least partially,

eliminate the sorbitol-decreasing effect of aldose reductase inhibition. Obviously this is not the case, since no significant inhibition of sorbitol dehydrogenase activity by 100 μM CMTI was recorded (I=0.9±2.7 %, n=3). Activity of sorbitol dehydrogenase was assayed spectrophotometrically by following NAD^+ reduction at 340 nm. The reaction mixture contained 2.5 mM sorbitol as a substrate, 0.12 mM NAD^+, 10 mM phosphate buffer, pH 7.4 and 0.75 U sorbitol dehydrogenase (S-3764, Sigma-Aldrich, St Luis, Mo, USA) in the total volume of 1.5 ml. The enzyme reaction was initiated by addition of sorbitol and was monitored for 4 min after an initial period of 1 min at 37 °C.

To conclude, the presented results showed the ability of the efficient aldose reductase inhibitor CMTI to affect the polyol pathway in diabetic rats *in vivo* and thus represent a further step in a complex preclinical evaluation of CMTI as a potential agent for treatment of chronic diabetic complications.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

References

ALEXIOU P, PEGKLIDOU K, CHATZOPOULOU M, NICOLAOU I, DEMOPOULOS VJ: Aldose reductase enzyme and its implication to major health problems of the 21(st) century. *Curr Med Chem* **16**: 734-752, 2009.

CHATZOPOULOU M, ALEXIOU P, KOTSAMPASAKOU E, DEMOPOULOS VJ: Novel aldose reductase inhibitors: a patent survey (2006-present). *Expert Opin Ther Pat* **22**: 1303-1323, 2012.

COSTANTINO L, RASTELLI G, GAMBERINI MC, BARLOCCO D: Pharmacological approaches to the treatment of diabetic complications. *Expert Opin Ther Pat* **10**: 1245-1262, 2000.

DEL CORSO A, CAPPIELLO M, MURA U: From a dull enzyme to something else: facts and perspectives regarding aldose reductase. *Curr Med Chem* **15**: 1452-1461, 2008.

HOTTA N: New approaches for treatment in diabetes: aldose reductase inhibitors. *Biomed Pharmacother* **49**: 232-243, 1995.

JUSKOVA-KARASOVA M, SOLTESOVA-PRNOVA M, STEFEK M: A novel zwitterionic inhibitor of aldose reductase interferes with polyol pathway in *ex vivo* and *in vivo* models of diabetic complications. *Pharmazie* **69**: 747-751, 2014.

MACCARI R, OTTANA R: Targeting aldose reductase for the treatment of diabetes complications and inflammatory diseases: new insights and future directions. *J Med Chem* **58**: 2047-2067, 2015.

MATSUMOTO T, ONO Y, KURONO M, KUROMIYA A, NAKAMURA K, BRIL V: Ranirestat (AS-3201), a potent aldose reductase inhibitor, reduces sorbitol levels and improves motor nerve conduction velocity in streptozotocin-diabetic rats. *J Pharmacol Sci* **107**: 231-237, 2008.

MIYAMOTO S: Recent advances in aldose reductase inhibitors: potential agents for the treatment of diabetic complications. *Expert Opin Ther Pat* **12**: 621-631, 2002.

MYLARI BL, LARSON ER, BEYER TA, ZEMBROWSKI WJ, ALDINGER CE, DEE MF, SIEGEL TW, SINGLETON DH: Novel, potent aldose reductase inhibitors: 3,4-dihydro-4-oxo-3-[[5-(trifluoromethyl)-2-benzothiazolyl] methyl]-1-phthalazineacetic acid (zopolrestat) and congeners. *J Med Chem* **34**: 108-122, 1991.

MYLARI BL, ARMENTO SJ, BEEBE DA, CONN EL, COUTCHER JB, DINA MS, O'GORMAN MT, LINHARES MC, MARTIN WH, OATES PJ, TESS DA, WITHBROE GJ, ZEMBROWSKI WJ: A highly selective, non-hydantoin, non-carboxylic acid inhibitor of aldose reductase with potent oral activity in diabetic rat models: 6-(5-Chloro-3-methylbenzofuran- 2-sulfonyl)-2-H-pyridazin-3-one. *J Med Chem* **46**: 2283-2286, 2003.

OATES PJ: Aldose reductase, still a compelling target for diabetic neuropathy. *Curr Drug Targets* **9**: 14-36, 2008.

OBROSOVA IG, KADOR PF: Aldose reductase/polyol inhibitors for diabetic retinopathy. *Curr Pharm Biotechnol* **12**: 373-385, 2011.

OBROSOVA IG, CHUNG SS, KADOR PF: Diabetic cataracts: mechanisms and management. *Diabetes Metab Res Rev* **26**: 172-180, 2010.

STEFEK M, SOLTESOVA-PRNOVA M, MAJEKOVA M, RECHLIN C, HEINE A, KLEBE G: Identification of novel aldose reductase inhibitors based on carboxymethylated mercaptotriazinoindole scaffold. *J Med Chem* **58**: 2649-2657, 2015.

TANG WH, MARTIN KA, HWA J: Aldose reductase, oxidative stress, and diabetic mellitus. *Front Pharmacol* **3**:87, 2012.

YABE-NISHIMURA C: Aldose reductase in glucose toxicity: a potential target for the prevention of diabetic complications. *Pharmacol Rev* **50**: 21-33, 1998.