Reactive aldehydes – Second messengers of free radicals in diabetes mellitus

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Bioactive 1,4-dihydroisonicotinic acid derivatives prevent oxidative damage of liver cells

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Abstract

1,4-Dihydroisonicotinic acid derivatives (1,4-DHINA) are compounds closely related to derivatives of 1,4-dihydropyridine, a well-known calcium channel antagonists. 1,4-DHINA we used were derived from a well-known antioxidant Diludin. Although some compounds have neuromodulatory or antimutagenic properties, their activity mechanisms are not well known. This study was performed to obtain data on antioxidant and bioprotective activities of: 2,6-dimethyl-3,5-diethoxycarbonyl-1,4-dihydroisonicotinic acid (Ia); sodium 2-(2,6-dimethyl-3,5-diethoxycarbonyl-1,4-dihydropyridine-4-carboxamido)glutamate (Ib) and sodium 2-(2,6-dimethyl-3,5-diethoxycarbonyl-1,4-dihydropyridine-4-carboxamido)ethane-sulphate (Ic). 1,4-DHINA's activities were studied in comparison to Trolox by: *N*,*N*-Diphenyl-*N*′-picrylhydrazyl ($DPPH^{\cdot}$), deoxyribose degradation, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) radical scavenging and antioxidative capacity assays; copper-induced lipid peroxidation of cultured rat liver cells (malondialdehyde determination by high performance liquid chromatography and 4-hydroxynonenal-protein conjugates by dot-blot); ^{3}H-thymidine incorporation and trypan blue assay for liver cells growth and viability. In all assays used Ia was the most potent antioxidant. Ia was also a potent antioxidant at non-toxic concentrations for liver cell cultures. It completely abolished, while Ic only slightly decreased copper-induced lipid peroxidation of liver cells. Thus, antioxidant capacities are important activity principle of Ia, which was even superior to Trolox in the cell cultures used, while activity principles of Ic and Ib remain yet to be determined.

Keywords: 1,4-Dihydropyridine; Antioxidants; Lipid peroxidation; Malondialdehyde; 4-Hydroxynonenal; Hepatoprotection

1. Introduction

Derivatives of 1,4-dihydropyridine (1,4-DHP) are a well-known class of cardiovascular remedies (nifedipine, amlodipine, etc.) achieving their bioactivity as calcium channel antagonists (Triggle, 2003). Many of the widely studied 1,4-DHPs are substituted at position four in the 1,4-dihydropyridine ring, especially with aryl groups (Krauze et al., 2004). There are only few studies on the antioxidant activity of these 1,4-DHP despite the fact that these compounds are widely used as calcium antagonists (Tirzitis et al., 2001; Wang et al., 2004). However, one 1,4-DHP derivative — Diludin (chemical name 2,6-dimethyl-3,5-diethoxycarbonyl-1,4-dihydropyridine), which is not substituted at position four in the 1,4-dihydropyridine ring, is a widely known antioxidant useful for the stabilization of edible oils (Kourimska et al., 1993, Tirzitis et al., 2001). This compound exhibits synergetic properties with other antioxidants, such as α-tocopherol (Tirzitis et al., 1983) and 2,6-bis(tert-butyl)-4-hydroxytoluene (Tirzitis and Kirule, 1999). This compound has very low toxicity; LD_{50} of Diludin is about 10 g/kg (mice, per os) (Tirzitis et al., 2001). By the introduction of a carboxyl group at position four in 1,4-

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dihydropyridine ring of Diludin, a whole new group of compounds was created, assigned due to a carboxyl group as 1,4-dihydroisonicotinic acid (1,4-DHINA) derivatives. Although scarce data are available for this class of partially hydrogenated nitrogen heterocycles, some of the derivatives possess very promising pharmacological activities for biomedical application. The parental compound, 2,6-dimethyl-3,5-diethoxycarbonyl-1,4-dihydroisonicotinic acid sodium salt (Ia), as well as 1,4-DHINA with glutamic acid residue — glutapyrone (Ib), possess antimutagenic properties (Goncharova and Kuzhir, 1989; Goncharova et al., 2001; Ryabokon et al., 2005), while Ib also shows a wide variety of pharmacological properties, amongst which the neuromodulatory one is the most pronounced (Karpova et al., 1993; Misane et al., 1998). Taurine derivative of 1,4-DHINA (Ic) shows anti-platelet properties and is active as an anti-aggregant at concentrations that are six times lower than of those for taurine and influences only the first, reversible phase of platelet aggregation (Poikans et al., 1994). It is necessary to underline that none of these compounds reveal calcium antagonistic properties (Misane et al., 1998). All compounds mentioned are water-soluble and retained a very low toxicity (for Ib, it is >8 g/kg, mice, i.p.) of Diludine (Misane et al., 1998). Mechanisms of these compounds' activities are not elucidated and require further studies. Although some indices suggest their influence on oxidative stress, possible antioxidant effects were uncertain and other mechanisms are suggested (Utno et al., 1989; Ryabokon et al., 2005). To clarify this, we have chosen several methods for the characterization of antioxidative properties of 1,4-DHINA. The liver cell model was chosen to test the influence on cell viability, taking into account possible future research direction for the development of new hepatoprotective compounds.

2. Materials and methods

2.1. Compounds

The tested compounds denoted as Ia–c (Fig. 1) were synthesized at the Latvian Institute of Organic Synthesis and according to high performance liquid chromatography (HPLC) data have at least 98% purity. We tested: 2,6-dimethyl-3,5-diethoxycarbonyl-1,4-dihydroisonicotinic acid sodium salt (Ia), glutamic acid derivative of 1,4-DHINA [Ib, glutapyrone, sodium 2-(2,6-dimethyl-3,5-diethoxycarbonyl-1,4-dihydropyridine-4-carboxamido)glutamate] and the taurine derivative of 1,4-DHINA [Ic, tauropyrone, sodium 2-(2,6-dimethyl-3,5-diethoxycarbonyl-1,4-dihydropyridine-4-carboxamido)ethane-sulphate]. The efficiency of these compounds was compared to water-soluble vitamin E analogue Trolox (Sigma, USA). Trolox was prepared as 100 mM stock solution by dissolving it in 17.2% methanol and 0.144 mM NaOH. For the purpose of the experiments with cell cultures, thus prepared solution of water dissolved Trolox was further diluted with the culture medium.

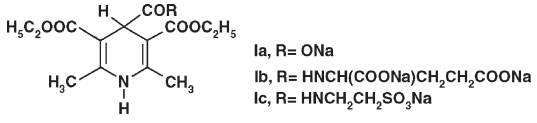

Fig. 1. Structure of 1,4-dihydroisonicotinic acid and its derivatives. Ia — 2,6-dimethyl-3,5-diethoxycarbonyl-1,4-dihydroisonicotinic acid sodium salt; Ib — glutamic acid derivative of 1,4-dihydroisonicotinic (glutapyrone), i.e. — sodium 2-(2,6-dimethyl-3,5-diethoxycarbonyl-1,4-dihydropyridine-4-carboxamido)glutamate; Ic — taurine derivative of 1,4-dihydroisonicotinic (tauropyrone), i.e. — sodium 2-(2,6-dimethyl-3,5-diethoxycarbonyl-1,4-dihydropyridine-4-carboxamido)ethane-sulphonate.

2.2. N,N-Diphenyl-N′-picrylhydrazyl (DPPH˙) assay

This assay is based on the measurement of the scavenging ability of compounds towards the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH˙) and performed according to Abdalla et al. (1999). Briefly, an ethanol solution (2.5 ml) of 100 μM DPPH (Aldrich, USA) was incubated at 30 °C with 50 μl of compound or Trolox solutions in deionized H_2O. The final concentration of compounds was 100 μM. A decrease in absorbance was measured at 517 nm (Hitachi 557 UV–VIS spectrophotometer). The rate constant was calculated as the average value of 5 to 6 time-points (intervals) until the absorbance diminished by 50%. The radical scavenging activity was expressed as the reduction rate constant (k) of DPPH and calculated according to the Eq. (1), where $[DPPH]_0$ is the starting concentration and $[DPPH]_t$ is the concentration at the time 't'.

$$k(M^{-1}s^{-1}) = ([DPPH]_0 - [DPPH]_t)/(t[DPPH]_0[DPPH]_t) \quad (1)$$

2.3. Deoxyribose degradation assay

Deoxyribose degradation by oxygen radicals generated by the Fenton reaction was used to test inorganic radical scavenging efficiency of compounds as described before (Gutteridge and Quinlan, 1992). Briefly, 0.2 ml of 10 mM solution of deoxyribose (Sigma, USA), 0.2 ml of phosphate buffered saline (0.1 M, pH 7.4; from Sigma tablets), 0.01 ml of compound or Trolox solution, 0.01 ml of ferric chloride (1 mM) (Aldrich, USA), 0.02 ml of ascorbic acid (7.5 mM) (Sigma, USA) (in blank instead of sample and ascorbic acid solution, 0.07 ml of H_2O was added) and 0.02 ml of H_2O_2 (8.8 mM) were mixed and incubated at 37 °C for 1 h. Then, 0.5 ml of 2.8% (v/v) trichloroacetic acid (Aldrich, USA) and 0.5 ml of 1% (w/v) 2-thiobarbituric acid (Aldrich, USA) were added to the solution and heated at 100 °C for 15 min, cooled and a further 1.5 ml of 1-butanol (Aldrich, USA) were added to the reaction mixture and vortexed, spun down and the absorbance of the butanol extract was read at 532 nm (Hitachi 557 UV–VIS spectrophotometer). The concentration of analyzed compounds was 5.0 mM. The % of reaction inhibition was calculated from test data according to Eq. (2).

$$\%_{inhib.} = 100 - [(A_{test} - A_{blank})/(A_{control} - A_{blank})] \times 100 \quad (2)$$

2.4. $ABTS^{\cdot+}$ radical cation scavenging assay

In this assay the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt ($ABTS^{\cdot+}$) radical cation was generated by oxidation of ABTS with hydrogen peroxide (Miller et al., 1993). A 1 ml spectrophotometer cell contained

0.3 ml of 0.5 mM ABTS (Sigma, USA) solution in phosphate buffered saline (5 mM, pH 7.4; from Sigma tablets, USA), 0.036 ml of 0.07 mM methaemoglobin (Sigma, USA) solution in phosphate buffered saline, 0.016 ml of compound or Trolox solution and 0.498 ml of phosphate buffered saline and 0.167 ml of 0.1 M H_2O_2. The absorbance at 734 nm was measured on a Hitachi 557 UV–VIS spectrophotometer at 2 min intervals and the % of reaction inhibition was calculated. Concentrations of Ia and Trolox were 0.5 mM and Ib and Ic were 5 mM.

2.5. Antioxidative capacity assay

The antioxidative capacity assay (ANTIOX-CAP, KEG Reagenzien and Recycling, Austria) was used to test hydrogen peroxide scavenging properties of compounds (Resch et al., 2002). Briefly, 5 μl of compounds Ia–c solutions or Trolox (final concentrations were 0.00025–1.25 mM) were pipetted into each well of the microwell plate and mixed with 100 μl of Reagent 2 containing 0.0003% (v/v) hydrogen peroxide. The absorbance was measured at 450 nm (Easy-Reader 400 FW, SLT Lab Instruments GmbH, Austria). Then, 100 μl of Reagent 1 containing 1.25 mU horse radish peroxidase and tetramethylbenzidine was added. After 15 min of incubation and addition of 50 μl of Stop Reagent, the absorbance was measured at 450 nm. Results were presented as a difference between the second and the first absorbance values.

2.6. Primary liver cell cultures

The liver was taken from sacrificed adult female Wistar rat anesthetized by ether in accordance with the ILAR Guide for the Care and Use of Laboratory Animals, Council Directive (86/609/EEC), USA National Institutes of Health and Croatian animal welfare law (NN 19/99). Liver was removed from the animal under sterile conditions and was minced in a beaker containing cold sterile Hanks' solution. The beaker was placed on ice so as to allow large particles to settle down. Five minutes later the supernatants were removed using a syringe with a 22-gauge needle and passed through a sterile gauge into test tubes. The specimen was spun down at 150 g for 10 min, the supernatant was discarded and the pellet resuspended. All visible clumps were removed using a Pasteur pipette so that the cells were dispersed as a single cell suspension. After washing the cells with cold RPMI medium (Sigma, USA) three times, at 350 g for 10 min, the viability of the cells was tested by trypan blue exclusion.

Liver cell cultures were maintained in RPMI medium with 20% (v/v) fetal calf serum (Sigma, USA) in an incubator (Heraeus, Germany) at 37 °C, with a humid air atmosphere containing 5% CO_2 as it was already described (Cipak et al., 2005).

2.7. ^{3}H-Thymidine incorporation and cell viability assay

Liver cells were cultured for 24 h in microwell plates (TPP, Switzerland) at a seeding density of 2×10^4 cells per well in a final volume of 200 μl composed of 20% (v/v) fetal calf serum and substances Ia–c (0–100 mM). Low water solubility of Trolox and presence of methanol for solubilisation did not allow the use of Trolox at concentrations higher than 10 mM. ^{3}H-thymidine (1 μCi/well, Amersham, USA) was added after 24 h to each culture and incubation was continued for the additional 24 h. The cells were then harvested over a glass filter by Skatron cell harvester, and the incorporation of the ^{3}H-thymidine was determined with a β-liquid-scintillation counter (Beckman, LS 3800 Series). Cell viability was determined parallel to the ^{3}H-thymidine incorporation after 3 and 24 h incubation upon trypan blue exclusion assay on a Bürker-Türk hemocytometer.

2.8. Copper ion catalyzed peroxidation of liver cells

To test the influence of copper ion catalyzed peroxidation of liver cells, the original method (Sugihara et al., 1999) was modified. Liver cells (1×10^6) were incubated in Krebs-Heneseleit buffer in the presence of 500 μM $CuSO_4$ (Kemika, Croatia) and Ia in a final volume of 200 μl in a shaking water bath on 37 °C for 3 h. The final concentrations of Ia–c were 1 and 10 mM and Trolox 0.2 and 2 mM. After incubation, Eppendorf tubes with cells were placed on ice and ethylenediaminetetraacetic acid disodium salt (Kemika, Croatia) was added to reach a final concentration of 1 g/l. Samples were frozen immediately and stored in liquid nitrogen. These samples were further analyzed for the presence of malondialdehyde and 4-hydroxynonenal. Cell viability was in parallel determined upon trypan blue exclusion assay on a Bürker-Türk hemocytometer.

2.9. Malondialdehyde determination by HPLC method

Malondialdehyde standards were prepared using 1,1,3,3-tetraethoxy-propane (Sigma, USA) by serial dilution: 0, 0.31, 0.63, 1.25, 2.5, 5 and 10 μM. Samples were analyzed as previously described (Khoschsorur et al., 2000). The total volume of sample (250 μl) or diluted standard (25 μl of standard + 225 μl of water) was mixed with 375 μl 0.44 M H_3PO_4 (Kemika, Croatia) and 125 μl 42 mM thiobarbituric acid (Sigma, USA). All samples were boiled at 100 °C for 60 min, cooled on ice and treated 1:1 with alkaline methanol (4.5 ml 1 M NaOH + 50 ml methanol). 50 μl of clear supernatant was analyzed after centrifugation by an HPLC method with fluorescence detection (527 nm ex, 550 nm em). The HPLC system consisted of Beckman System Gold, Midas Spark Holland autosampler and a Shimadzu RF-535 fluorescence detector. The mobile phase consisted of 50 mM KH_2PO_4 (Kemika, Croatia) pH 6.8 with 40% (v/v) methanol (Merck, Germany). The flow was set to 1 ml/min and the samples were analyzed on a Waters Spherisorb ODS2, 5 μm, 4.6 × 150 mm column.

2.10. Determination of cellular 4-hydroxynonenal-protein adducts

The dot-blot method based on the affinity of proteins to bind to the nitrocellulose membrane was performed to determine the effect of Ia–c on the formation of stable 4-hydroxynonenal-protein conjugates. These conjugates were determined by

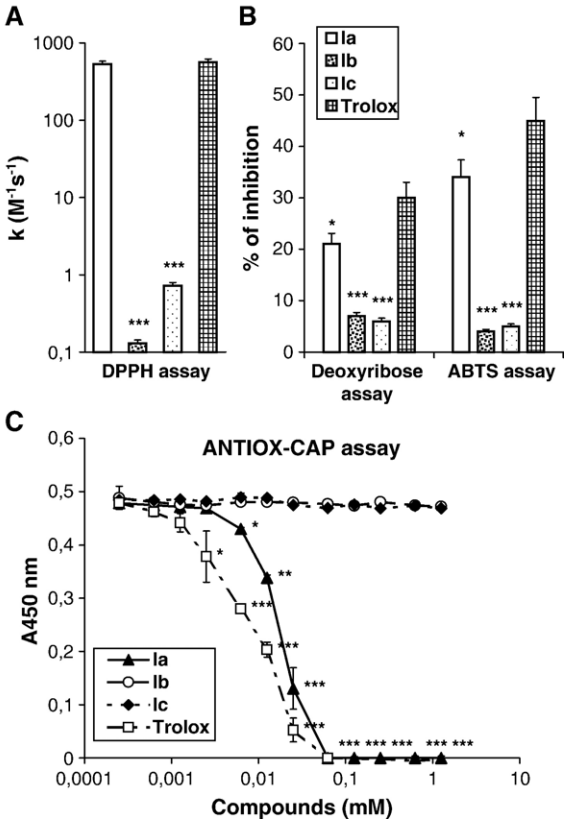

Fig. 2. Radical scavenging activity of Ia–c and Trolox in radical scavenging assays. *N*,*N*-diphenyl-*N*′picrylhydrazyl (DPPH) assay was presented on (A). Results were expressed as reduction rate constant (*k*) of DPPH±S.D. Deoxyribose degradation assay was used to test inorganic radical scavenging efficiency in Fenton reaction, while 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) radical scavenging assay was used to test scavenging efficiency toward cation-centered radicals (B). Results were expressed as % of reaction inhibition±S.D. Hydrogen peroxide scavenging activity was presented in antioxidative capacity (ANTIOX-CAP) assay (C). Results were presented as $A_{450\ nm}$ mean values±S.D. Significance was calculated according to the Student's *t*-test, $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

specific monoclonal antibodies to 4-hydroxynonenal-histidine (Waeg et al., 1996). Cell samples each containing 0.15×10^{6} lysed liver cells were applied as triplicates of independent, equally treated cell cultures to the nitrocellulose membrane. The primary antibody against 4-hydroxynonenal-histidine conjugates was added and the possible endogenous peroxidase activity of samples was blocked with 1.5% hydrogen peroxide, 0.1% sodium azide (Kemika, Croatia) and 2% bovine serum albumin (Sigma, USA) before secondary antibody addition. The detection of antibodies was conducted by measurement of peroxidase activity of enzyme-marked antibody (EnVision, Dako) applying diaminobenzidine staining (Sigma, USA) (Sovic et al., 2001). The colour intensity was evaluated by IPLab 3.52 software.

2.11. Statistical analyses

All assays were carried out in triplicates. The comparison of the mean values was made using the two tailed Student's *t*-test considering values of $p<0.05$ as significantly different.

3. Results

Radical scavenging activities (reactivity towards the individual radicals) of 1,4-DHINA are presented in Fig. 2A and B. Only Ia exhibited radical scavenging activity in a stable free radical DPPH, ABTS and mixed oxygen radicals in deoxyribose degradation assays. The glutamic acid derivative (Ib) and taurine derivative (Ic) did not react with any of the tested radicals. Ia in reaction with DPPH showed the same radical scavenging activity as Trolox, but in deoxyribose degradation and ABTS assays showed about two thirds of Trolox activity.

The hydrogen peroxide scavenging efficiency of tested compounds in antioxidative capacity assay is shown in Fig. 2C. Trolox had the best efficiency, being effective already at 0.25 μM, $p<0.05$, while Ia acted at 6.25 μM, $p<0.05$. Other compounds (Ib, Ic) were not effective in hydrogen peroxide scavenging assay in the concentration range of 0.00025 to 1.25 mM.

The effect of Ia–c and Trolox on liver cell viability was shown in Fig. 3A. All substances caused dose-dependent decrease in cell viability. Trolox exerted the most prominent toxic effect, decreasing viability of the cultured liver cells already at 2.5 mM ($p<0.001$), while 10 mM was completely cytotoxic. Methanol containing solvent used to dissolve Trolox in water before addition to the cell culture medium was also tested in

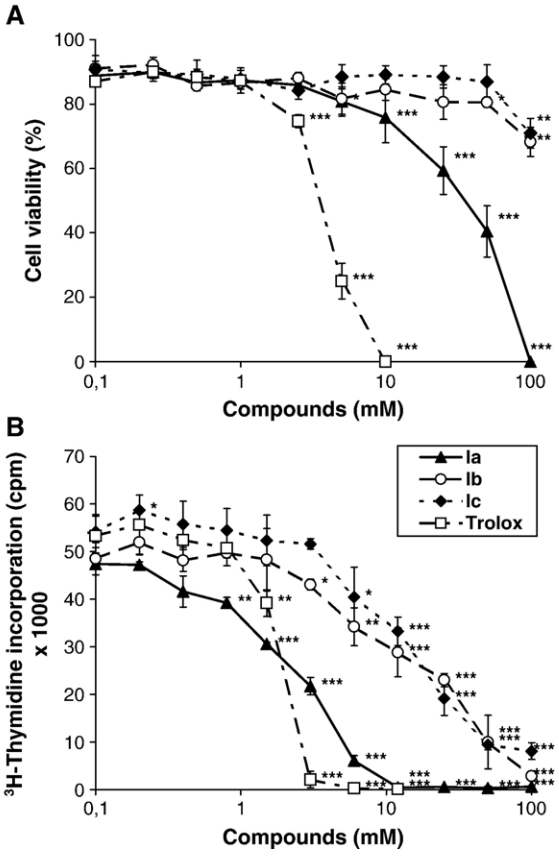

Fig. 3. The influence of Ia–c and Trolox on liver cells viability in trypan blue assay (A) and ^{3}H-thymidine incorporation assay (B). Results were expressed as mean values±S.D. Significance was calculated according to the Student's *t*-test, $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

corresponding dilution but it did not influence cell viability (data not presented). Out of DHINA derivatives, Ia had the strongest effect, decreasing cell viability at 5 mM ($p<0.05$), while Ib was effective at 50 mM ($p<0.05$) and Ic at 100 mM ($p<0.05$).

The effect of tested compounds on ^{3}H-thymidine incorporation in liver cell cultures was shown in Fig. 3B. All substances caused a dose-dependent decrease in ^{3}H-thymidine incorporation, but in different concentration ranges. Ia had the strongest effect, causing inhibition at 0.8 mM ($p<0.05$), while Ib and Ic were effective at 6.25 mM ($p<0.05$). Trolox caused very rapid decline in ^{3}H-thymidine incorporation starting at 1.5 mM ($p<0.01$) and leading to complete abolishment of ^{3}H-thymidine incorporation at 6 mM.

Because Ia showed strong hydrogen peroxide scavenging activity at 1 mM concentration in antioxidative capacity assay (Fig. 2C), while it did not show any cytotoxic effects during the 24 h incubation (Fig. 3A), we used this particular concentration, and 10 times higher, to test potential antioxidant effects of the substance in the cell culture models. Due to the higher cytotoxicity of Trolox than was observed for DHINAs, it was not possible to use the same concentrations of all substances. Hence, the concentrations of Trolox with equivalent efficiencies in cell cultures as Ia were chosen to test antioxidant effects in liver cell cultures.

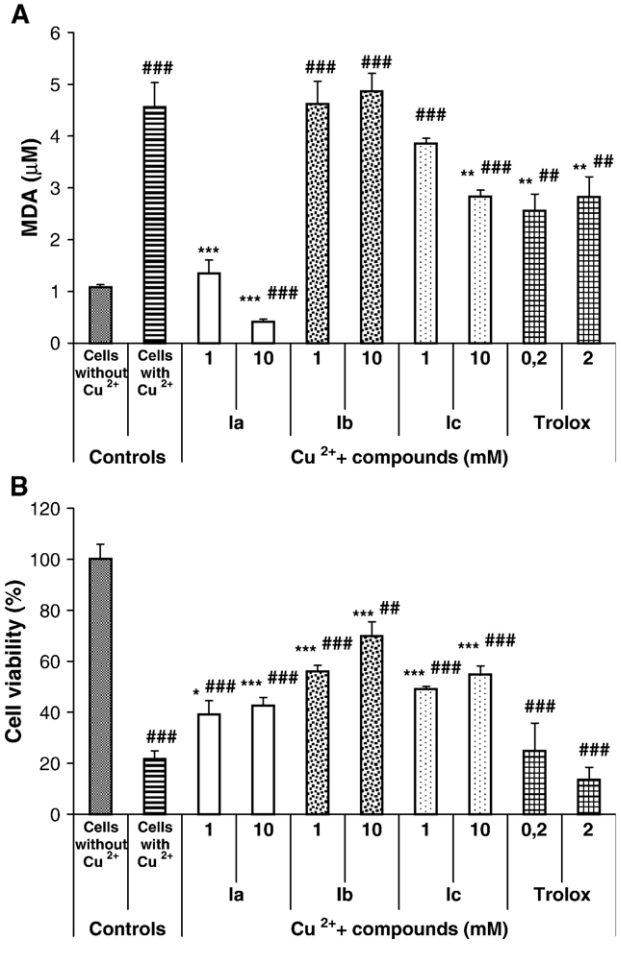

Fig. 4. Antioxidative activity of Ia–c and Trolox in copper ion catalyzed peroxidation of liver cells. Malondialdehyde (A) was analyzed by HPLC method using fluorescence detector set at 527 nm ex and 550 nm em, while cell viability (B) was determined by trypan blue assay. Results were expressed as mean values±S.D. Significance was calculated according to the Student's *t*-test, with respect to control cells ($^{\#}p<0.05$; $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$) and copper only treated cells (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

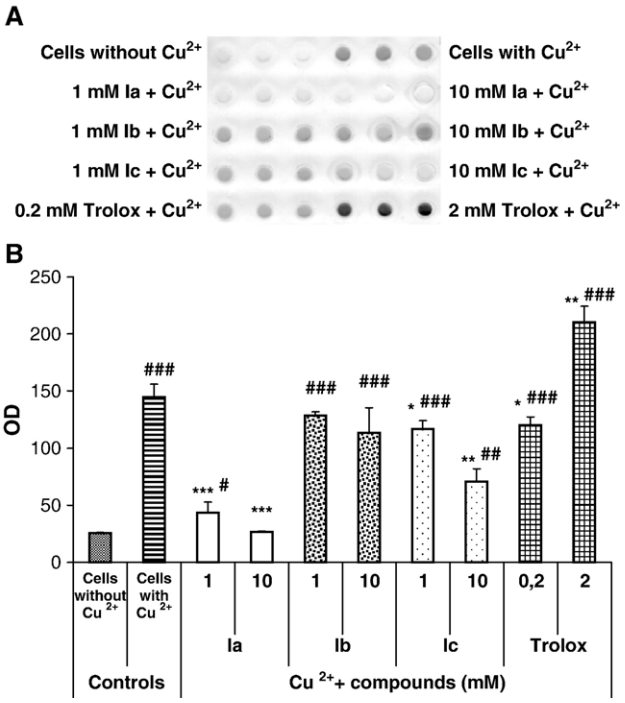

Fig. 5. Antioxidative activity of Ia–c in copper ion catalyzed peroxidation of liver cells, determined as the amount of 4-hydroxynonenal-histidine conjugates by dot-blot method. Each spot presented individual cell culture (A). Control cell cultures were not treated with either copper or Ia. Results were analysed by optical densitometry and expressed as OD mean values±S.D. (B). Significance was calculated according to the Student's *t*-test, with respect to control cells ($^{\#}p<0.05$; $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$) and copper only treated cells (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

The effect of Ia–c or Trolox on copper ion-induced peroxidation of liver cells measured as concentration of malondialdehyde produced in cell cultures is shown in Fig. 4A. Copper increased malondialdehyde production in cell cultures ($p<0.001$) while Ia completely abolished malondialdehyde production in liver cell cultures to the control levels. Ib was not effective, while Ic decreased malondialdehyde production only when used at higher, 10 mM concentration ($p<0.001$). Trolox also reduced malondialdehyde generation in the cell cultures ($p<0.01$), but was less efficient than Ia ($p<0.01$).

The bioprotective effect of Ia–c and Trolox for liver cells damaged by copper-induced lipid peroxidation is shown in Fig. 4B. Copper showed strong toxic effects for liver cells (80% cytotoxicity) ($p<0.001$), while all DHINAs showed a concentration-dependent attenuation of copper cytotoxicity. 10 mM Ia increased more than two-fold ($p<0.001$) the incidence of viable cells (43% of control values), while other compounds had similar effects. Trolox was not effective in attenuation of copper-induced liver cell damage (for both concentrations used

in comparison to the copper-treated control cell cultures, $p>0.1$).

The effects of substances on copper ion-induced peroxidation of liver cells measured as the amount of 4-hydroxynonenal-protein conjugates produced in cell cultures is presented in Fig. 5A,B. Copper increased the amount of 4-hydroxynonenal-protein adducts in cell cultures ($p<0.001$) while Ia strongly decreased it ($p<0.001$). Ib was not effective, but Ic decreased the amount of 4-hydroxynonenal-protein adducts in both concentrations used ($p<0.05$). Trolox decreased the amount of 4-hydroxynonenal-protein adducts if used in lower, 0.2 mM concentration, while if used in higher, 2 mM concentration it enhanced production of the 4-hydroxynonenal-protein adducts in the cultured liver cells ($p<0.001$).

4. Discussion

Among the tested 1,4-DHINA derivatives, only the free acid (Ia), i.e. the corresponding anion, possessed a radical scavenging activity in stable free radical DPPH, ABTS cation centered radical and mixed oxygen radical assays (deoxyribose degradation assay) (the last test system contains predominantly so-called "crypto-$HO^{\cdot}$" type oxygen radicals) (Halliwell and Gutteridge, 1999). Amide derivatives of Ia, i.e. amino acid residues containing Ib and Ic, showed no reaction with any of tested radicals. This observation suggests that only the compound containing a free (unsubstituted) carboxyl group at a position four has the radical scavenging activity. Similarly, derivatives of 1,4-DHP substituted at the position four, have shown less pronounced electron and hydrogen donating properties than its unsubstituted form (Stradins et al., 2000). The 1,4-DHINA derivative Ia, containing a carboxyl group at the position four in dissociated form, shows a good electron and hydrogen donating properties. For example, Ia easily interacts with oxygen radicals in the Fenton system (Rubene et al., 1982) and with the peroxynitrite anion, an active nitrogen form, while at the same time Ib and Ic do not react with the peroxynitrite anion (Tirzitis et al., 1998). Ia in reaction with DPPH showed the same radical scavenging activity as Trolox, a water-soluble vitamin E analogue. In deoxyribose degradation and ABTS assays its effect was also comparable with Trolox. Furthermore, Ia and Trolox were active as hydrogen peroxide scavengers in the antioxidative capacity test-system.

Growth inhibitory effect for culture liver cells was present for all tested 1,4-DHINA compounds and it was concentration-dependent, while Ia had the strongest effect. Trolox was more cytotoxic than DHINA substances. Thus, it was not possible to use Trolox in liver cell cultures in concentrations higher than 2.5 mM, so we used 0.2 and 2 mM concentrations in liver cell cultures. Although Trolox is declared as water-soluble analogue of vitamin E, it is not completely soluble in water. Therefore, methanol should be used to dissolve Trolox in cell culture medium, which might contribute to the observed cytotoxicity of Trolox. However, the methanol-containing solvent alone did not have such a toxic effect, hence cytotoxicity should be mostly related to Trolox. Mild growth inhibitory effect of Ia at 1 mM concentration observed for liver cells by the ^{3}H-thymidine incorporation did not cause any decrease in cell viability, according to the trypan blue assay. However, this growth inhibition occurred only after long-time incubation (24 h), while after a short incubation (3 h, data not presented), even 100 mM concentration did not decrease cell viability (the same for all compounds). Attempting to prevent lipid peroxidation and cell damage, we further used 1 and 10 mM compounds, as Ia was very efficient in hydrogen peroxide scavenging at these concentrations. In liver cell cultures, Ia was able to prevent completely copper-induced lipid peroxidation and, as a consequence, keep amounts of free malondialdehyde as well as 4-hydroxynonenal-protein conjugates on control level. Ic was also able to diminish production of free malondialdehyde and 4-hydroxynonenal-protein conjugates, but impact was not so striking as this of Ia. Trolox diminished production of free malondialdehyde with efficiency weaker than Ia but similar to that of Ic. On the other hand, Trolox diminished production of 4-hydroxynonenal-protein conjugates only if used at lower, 0.2 mM concentration. If used at higher, 2 mM concentration, it increased the amount of 4-hydroxynonenal-protein conjugates in copper-treated cells, indicating ability of Trolox to act as prooxidant in the presence of copper ions. The ability of Trolox to act as prooxidant was already described in other cell culture models as well as in cell-free systems (Ko et al., 1994; Albertini and Abuja, 1999; Tafazoli et al., 2005). Although Ia had antioxidant activity equal to Trolox or even stronger than this water-soluble vitamin E analogue, Ia did not show such undesirable prooxidant effect thus showing antioxidant bioactivity of Ia superior to Trolox.

On the other hand, observed growth modifying effects of Ia could be related to its effects on production of 4-hydroxynonenal-protein conjugates, as 4-hydroxynonenal is not only the cytotoxic product of lipid peroxidation, but it also acts as a growth regulating factor interfering with the effects of cytokines and acting as a fibrogenic stimulant (Zarkovic et al., 1993, 1994; Poli and Parola, 1997; Kreuzer et al., 1998; Zarkovic et al., 1999; Zarkovic, 2003). By suppressing lipid peroxidation and production of 4-hydroxynonenal, Ia could act as a bioprotective antioxidant but could also influence the growth of the cells. On the other hand, 4-hydroxynonenal-modified proteins are hardly metabolized and cause degeneration of the affected cells (Grune and Davies, 2003). Hence, in living cells under oxidative stress and thus induced lipid peroxidation, Ia acted as the most efficient antioxidant.

The most used 1,4-DHP derivatives include important cardiovascular drugs, acting as L-type calcium channel antagonists (nifedipine, nitrendipine, amlodipine, and nisoldipine), which exert antihypertensive and antianginal effects (Triggle, 2003). They also act at a specific receptor site for which defined structure–activity relationships exist, including stereoselectivity (Mikus et al., 1995). When properly substituted, 1,4-dihydropyridine structures can interact with diverse receptors and ion channels, including potassium and sodium channels and receptors of the G-protein class (Triggle, 2003). Some of those compounds also possess radical scavenging activities (Kagedal et al., 1999; Valenzuela et al., 2004). However, only very limited data exist for the closely related 1,4-

DHINA derivatives we used in our research. The original compound from which this group of compounds was derived, Diludin, possesses well-known antioxidant property (Kourimska et al., 1993, Tirzitis et al., 2001). Liposolubility of Diludin limited its use in systems other than oils. Apparently, by introducing carboxyl group at position four in 1,4-dihydropyridine ring a water-soluble compound was created, but antioxidative activity of Diludin was at least retained.

Previously published data showed the ability of 1,4-DHINA derivatives to act as antimutagens in *Drosophila melanogaster* model (Kuzhir et al., 1999). Furthermore, Ia and Ib are able to act as antimutagens in experiments performed on mice in vivo, decreasing frequencies of micronuclear cells in mouse bone marrow (Goncharova et al., 2001). Ia proved to be very efficient in preventing alkylation and radiation-induced mutations in animal models, as well as in cell culture models. In the cell culture model, Ia reduced the number of DNA strand-brakes caused by γ-irradiation, ethyl methane sulfonate and hydrogen peroxide. It is suggested that Ia modulates DNA-repair by influencing poly(ADP)ribosilation (Ryabokon et al., 2005). However, due to our results, and the fact that γ-irradiation, ethylmethane sulfonate and hydrogen peroxide can induce mutations by creating free radicals, we propose that the radical scavenging activity of Ia should be responsible for such bioactivity. The question remained why Ib, which was not effective in our model as antioxidant, acts in vivo as efficient anticarcinogenic compound, decreasing malignancy rate induced by γ-irradiation (Vartanyan et al., 2004). This compound, called glutapyrone, is made by the reaction between the carboxyl group of Ia and amino group of glutamic acid and as a result a peptide bond is created. Different enzymes called carboxypeptidases hydrolyse peptide bonds release free amino acids. These enzymes could be specific for only one amino acid, so, in the case of glutamic acid, glutamate carboxypeptidase should cleave peptide bond (Goldman and Levy, 1967) and liberate the parental compound Ia, which proved to be very effective as an antioxidant and an antimutagen.

All compounds were effective in attenuating liver cell damage caused by copper (evaluated by trypan blue assay). It seems that this effect is not necessarily related to antioxidative capacities of these compounds. The low protective effect of Ia when compared to Ib and Ic may be mainly due to its ability to affect cell growth. Results could be also explained partly by taking into account the data for location of 1,4-DHP derivatives in cell membranes. It is found by means of electron spin resonance probes that Ia, affects only the exposed portions (surface) of erythrocyte membranes increasing the mobility of phospholipid polar head groups and decreasing the polarity of the bilayer at the depth of 6–8 Å (Panasenko et al., 1984). Furthermore, Ia does not inhibit both osmotic or dialuric acid erythrocyte haemolysis (D.Tirzite, unpublished results) while close related 1,4-DHP such as 2,6-dimethyl-3,5-diethoxycarbonyl-4-phenyl-1,4-dihydropyridine, which contains a phenyl ring in position four instead of carboxylic group in Ia, is a good erythrocyte membrane stabiliser (Tirzite et al., 1982). Thus, by introducing new groups, bioactive properties of these substances could be changed. This is especially important due to the fact that copper induces cell damage not only by the creation of oxygen free radicals, but also by destabilisation of cellular membranes (Marchi et al., 2004).

Results obtained showed that the most promising antioxidant and bioactive compound, even superior to Trolox, would be Ia, as it was able to protect liver cells from copper-induced lipid peroxidation and increased cell viability. Ia could therefore be studied further as a potential antioxidant and hepatoprotective agent, attenuating the onset of oxidative stress and lipid peroxidation based diseases. The activity principles of other compounds that had weak antioxidant effects (Ic) or did not act as antioxidants (Ib) but increased viability of the liver cells damaged by lipid peroxidation should be further studied, too.

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