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Stability and antioxidant activity of gossypol derivative immobilized on N-polyvinylpyrrolidone

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ABSTRACT

The objective of this study is analysis of stability and antioxidant and antiradical activities of the gossypol derivative – megosin conjugated with N-polyvinylpyrrolidone (PVP). The results of study have shown the greater stability of megosin + PVP than megosin in aqueous solution of wide range of pH. Here we also demonstrated that megosin + PVP, named rometin, possess high antioxidant activity in the same range as well known antioxidant trolox as determined by its ability to scavenge free $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radicals *in vitro*. In addition, megosin + PVP was able to prevent accumulation of products of lipid peroxidation (thiobarbituric acid reactive substances and diene conjugates) and lysophospholipids formation in mitochondria membranes caused by CCl_4-induced oxidative stress in rat liver *in vivo*. Furthermore, megosin + PVP rescued mitochondrial functions, such as respiration and oxidative phosphorylation, which declined after CCl_4 administration. Thus we present that the conjugation of megosin to PVP increase its stability and remain antioxidant activity *in vivo* and *in vitro*.

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1. Introduction

Development of new medicines and modification of the existing ones is driven by the need to enhance and to prolong their therapeutic effects, which are often related to the water-solubility of compounds and their stability. Various carriers are used for preparation of medicines with prolonged action, including synthetic polymers which meet such medical criteria as absence of side effects, as well as quick and nontoxic biodegradation and removal from the organism. Water-soluble low-molecular-weight fractions of N-polyvinylpyrrolidone (PVP) (MM 5000–15,000) are polymers that satisfy these requirements. PVP is among the most widely used polymers in production of various medicinal forms (tablets, pills, injection solutions) and contrast agents [1–5]. One of the first successful applications of PVP was development of Povidone-iodine, complex of polyvinylpyrrolodone and elemental iodine [5]. PVP can form complexes with various organic compounds. When a poorly soluble drug forms a complex with a PVP macromolecule, its solubility in water is increased, and so are its stability in the solution and the long blood half-life duration of its action within the organism [1,6–8].

Gossypol (GP) is a natural polyphenolic compound extracted from cotton plants (*Gossypium* spp.). The studies of gossypol and its derivatives as promising pharmaceutical compounds are widely held around the world. It was shown that gossypol holds medicinal potentials as a male antifertility agent [9,10], and as an antiproliferative [11–16], antiviral [17,18] or immunomodulatory [19,20] drug. The diversity of these pharmacological effects of GP is attributable to a variety of its cellular effects. One of them, in common with many other aromatic phenols, is an antioxidant activity. However, GP *per se* is a toxic compound, which displays both prooxidant and antioxidant properties depending on a concentration used [21–23]. In contrast, the GP derivative – disodium salt – 2,2′{[(7,7′,8,8′-tetrahydro-1,1′,6,6′-tetrahydroxy-5,5′-diisopropyl-3,3′-dimethyl-7,7′-dioxo)-2,2′-dinaphthyl]-8,8′-methylenimino}-sulfonic acid (megosin), while preserving the GP original pharmacological properties, is less toxic and could be of great interest as a novel medicinal preparation [24–26]. However, megosin, like other gossypol derivatives, has low

Abbreviations: PVP, N-polyvinylpyrrolidone; GP, gossypol; CCl_4, carbon tetrachloride; TBA, thiobarbituric acid; $DPPH^{\bullet}$, 1,1-diphenyl-2-picrylhydrazyl; $ABTS^{\bullet+}$, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); HRP, horseradish peroxidase; LPO, lipid peroxidation; TBARSs, thiobarbituric acid reactive substances; DCs, diene conjugates; LPC, lyso-phosphatidylcholine; LPE, lyso-phosphatidylethanolamine; LCL, lyso-cardiolipin; ROS, reactive oxygen species.

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solubility in water, making it difficult to use for clinical applications. Previously we showed that immobilization of megosin on PVP resulted in increase in its water solubility and bio-accessibility and reduction of its membrane-destructive properties [26–29]. The same approach was applied by us for obtaining of water-soluble form of another derivative of gossypol, batriden [27]. At present water-soluble form of batriden is used in dermatology [30].

Aim of this study was to investigate stability, antioxidant and antiradical activities of megosin under its immobilization on N-polyvinylpyrrolidone *in vitro*. We examined also the ability of immobilized on N-polyvinylpyrrolidone megosin to prevent oxidative changes caused by carbon tetrachloride at subcellular level (rat liver mitochondria) *in vivo*.

2. Experimental

2.1. Materials

Trolox (6-hydroxyl-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) was obtained from Fluka Chemical Co. (Ronkonkoma, NY, USA). Carbon tetrachloride (CCl_4), thiobarbituric acid (TBA), 1, 1-diphenyl-2-picrylhydrazyl (DPPH•), 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$), and horseradish peroxidase (HRP) were from Sigma–Aldrich (St. Louis, MO). All other reagents were purchased from Reakhim (Russia) and POCH (Poland).

2.2. Chemical synthesis

Megosin, disodium salt – 2,2′{[(7,7′,8,8′-tetrahydro-1,1′,6,6′-tetrahydroxy-5,5′-diisopropyl-3,3′-dimethyl-7,7′-dioxo)-2,2′-dinaphthyl]-8,8′-methylenimino}-sulfonic acid, was synthesized and immobilized on N-polyvinylpyrrolidone (8000 MM), as described previously (Fig. 1) [26,28,29]. The content of megosin in complex with N-polyvinylpyrrolidone was 9–10%. The identity and purity of megosin and megosin + PVP complex (named rometin) were determined by TLC analysis using Silufol UV-254 thin layer plates in acetone:diethyl ether (5:1) systems. In addition, the percent content of the preparation was determined by spectrophotometry. Maximal yield of complex was 88% and purity of the compound was equal to or greater than 96%. DMSO and water were used as a solvent for megosin and megosin + PVP, respectively. Used concentration of megosin + PVP was equimolar with respect to that of megosin in the complex.

2.3. Radicals scavenging activity assay in vitro

The DPPH• radicals scavenging activity assay was measured according to Yang et al. [31]. 5 μL of tested compounds was added to 995 μL of 100 μM DPPH• ethanol solution. After a 3 min incubation period at room temperature, the decrease in absorbance of DPPH• solution was evaluated at 520 nm. The decrease at 520 nm between the blank (ethanol) which was set as 100% and a sample was used for calculating the scavenging activity.

The $ABTS^{\bullet+}$ assay was based on the method described by Celik et al. [32] slightly modified. The $ABTS^{\bullet+}$ radical cation was produced by reacting 720 μL 2 mM ABTS in aqueous solution and 720 μL 0.95 nM horseradish peroxidase in 50 mM KH_2PO_4 (pH 7.0) with 75 μL 1 mM H_2O_2 in 50 mM KH_2PO_4 (pH 7.0). The formation of $ABTS^{\bullet+}$ was evaluated at 414 nm for 10 min. The decrease in absorbance at 414 nm between the blank (without antioxidant compound), which was set as 100%, and a sample containing antioxidant compound was used for calculating the scavenging activity. The EC50 value was defined as the concentration of compound required to scavenge 50% of DPPH• and $ABTS^{\bullet+}$ radicals. Trolox was used as a positive control.

2.4. Animals and carbon tetrachloride-induced hepatic oxidative damage

Experiments were carried out on male Wistar rats (120–140 g). The rats were housed in cages with controlled temperature (23 ± 1 °C) and light cycle (12 h light and 12 h dark). The animals had free access to standard laboratory diet and tap water. The protocol was approved by the Animal Ethical Committee of the National University of Uzbekistan constituted for animal study.

The rats were divided into three groups of six animals each. The first group served as a control. The second and the third groups received 1 mL/kg 50% oil solution of carbon tetrachloride (CCl_4) intraperitoneal injections in every other day during 10 days. Animals in the third group received intraperitoneal injections of rometin in the amount of 50 mg/kg (corresponds to 5 mg of megosin in the complex) in addition to CCl_4.

2.5. Isolation of mitochondria and determination of phospholipids composition and products of lipid peroxidation in the chloroform–methanol extracts of mitochondria

Mitochondria were isolated from rat liver by standard protocol [33]. Isolation medium A contained 250 mM sucrose, 5 mM Tris–HCl, 0.5 mM EDTA, pH 7.4. Immediately after decapitation, the liver was excised, chopped, and homogenized in 40 mL medium A with a Potter–Elvehjem homogenizer. The homogenate was centrifuged at 800 × *g* for 7 min. The supernatant was separated and recentrifuged at 6000 × *g* for 15 min. The resulting pellet was resuspended with EDTA-free isolation medium B contained 250 mM sucrose, 5 mM Tris–HCl, pH 7.4 and centrifuged at 6000 × *g* for 15 min. The final pellet was resuspended again in medium B at approximately 50 mg/mL of protein (measured by the Biuret method) [34], stored on ice, and used within a few hours. All procedures were performed at 0–4 °C.

Phospholipids were extracted from mitochondria by the method of Blay–Dyer with modifications. 3 mL of mixture of chloroform–methanol (1:2) was added to 0.8 mL incubation medium contained 120 mM KCl, 10 mM Tris–HCl pH 7.5 and 8 mg of mitochondrial protein. The samples were stirred for 15 min followed by addition of 1 mL of chloroform and 1 mL of 150 mM NaCl. To separate the chloroform and methanol–water phases, samples were centrifugated 10 min at 2000 × *g*. The water–methanol layer was used for measurements of the level of TBA reactive substances (TBARS). The chloroform phase was used for measurements of diene conjugates (DC), general phosphorous and phospholipids composition. Phospholipids were separated on silica gel plates by a two-dimensional micro-thin-layer chromatography using chloroform–methanol–liquid ammonia (65:25:5) and chloroform–acetone–methanol–acetic acid–water (60:80:20:20:10) solvent systems. Lipids were visualized by iodine staining. The spots were scraped from plates to test tubes and quantified for the content of inorganic phosphate. 0.2 mL of 72% chloric acid was added to test tubes and then samples were burned at 200 °C for 60 min. After cooling 1.3 mL Vaskovsky's reagent contained $NH_2NH_2{\cdot}HCl$, $Na_2MoO_4{\cdot}2H_2O$, HCl and H_2SO_4 [35] was added and the samples were incubated at 95 °C for 20 min. The absorbance of samples was measured at 815 nm. The amount of phosphorous was calculated using standard curve. KH_2PO_4 was used as a standard. The amount of phospholipids was estimated as phosphorous content. The total phosphorous was determined in 50 μL of chloroform phase by method described by Vaskovsky et al. [35].

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