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Effect of carboxymethylated pyridoindoles on free radical-induced haemolysis of rat erythrocytes *in vitro*★

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Recently novel carboxymethylated pyridoindoles, analogues of the efficient chain-breaking antioxidant stobadine, have been designed, synthesised and characterised as bifunctional compounds with joint antioxidant/aldose reductase inhibitory activities with the potential of preventing diabetic complications. The critical property for the efficacy of the novel aldose reductase inhibitors *in vivo* is their ability to penetrate into target tissues. In this study, the issue was addressed by measuring the antioxidant activity of compounds 1 [(2-benzyl-2,3,4,5-tetrahydro-1*H*-pyrido[4,3-*b*]indole-8-yl)-acetic acid] and 2 [(±)-2-benzyl-(4a,9*b*)-*cis*-1,2,3,4,4a,9*b*-hexahydro-1*H*-pyrido[4,3-*b*] indole-8-yl acetic acid] in the cellular system of intact erythrocytes exposed to peroxyl radicals generated by thermal degradation of the azoinitiator 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH) *in vitro*. Isolated washed rat erythrocytes were incubated in the presence of the azoinitiator AAPH and the compounds tested for increasing periods of time up to 4 h at 37 °C. The degree of haemolysis was determined by absorbance of the haemoglobin released. The onset of AAPH-induced haemolysis was found to be shifted from the starting zero point by the time interval assigned as a lag period. In the presence of the compounds studied the lag period was prolonged significantly. The free radical-initiated haemolysis was retarded by the compounds studied with decreasing efficiency: stobadine > compound 1 ~ Trolox > compound 2. The results have demonstrated an antioxidant activity of the novel carboxymethylated pyridoindoles developed as potential agents for multitarget pharmacology of diabetic complications.

Keywords: pyridoindoles, antioxidants, aldose reductase inhibitors, erythrocytes, haemolysis, oxidative stress

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INTRODUCTION

Oxidative stress in diabetes may result from various sources, of which the most prevalent ones could be the processes triggered by non-enzymatic glycation (Brownlee, 2005; Monnier *et al.*, 2005) and the polyol pathway (Obrosova, 2005). Irreversible advanced glycation endproducts (AGEs) have been shown to be formed *via* a sequence of glycation and oxidation reactions. Under physiological conditions, glucose, free or bound in the form of the ketoamine Amadori product, undergoes auto-oxidative reactions accompanied by the formation of reactive oxygen species which contribute to the oxidative damage of proteins exposed to hyperglycaemia. Proteins exposed to elevated levels of glucose are extensively fragmented and cross-linked in processes dependent on the presence of oxygen and free transition metal ions. Under hyperglycaemia, the increased flux of glucose through the polyol pathway and consequent depletion of NADPH and NAD^+ may lead to a redox state change and a cascade of interrelated metabolic imbalances (Hamada *et al.*, 1996). The depletion of NADPH cell stores by aldose reductase (ALR2, EC 1.1.1.21), the first enzyme of the polyol pathway, may inhibit the activity of other NADPH-requiring enzymes, including those of the glutathione redox cycle. In turn, the decreased levels of reduced glutathione increase the susceptibility of cells to damage by reactive oxygen species. The hyperglycaemia-induced increase in the NADH-to-NAD^+ ratio is referred to as hyperglycaemic pseudohypoxia and is thought to play a role in diabetic complications. The polyol pathway enhances the intracellular glycation process supplying a reactive glycation agent — fructose. Various studies have documented elevated blood and tissue levels of markers of oxidative stress in diabetic patients and demonstrated the ability of antioxidant supplementation to attenuate complications in diabetic animals. These data provide support for the use of antioxidants, which minimise biological auto-oxidations and their consequences, in prevention of diabetic complications. In addition, under hyperglycaemic conditions, aldose reductase reduces some of the excess glucose to the organic osmolyte sorbitol in an NADPH-dependent manner. The accumulation of sorbitol increases cellular osmolarity leading to a deleterious hyperosmotic swelling (Del Corso *et al.*, 2008).

Since inhibition of both oxidative stress and the polyol pathway is desirable, a bifunctional compound with joint antioxidant/aldose reductase inhibitory (AO/ARI) activities could be multifactorially beneficial. Compounds such as pyridazines (Coudert *et al.*, 1994), benzopyranes (Costantino *et al.*, 1999) and pyridopyrimidines (La Motta *et al.*, 2007) have been synthesised and have been shown to display antioxidant as well as aldose reductase inhibitory activities under *in vitro* conditions.

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Abbreviations: AAPH, 2,2'-azobis(2-amidinopropane) hydrochloride; AGEs, advanced glycation endproducts; ALR2, aldose reductase; AO, antioxidant; ARI, aldose reductase inhibitor; D, distribution ratio; DPPH, 1,1'-diphenyl-2-picrylhydrazyl; PC, L-α-phosphatydilcholine dioleoyl.

Recently novel carboxymethylated pyridoindoles, analogues of the efficient chain-breaking antioxidant stobadine (Fig. 1), have been designed, synthesised and characterised as bifunctional compounds with joint antioxidant/aldose reductase inhibitory activities, with the potential of preventing diabetic complications (Djoubissie *et al.*, 2006;Stefek *et al.*, 2008). Among the novel compounds developed, (2-benzyl-2,3,4,5-tetrahydro-1*H*-pyrido[4,3-*b*]indole-8-yl)-acetic acid (compound **1**) was characterised as an aldose reductase inhibitor efficient in the micromolar region, while its saturated analogue (±)-2-benzyl-(4a,9*b*)-*cis*-1,2,3,4,4a,9*b*-hexahydro-1*H*-pyrido[4,3-*b*] indole-8-yl acetic acid (compound **2**) (Fig. 1) showed only marginal aldose reductase inhibitory activity (Stefek *et al.*, 2008). The critical property for the efficacy of the novel aldose reductase inhibitors *in vivo* is their ability to penetrate into target tissues. Under *in vitro* conditions, compound **1** was found to be avidly taken up by red blood cells, yet it did not significantly affect glucose consumption and lactate production, nor did it affect osmotic fragility of the erythrocytes (Juskova *et al.*, in press).

In the present work we evaluated the efficiency of compounds **1** and **2** in scavenging stable free radicals of 1,1'-diphenyl-2-picrylhydrazyl (DPPH) in ethanolic solution. Further we studied the antioxidant action of the compounds in the cellular system of intact erythrocytes exposed to peroxyl radicals generated by thermal degradation of the water-soluble azoinitiator 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH) *in vitro*. The activities of the compounds tested were compared with the activity of stobadine and a water-soluble derivative of vitamin E, Trolox.

MATERIALS AND METHODS

Chemicals and instruments. Compound **1** (2-benzyl-2,3,4,5-tetrahydro-1*H*-pyrido[4,3-*b*]indole-8-yl)-acetic acid, compound **2** (±)-2-benzyl-(4a,9*b*)-cis-1,2,3,4,4a,9*b*-hexahydro-1*H*-pyrido[4,3-*b*] indole-8-yl acetic acid and stobadine (Fig. 1) were synthesised at the Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences (Stefek *et al.*, 2008) and were available as respective hydrochloride and potassium carboxylate salts. 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH) was obtained from Fluka Chemie GmbH (Steinheim, Germany). Trolox was purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). 1,1'-diphenyl-2-picrylhydrazyl (DPPH) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals were purchased from local commercial sources and were of analytical grade quality. Spectrophotometric analysis was performed using a Hewlett-Packard Diode Array Spectrophotometer 8452A.

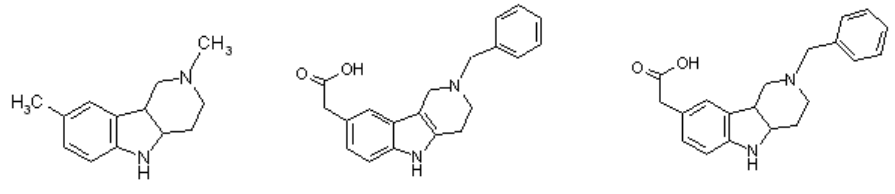
Animals. Male Wistar rats, 8–9 weeks old, weighing 200–250 g, were used. The animals came from the Breeding Facility of the Institute of Experimental Pharmacology (Dobra Voda, Slovak Republic). The study was approved by the Ethics Committee of the Institute and performed in accordance with the Principles of Laboratory Animal Care (NIH publication 83-25, revised 1985) and the Slovak law regulating animal experiments (Decree 289, Part 139, July 9th 2003).

DPPH test. To investigate antiradical activity of the pyridoindole derivatives, ethanolic solution of DPPH (50 μM) was incubated in the presence of the compound tested (50 μM; water solution) at laboratory temperature. The absorbance decrease at λ_{max} 518 nm during the first 1-min interval was taken as a measure of the antiradical activity.

Preparation of packed erythrocytes. The animals in light ether anaesthesia were killed by exsanguination of the carotid artery. The blood was collected in 3.8% sodium citrate (sodium citrate/blood, 1:9 v/v) 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 0 °C to 4 °C. After the last washing, the red blood cells were used for further studies. The erythrocyte suspensions used in the experiments were prepared daily.

Haemolysis measurements. The haemolysis studies were performed in rat erythrocyte suspensions in PBS with the haematocrit of 1.5%. Compounds **1**, **2**, stobadine and AAPH were dissolved in PBS directly. Trolox was dissolved in distilled water and titrated with 5 mM KOH. Further dilutions were made with PBS.

Compounds **1**, **2**, Trolox and stobadine were added from stock solutions in PBS to the erythrocyte suspensions to the final concentrations as reported below. Controls received an equivalent volume of PBS alone. Samples were than incubated for 5 min at 37°C. AAPH solution was added to samples to the final concentration of 30 mM and incubation continued at 37 °C up to 4 h. 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 haemolysis was estimated by spectrophotometry of the haemoglobin released into the supernatant fraction as described by Winterbourn (1990). The results were calculated as percentage of haemolysis. Total haemolysis (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.



Stobadine **1** **2**

Figure 1. Chemical structure of stobadine and its structural analogues compound 1 and 2.

Statistical analysis was performed using Student's *t*-test. Data are presented as means ± S.D. from at least four measurements.

RESULTS AND DISCUSSION

Oxidative stress and the polyol pathway are widely accepted as major agents in the aetiology of diabetic complications. Since inhibition of both processes is desirable, a bifunctional compound with joint antioxidant/aldose reductase inhibitory (AO/ARI) activities could be multifactorially beneficial. In the current study, we tested new carboxymethylated pyridoindoles **1** and **2** structurally based on the antioxidant drug stobadine (Fig. 1). They were previously characterised as compounds with joint antioxidant/aldose reductase inhibitory activities (Stefek *et al.*, 2008). According to *in vitro* studies of **1**, the more efficient inhibitor of aldose reductase, the compound was avidly taken up by the red blood cells, yet it did not significantly affect glucose consumption and lactate production (Juskova *et al.*, in press). The aldose reductase inhibitor **1** was thus found selective in relation to the glycolytic pathway of glucose elimination. At the concentrations studied up to 100 μM, compound **1** did not affect osmotic fragility of the erythrocytes. These findings support the current preclinical development of novel carboxymethylated tetrahydropyridoindoles as promising aldose reductase inhibitors for potential pharmacological prevention and treatment of diabetic complications.

The present study was conducted in order to determine the antiradical and antioxidant activity of compounds **1** and **2** in ethanolic solution of DPPH and in the system of intact erythrocytes.

Stobadine has been postulated as a chain-breaking antioxidant characterised by an ability to scavenge chain-propagating peroxyl radicals (Steenken *et al.*, 1992; Stefek *et al.*, 1992). DPPH, as a weak hydrogen atom abstractor, is considered a good kinetic model for peroxyl ROO$^{\bullet}$ radicals (Blois, 1958). In the homogeneous system of DPPH in ethanol, the antioxidant activity of a compound tested stems from its intrinsic chemical reactivity towards radicals. An absorbance decrease at 518 nm during the first 1-min interval was used as a measure of the antiradical activity of the compounds tested. As shown in Table 1, the carboxymethylated hexahydropyridoindole 2 rapidly reacted with DPPH, its antiradical activity being comparable to that of the parent stobadine but significantly higher than that of the tetrahydropyridoindole 1. This is in accordance with our previously published findings showing higher antiradical activity of stobadine compared with that of its tetrahydro analogue (Rackova *et al.*, 2002).

In membranes, however, the relative reactivity may be different since it is determined also by additional factors, such as location of the antioxidant and the radicals, ruled predominantly by their partition ratios between water and lipophilic compartments. Because of the erythrocyte susceptibility to peroxidation, red blood cells have been used as a model to investigate oxidative damage in biomembranes. Exposure of erythrocytes to free radicals may lead to a number of membrane changes, including lipid peroxidation, reduction in deformability, changes in cell morphology, protein cross-linking and fragmentation, and haemolysis. Lipid peroxidation and protein oxidation are both likely to play a key role in the haemolytic process. At physiological temperature, the decomposition of the water-soluble azo-compound AAPH generates free radicals that could attack the erythrocyte membrane to induce lipid peroxidation and cause haemolysis. Since the rate of free radical generation from AAPH can be easily controlled without the addition of potentially interfering cofactors and transition metals (Landi *et al.*, 1995), the haemolysis induced by AAPH provides a good approach to study the free radical-induced membrane damage. AAPH does not easily penetrate into the cell, thus oxidation of haemoglobin does not play a critical role in the overall process of oxidation itself, and formation of methaemoglobin is only regarded as a secondary associated phenomenon in the AAPH-induced oxidative process. Haemolysis induced by the azo-compound provides the clearest means for studying the oxidative membrane damage induced by free radical attack from outside the membrane.

Rat erythrocytes were treated with 30 mM AAPH with or without the compounds studied at 37 °C up to 4 h. As shown in Fig. 2, rat erythrocytes exposed to AAPH underwent progressive haemolysis, determined by measuring the release of haemoglobin. The degree of haemolysis in the samples without the compounds studied approached 100% after 3 h.

The onset of AAPH-induced haemolysis was shifted from the starting zero point by the time interval assigned as a lag period. In the presence of the compounds studied, the lag period increased significantly (Table 2). Based on the lag phase prolongation, the erythrocytes were found to be protected against the free-radical-induced haemolysis by the compounds studied with a decreasing efficiency as follows: stobadine > compound **2** ~ Trolox > compound **1**.

Our results are in good agreement with those of Racková *et al.* (2002) and Stefek *et al.* (2008) who found higher antioxidant activity of stobadine in comparison with Trolox in the system of unilamellar PC liposomes. With approximately one third of the antiradical activity of Trolox, based on the DPPH test (Table 1), the higher overall antioxidant activity of stobadine recorded in the cellular system of red blood cells (Table 2) was explained by its higher lipophilicity and thus correspondingly higher penetration through the membrane; the distribution ratio (D) of stobadine in the system water/octanol at pH 7.4 was D = 3.72 (Kagan *et al.*, 1993), while that of Trolox was D = 0.33 in the liposomes/water system at pH 7 (Barclay *et al.*, 1995). Analogically, the lower antioxidant activity of compound **2** in the cellular system of isolated erythrocytes compared with that of stobadine can be explained by its lower lipophilicity, as exemplified by its low distribution ratio D = 0.20 in the system

Table 1. Free radical scavenging activity in DPPH test
Effect of carboxymethylated pyridoindoles in comparison with stobadine and the standard Trolox.

Compound	DPPH test (-Δ A/min)
Compound **1**	< 0.030
Compound **2**	0.224 ± 0.002
Stobadine	0.156 ± 0.002
Trolox	0.494 ± 0.009

Ethanolic solution of DPPH radical (50 μM) was incubated in the presence of the compound tested (50 μM). Absorbance decrease at 518 nm during the first 1-min interval was determined. Results are mean values ± S.D. from at least three parallel experiments.

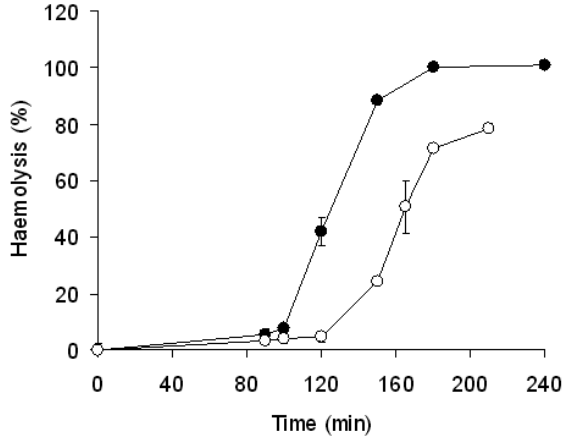


Figure 2. Time course of haemolysis of rat erythrocytes induced by AAPH
Erythrocyte suspensions (1.5%) were incubated with 30 mM AAPH alone (solid circles) or in the presence of 100 μM of compound **1** (empty circles). Results are means ± S.D. values (error bars show S.D.; where not shown, error bars fall within the symbol) from at least 4 experiments.

Table 2. AAPH-induced haemolysis of rat erythrocytes
Effect of substituted pyridoindoles in comparison with the standard Trolox and stobadine.

Compound	Lag period (min)	
Control erythrocytes	88.6 ± 2.2	(6)
Compound **1** (100 μM)	120.6 ± 0.5***	(4)
Trolox (100 μM)	143.5	(2)
Compound **2** (100 μM)	144.4 ± 4.2***	(4)
Stobadine (10 μM)	145.0 ± 1.1***	(3)
Stobadine (100 μM)	> 300	(2)

Erythrocyte suspensions (1.5%) were incubated with 30 mM AAPH alone or in the presence of the compounds tested. The onset of AAPH-induced haemolysis was shifted from the starting zero point by the time interval assigned as a lag period. Results are mean values ± S.D. from the number of experiments in parentheses. ***$P < 0.001$ *vs*. control.

1-octanol/phosphate buffer at pH 7.4 (Stefek *et al.* 2008), which is comparable to the distribution ratio of Trolox. The lower antioxidant activity of the unsaturated tetrahydropyridoindole **1** compared with that of **2** was in good agreement with the results of Racková *et al.* (2002) and Stefek *et al.* (2008), who found a much lower antioxidant activity of didehydrostobadine, an unsaturated tetrahydro-analogue of stobadine, in comparison with that of stobadine.

To conclude, our results demonstrated the ability of new carboxymethylated pyridoindoles, inhibitors of aldose reductase, to protect intact erythrocytes against oxidative damage induced by peroxyl radicals generated in a solvent. The novel compounds are thus potential agents for multi-target pharmacology of diabetic complications.

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