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Research Article

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In vitro and *in vivo* evaluation of antioxidant activity of ARIs: Benzothiadiazine and pyridothiadiazine derivatives

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ABSTRACT

Increased oxidant stress plays an important role in the chronic complications of diabetes. This study aimed to examine if our previously synthesized aldose reductase inhibitors(ARIs), benzothiadiazine 1,1-dioxide derivatives and the structural analogues pyrido[2,3-e]-[1,2,4]-thiadiazine 1,1-dioxide derivatives, have the potent as antioxidants. Several assays have been used to estimate antioxidant capacities including DPPH, MDA and Sorbitol assays. Effect of concentration, PH and sunlight on DPPH scavenging activity were determined, and results showed Benzothiadiazine 1,1-dioxide derivatives(compound 7a, 7b, 7c) at both concentration of 10^{-5}M and10^{-4}M had notable scavenging activity against DPPH, significantly higher than that of pyrido[2,3-e]-[1,2,4]-thiadiazine 1,1-dioxide derivatives(compound 7'a,7'b,7'c). The PH demonstrated no effect on the activity and sunlight was essential for the activity. The brain MDA levels (MDA, an index of lipid peroxidation) for compounds 7a, 7b, 7c significantly decreased comparing with the negative control, the brain homogenate sample induced by oxidant system Fe(Ⅲ) ascorbic acid. Oral administration of compound7'a,7'b and7'c to groups of STZ-induced rats for 5 days decreased the level of sciatic nerve sorbitol, The inhibition of sorbitol level in the range of 24%-35% for7'a,7'b and7'c, showing some difference with the in vitro study.

Key words: Oxidative stress; DPPH free radical; Lipid peroxidation; MDA (malondialdehyde)level; Sorbitol level

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INTRODUCTION

Diabetes mellitus (DM) is constantly growing chronic disease. 220 million people are suffering from DM worldwide and expected to increase in cases to 400 million by 2030[1].

The serious pathologies of diabetic complications such as retinopathy, nephropathy, neuropathy or cataract[2] are associated with DM. Hyperglycemia plays an essential role in the advancement and the development of these complications. It is reported that the progression of diabetic complications can be delayed the onset or slow down by controlling the blood glucose level. Even though, a large number of antidiabetic drugs are available but to keep glycemia under control is difficult and therefore, it resulted in long-term damage. Among the numerous biological mechanisms which are closely related to hyperglycemia are the polyol pathway[3，4，5], non-enzymatic glycation of proteins, the activation of protein kinase C (PKC) isoform and glucose auto-oxidation.

Aldose reductase, has been identified as the first enzyme of the polyol pathway that catalyzed the NADPH-dependent reduction of glucose to sorbitol, which is then oxidized to fructose by sorbitol dehydrogenase [3]. Sorbitol is formed more rapidly than it is converted to fructose, and its polarity hinders its penetration through membranes. Subsequent prevents its removal from tissues by diffusion, thus elevating intracellular concentration of sorbitol. In addition, the activated polyol pathway causes a substantial imbalance in the free cytosolic coenzyme ratios NADPH/$NADP^+$ and NAD^+/NADH. This alternation induces a state of pseudohypoxia, which contributes to the onset of hyperglycemic oxidative stress through the accumulation of reactive oxygen species (ROS). The

generated excessive ROS (Reactive Oxygen Species) can damage the balance of the oxidation/antioxidant system, causing oxidative stress both intracellular and extracellular. ROS also damage the structure and function of biological macromolecules, such as DNA, protein, carbohydrate and lipid, leading to diabetes and its complications[6-8]. Diabetes is a state of increased oxidant stress and there is accumulating evidence that oxidative damage may play a role in the pathogenesis of diabetes mellitus and its complications[9，10].

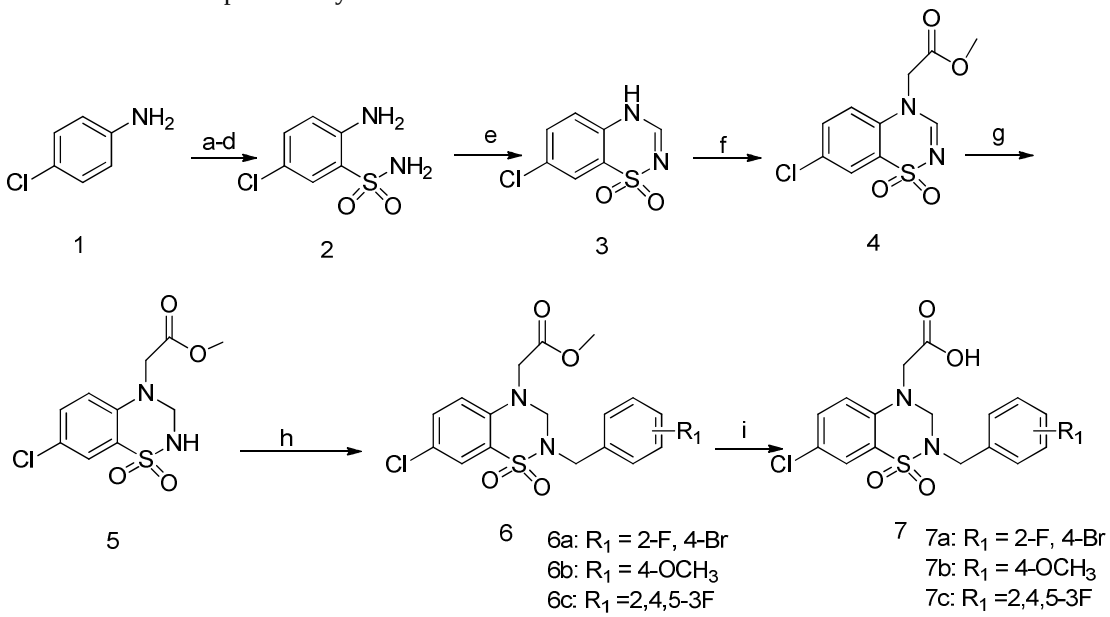
Therefore, synthesis of aldose reductase inhibitors (ARIs) possessing antioxidant activity seems to be desirable for the amelioration of diabetes and related complications.

In our previous research, a series of Benzothiadiazine and Pyridothiadiazine Derivatives have been synthesized and the aldose reductase inhibitory activity of the compounds has been determined. in present work, we selected the best ARIs among them and assess the antidiabetic activity in vitro and in vivo.

EXPERIEMENTAL SECTION

Chemistry

All benzothiadiazine 1,1-dioxide derivatives compounds were obtained by synthesis starting from substituted aniline(p-Chloroaniline), as showed in scheme 1[11]. The synthetic procedures and biological activity data of compound 7b has not been published yet.



Reagents and conditions: (a) $ClSO_2NCO$, CH_3NO_2, -40 ℃; (b) $AlCl_3$, 110 ℃; (c) 50% H_2SO4, 140 ℃; (d) aq NaOH; (e) $HC(OEt)_3$, reflux; (f) $BrCH_2COOCH_3$, K_2CO_3, CH_3CN, 70 ℃; (g) $NaBH_4$, 2-propanol; (h) Bn-Br, K_2CO_3, 70 ℃; (i) 1,4-dioxane, NaOH.

Scheme 1 Synthesis of benzothiadiazine 1,1-dioxide derivatives

All Pyrido[2,3-e]-[1,2,4]-Thiadiazine 1,1-Dioxide Derivatives were obtained by synthesis starting from substituted 2-Amino-5-chloropyridine[12].

Biological activity

Materials and methods

Vista rats (male, Grade II, weighing200-300g) were purchased from our Experimental Animal Center, Beijing Institute of Technology. Compounds 7a-7c and 7'a-7'c were synthesized in our lab. Trolox and DPPH were purchased from Acros and Sigma respectively. MDA Kit was purchased from Institute of Biological Engineering of Nanjing Jiancheng, Nanjing, China. Other reagents were analytical grade.

In vitro antioxidant activity

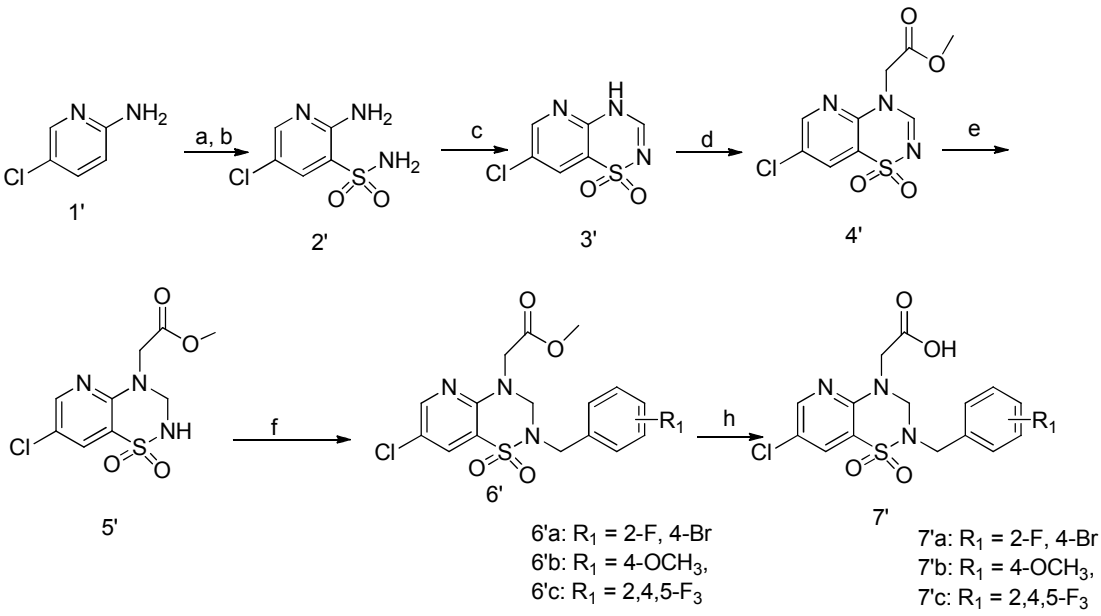
1. Scavenging activity against DPPH· radical

The stable free radical DPPH was dissolved in methnol to give a 2.5×10^{-2} mg/ mL stock solution; then stock solution was diluted to final concentrations of 2.5-25μg/mL. 0.1mL compound solutions in methnol at 10 μM or 100μM were added to 1ml of the methnol DPPH solution, and were diluted to 3ml with methnol. The mixture was shaken vigorously and allowed to stand at room temperature for 120 min. The absorbance was measured at 517 nm in a UV-Vis spectrophotometer; The lower absorbance of the reaction mixture indicates higher free radicals scavenging

activity. Methanol was used as the solvent and Trolox as the standard. The radical scavenging activity was calculated using the following equation:

Scavenging effect % =[(A_0 -A_1) /A_0]×100%

Where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of the samples or standards.



Reagents and conditions: (a) $ClSO_3H$, reflux; (b) NH_3, THF; (c) $HC(OCH_2CH_3)_3$, reflux; (d) $BrCH_2COOCH_3$, K_2CO_3, CH_3CN, 70 ℃; (e) $NaBH_4$, 1,4-dioxane; (f) Bn-Br, K_2CO_3, 70 ℃; (h) 1,4-dioxane, aqueous NaOH, then aqueous HCl.

Scheme 2 Synthesis of Pyridothiadiazine 1,1-Dioxide Derivatives

1.1 Effect of concentration on the free radical scavenging capacity of the compounds

Fig. 1.1a Antioxidant activity of all the 6 compounds on DPPH at concentration of 10μM

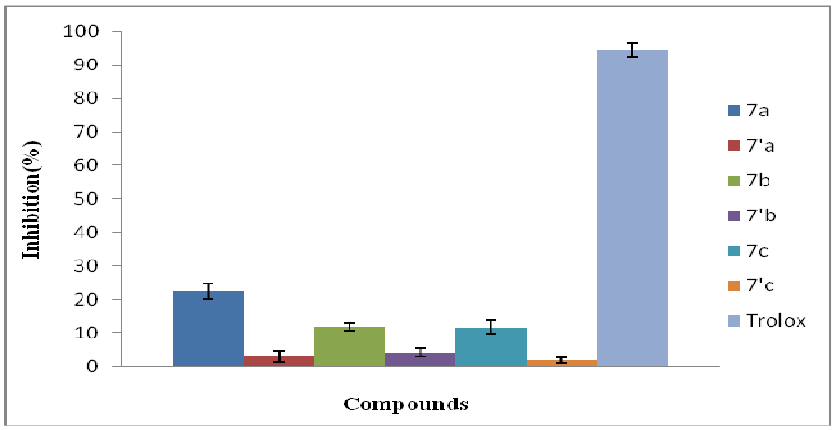
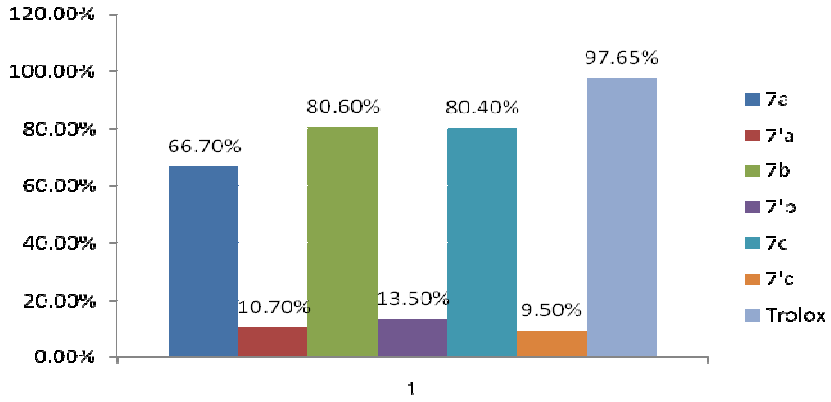


Fig. 1.1b Antioxidant activity of all the 6 compounds on DPPH at concentration of 100μM



As shown in Fig. 1a, all compounds at 10μM have low scavenging rate, with the highest 22%, but the overall trend is compound 7a, 7b and 7c having higher scavenging rate than their structural analogues compound 7'a, 7'b and 7'c. While at the concentration of 100μM(See Fig. 1b), compounds 7a, 7b and 7c showed sound antioxidant activity of 66.7%, 80.6% and 80.4%, respectively, significantly stronger than their structurally correspond compound 7'a(10.7%), 7'b (13.5%), 7'c(9.5%). Trolox are around 95% at both concentration.

1.2 Effect of PH on the free radical scavenging capacity of compound 7a and 7'a

Compound 7a and 7'a were only taken for this test.

Fig.1.2a Effect of PH on the free radical scavenging capacity of compound 7a

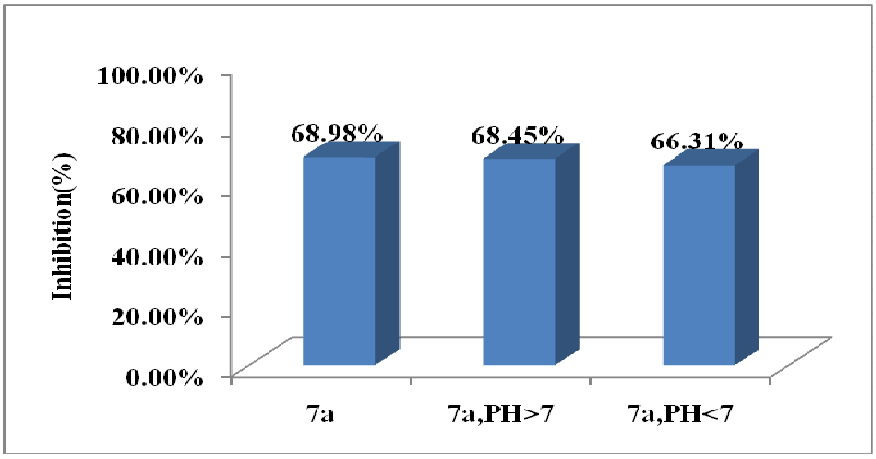
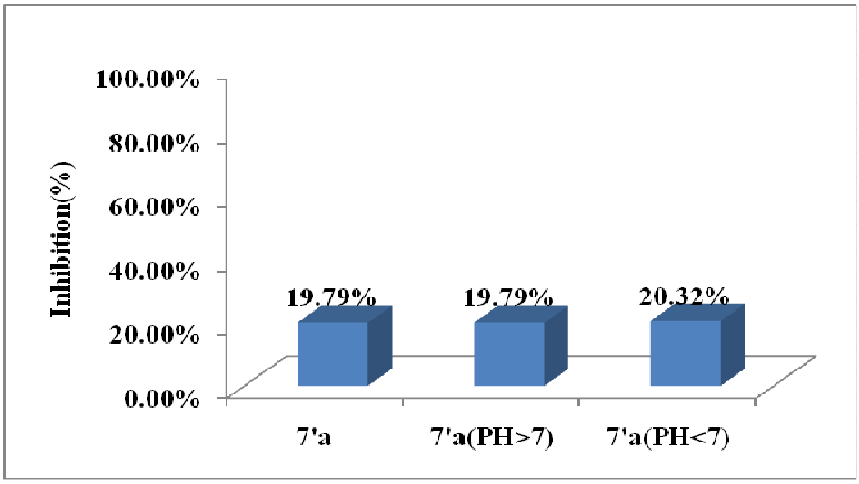


Fig.1.2b Effect of PH on the free radical scavenging capacity of compound 7'a



1.3 Effect of sunlight on the free radical scavenging capacity of compound 7a

Compound 7a was only taken for this test.

Table 1.Effect of sunlight on the free radical scavenging capacity of compound 7a

Condition	Under sunlight	Sunlight proof	Blank
OD value	0.048	0.185	0.191
	0.052	0.194	0.193
	0.059	0.201	0.193
Average inhibition (%)	72.4	0	--

2 Measurement of MDA level in rat brain homogenate

Male Wistar rats (200±40g body wt) were anesthetized and perfused through the heart with 0.9% NaCl(4℃). The brain was removed and frozen in -80℃ immediately.

Freshly isolated rat brain was homogenized in ice-cold 0.9%NaCl. $FeCl_3$ (20 μM) and 100 μM ascorbic acid (final concentrations) were added to rat brain homogenate, in the absence or presence of the test compounds or reference scavenger trolox, and were diluted to 0.5ml with distilled water or methanol. Samples were incubated for 30 min at 37 °C in a water bath under slight stirring, then the incubated brain homogenates were mixed with the solutions in the MDA kit and all the subsequent steps were according to the manufacturer's instructions. All of the experiments were performed in triplicate.

MDA levels in the brain homogenates were determined at 532nm using UV-Vis spectrophotometer. Protein concentrations were determined using Kit. The data were analyzed using a one-way analysis of variance (ANOVA)

Table 2 MDA level treated with different compounds

Compound	MDA (nmol/mgpro)	MDA (Inhibition%)
7a	0.202±0.049	88.4%
7'a	1.065±0.035	10.8%
7b	0.195±0.009	89%
7'b	1.079±0.023	9.5%
7c	0.206±0.031	88%
7'c	1.078±0.068	9.6%
Trolox	0.076±0.024	99.7%
Blank	0.073±0.036	--
Control	1.185±0.077	--

MDA level in rat brain homogenates are illustrated in Fig. 3. The MDA level was 0.073 nmol/mgpro for the blank, 0.076 nmol/mgpro for Trolox and 1.185 nmol/mgpro for control, 0.202±0.049 nmol/mgpro 0.195±0.009 nmol/mgpro, 0.206 ±0.031nmol/mgpro for compound 7a-7c, respectively. 1.065±0.035 nmol/mgpro, 1.079±0.023 nmol/mgpro, 1.078±0.068nmol/mgpro for compound 7'a-7'c, respectively., The brain MDA levels significantly decrease for compound 7a-7c compared with the negative control ($P<0.01$). And the MDA level for compound 7'a-7'c show no significant difference with the negative control. The MDA levels for all compounds, including the positive control Trolox, were slightly higher than that of the blank.

3. In vivo antioxidant activity

Animal model and experimental design

The Wistar rats weighing 200±20g were housed in an air-conditional room with constant humidity and a 12/12-h light/dark cycle. All the rats were fed standard laboratory chow and tap water. After the animals had been kept 5 days for adaptation to the laboratory environment, the rats were fasted for 12h and were intraperitoneally injected with STZ (55mg/kg body weight) to induce diabetes(except the control group). After 24h of STZ application, the body weights and blood glucose were determined. The animals with blood glucose exceeding 16.7mmol//L were accepted as diabetic. Then the rats were divided into groups according to blood glucose and body weights. The groups were as follows: group 1(control, n=6), group 2(diabetes, n=8), group 3-8(diabetes + compound, n=8). Rats in all the groups were fed the normal laboratory diet and tap water. Rats in group 3-8, in addition to normal diet and water, were orally administered the compound vehicle for 5 days. In the fifth day, six hours after the final dose was

administered, the rats were sacrificed and sciatic nerves were removed, weighed and stored at -80℃ for sorbitol analysis.

Determination of sorbitol content in rat Sciatic nerve

The STZ-induced diabetic rat model firstly were established by an intraperitoneal injection of STZ (55mg/kg), then the diabetic rats were allocated into control group, model group (diabetic group) and test group(diabetic rats fed with compound groups)(n = 6-8). The rats in those test groups were administered the compound vehicle of 0.5% sodium carboxymethylcellulose, the control group and the model group received vehicle during this period.

Sciatic nerve sorbitol levels are measured by a standard enzymatic method that uses sorbitol dehydrogenase. Tissues are homogenized in ice-cold 1.5ml 6% perchloric acid using a tissue homogenizer and centrifuged at 3000 rpm for 15 min at 4℃.The supernatant is transferred to a tube, then neutralized by 3M K_2CO_3. This mixture then was centrifuged at 1200 rpm for 10min at 4℃, and supernatants were kept in tubes for use. A reaction mixture combining buffer, NAD^+, and distilled water is added to each supernatant. Sorbitol dehydrogenase is added to initiate the reaction. Tubes are incubated at 37℃ for 45min. The absorbance was measured at 460 nm using a Fluorescence Spectrophotometer. Sciatic nerve sorbitol content is calculated as μmol of sorbitol/mg of wet tissue weight by comparing to a sorbitol standard curve.

Table 3 Effect of compounds on the level of sorbitol in rat sciatic nerve

Group	Sorbitol level (μmol/g)	Inhibition of sorbitol level（%）
Normal	0.0943±0.0245	--
Diabetic	0.2791±0.0700	--
Diabetic+7’a	0.2356±0.0222	24.06%
Diabetic+7’b	0.2402±0.0506	21.02%
Diabetic+7’c	0.2145±0.0641	35.01%
Diabetic+7a	0.1792±0.0111	54.07%

As shown in Table 3, all diabetic groups showed a significant increase in sorbitol level compared to control group ($P<0.01$); while all diabetic groups showed inhibitory effect on sorbitol level to some extent compared to model group. The inhibition of sorbitol level in the range of 24%-35% for7’a,7’b and7’c, showing some difference with the in vitro study.

DISCUSSION

According to the DPPH free radical scavenging test results, the following conclusions can be drawn. Derivatives containing benzene ring are good scavenger, while the Pyridine ring-containing derivatives had slightly DPPH free radical scavenging activity. For better understand the mechanism leading to the activity difference. Simple tests were performed. Firstly, we kept the reaction solution of compound 7a in dark to avoid the sunlight, 120min later, the solution color showed no difference with the blank (see table 1.), which indicated that the reaction is the sunlight induced free radical reaction. To prove our hypothesis that if the H atom of the acetic group plays an important role in the significant activity difference between the compounds. We selected compound 7a and 7’a for test. The compared samples were treated by NaOH or HCl solution, then the DPPH scavenging tests were performed. The result showed the scavenging rates of compound 7a and 7’a are similar with that no treatment, meaning H atom of acetic group didn’t cause the difference of the anti-oxidant activity. More study is needed to obtain the overall picture of the mechanism.

It has been hypothesized that one of the principal causes of diabetes-induced injury is the formation of lipid peroxides by free radicals. Thus, the antioxidant activity or the inhibition of generation of free radicals is important in the protection against diabetes and its complications.

The level of lipid peroxide (MDA) is a measure of membrane damage and alterations in the structure and function of cellular membranes. In the present study, the elevation of lipid peroxidation in the brain of diabetic rats was observed. The increase in MDA levels in the control samples, compared to MDA level in the blank sample, suggests an enhanced lipid peroxidation due to being induced by $FeCl_3$ and Vitamin C. While the significant decreases in MDA levels in the test samples, compared to MDA levels in the control samples showed that those compounds significantly reversed these changes.

The result of the in vivo experiment showed sound inhibitory effect of compounds on sorbitol accumulation in sciatic nerve. These compounds 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 sciatic nerve of diabetic animals after administration of compounds appears to be the inhibitory effect of the compounds on aldose reductase or on the excessive ROS generated in the polyol pathway, or both.

The reasons for the in vivo results showing some difference with the in vitro study by using DPPH and rat brain homogenate is possible attributed to absorption, distribution, metabolism and excretion properties, Further biological study on those compounds are needed.

In summary, benzothiadiazine 1,1-dioxide derivatives and Pyrido[2,3-e]-[1,2,4]-Thiadiazine 1,1-Dioxide Derivatives, 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, which are further proved by in vivo study on the sorbitol level in sciatic nerve. This findings prompted us to screen and evaluate of the series of derivatives, moreover, it allowed us get the insight into how to modify the present series of compounds.

Acknowledgments
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