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Effect of Pr(III) nitrate and Pr(III) complex on the accumulation of free radicals in rat blood serum

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The success of the cancer treatment depends on the good control over the oxidative stress (OS). Lanthanides and their compounds are promising anticancer agents due to the prooxidant activity of the Ln(III) cations. A complex formed by coordinatively bonding lanthanide ion with antioxidant ligand is promising strategy in the search of efficient anticancer medications. The 5-aminoorotic acid (HAOA) proved to be antioxidant at homeostatic pH. In this investigation, the effect of HAOA, Pr(III), and their complex, PrAOA, on the free radicals accumulation in rat blood serum was estimated. Pr(III) exhibited prooxidant properties. Below concentrations of 10^{-6} M both HAOA and PrAOA did not influence the $OH^{\bullet}$-initiated OS in the serum. Above this concentration, both compounds were antioxidants, the complex being weaker than the ligand. It was proposed that the antioxidant effect of PrAOA resulted from both antioxidant properties of the ligands and prooxidant properties of Pr(III).

Keywords: Praseodymium cation, 5-Aminoorotic acid, Praseodymium complex, Antioxidant properties, Free radicals accumulation, Rat blood serum.

INTRODUCTION

5-Aminoorotic acid (HAOA) is the amino-derivative of naturally occurring orotic acid (vitamin B_{13}), latter being intermediate in the biosynthesis of the pyrimidine nucleotides of DNA and RNA. Orotic acid (HOA) and its metal complexes have attracted growing attention in medicine [1-5], their structural and spectroscopic properties being comprehensively studied [6,7]. The HOA molecule is related to the molecules of uracil or thymine. Various theoretical studies on these types of molecules have been performed [8-10] and these results are very helpful in the characterization of newly synthesised complexes of HOA and HAOA. We have reported promising results on the significant cytotoxic activity of such types of Ln(III) complexes in different human cell lines [11-15]. The present work can be regarded as a continuation of our efforts in the bioinorganic chemistry of Ln(III) complexes with a number of biologically active ligands.

The coordination chemistry of lanthanides, relevant to the biological, biochemical and medical aspects, makes a significant contribution to understanding the basis of application of lanthanides, particularly in biological and medical systems. The lability of lanthanide complexes, strong oxyphilicity, very fast water exchange reaction, no directionality of lanthanide ligand bond and varying coordination number, all contribute towards lanthanide interaction with biomolecules. The ionic size of Ln(III) varies from one lanthanide to another; in addition, the ionic size of a particular lanthanide also varies significantly with the coordination number. Smaller size of chelating biologically active ligand can even suit larger lanthanides with lowered coordination number. Similarly small lanthanides can expand their coordination number and can form stable chelates with larger biomolecules. This can explain the different coordinating potential and biological behavior of different lanthanides under various physiological conditions. It has been reported by us earlier that Ln(III) ions attack cancer cells and induce apoptosis, considered as the core of the lanthanide potential as anticancer activity [11-15]. Along with apoptosis, there are several synergic related effects, ROS scavenging, cell protection, cytoskeleton stabilization and also immunologic enhancement [16-24].

Reactive oxygen species (ROS) are involved in the development of many diseases and the antioxidants are used to limit the ROS overproduction. Recently the antioxidant activity of Ln(III) complexes was determined by DPPH radical scavenging method. Some of these complexes exhibited more effective antioxidant activity than the respective ligands [17,18,23,24]. Their considerable radicals scavenging activity was explained by the chelation of organic molecules to rare earth ions. Ln(III) ions such as La(III), Sm(III), Eu(III) and Dy(III) exerted differential and

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selective effects on scavenging radicals of the biological system. Therefore, the studied Ln(III) complexes of biologically active derivatives of HAOA deserve to be further explored.

The aim of this work was to synthesize and characterize a new praseodymium(III) complex of HAOA and to evaluate its antioxidant activity. HAOA, $Pr(NO_3)_3.6H_2O$, and their complex (PrAOA) were investigated for possible involvement in the free radicals homeostasis, using rat blood serum as a model system. The effect of Pr(III) was estimated by subtracting the effect of the NO_3^- from this of $Pr(NO_3)_3.6H_2O$. The $OH^\bullet$-induced oxidative stress (OS) in the blood serum was initiated by the $Fe(II)/H_2O_2$/EDTA/ascorbate model system. The free radicals accumulation (FRA) was estimated spectrophotometrically, using the transformation of the yellow marker molecule 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into the purple MTT formazan (λ=576 nm in phosphate buffer). The solvent effect on the compounds investigated was illustrated on the example of UV spectra in water and phosphate buffer.

MATERIALS AND METHODS

Chemicals and solutions: The compounds used for preparing the solutions for the synthesis were Sigma-Aldrich products, p.a. grade: $Pr(NO_3)_3.6H_2O$ and 5-aminoorotic acid. Latter was used as a ligand for the preparation of the metal complex.

The carbon, hydrogen and nitrogen contents of the compound were determined by elemental analysis.

Stock solutions of 10^{-3} M $NaNO_3$, $Pr(NO_3)_3.6H_2O$, HAOA and PrAOA in distilled water or PBS were prepared. Before the experiment they were diluted further to desired concentrations. Ice-cold aqueous solution of 3 mM $FeCl_2$, 3 mM H_2O_2, 0.3 mM EDTA and ice-cold aqueous solution of 4 mg/ml ascorbate were prepared and stored in ice bath. MTT was dissolved in distilled water to concentration of 3mg/ml.

Infrared spectra: The solid-state infrared spectra of the ligand and its Pr(III) complex were recorded in KBr in the 4000-400 cm^{-1} frequency range by FT-IR 113V Bruker spectrometer.

Raman spectra: The Raman spectra of HAOA and PrAOA were recorded with a Dilor microspectrometer (Horiba-Jobin-Yvon, model LabRam) equipped with 1800 grooves/mm holographic grating. The 514.5 nm line of an argon ion laser (Spectra Physics, model 2016) was used for the probes excitation. The spectra were collected in a backscattering geometry with a confocal Raman microscope equipped with an Olympus LMPlanFL 50× objective and with a resolution of 2 cm^{-1}. The detection of Raman signal was carried out with a Peltier-cooled CCD camera. Laser power of 100 mW was used in our measurements.

UV-spectra: UV spectra were recorded in quartz cuvette, using Shimadzu 1600 apparatus equipped with software package, within 400-200 nm, at very low speed (step of 0.5 nm). The instrumental errors were eliminated by recording the spectrum of solvent (water or PBS) against solvent and subtracting this spectrum from the experimental spectrum of the corresponding solution. By scanning of the same spectrum three times we found that the experimental error for λ was ±1.0 nm in position and ±0.001 a.u. in intensity.

Blood serum preparation: The serum was separated from rat total blood as described in [25,26] and stored at -86°C for biochemical analysis. The proteins concentration in the serum was measured [27]. Serum containing 1 mg/ml protein was left in contact with solution investigated (10^{-7}, 10^{-6}, 10^{-5} or 10^{-4} M) or pure solvent (0 M, controls). Five test tubes with identical content were prepared for each concentration. All flasks were kept at 4°C for 30 min. Before FRA assay, the samples were conditioned to room temperature.

FRA assay: The content of each flask was transferred in a quartz cuvette, where MTT was introduced and $OH^\bullet$-induced OS was generated. The relative change of the absorption at 576 nm was measured for 10 minutes, using the kinetics software of the Shimadzu 1600, against solvent.

For FRA in the serum alone: one ml of the sample cuvette contained serum corresponding to 1 mg proteins, 0.10 ml MTT, 0.1 ml $Fe(II)/H_2O_2$/EDTA/ascorbate, and PBS to 1 ml; the control measurement was performed in absence of $Fe(II)/H_2O_2$/EDTA/ascorbate; for the background the serum was omitted.

For FRA in the serum, in presence of a solution, in 1 ml of the sample cuvette: serum corresponding to 1 mg proteins, 0.10 ml MTT, 0.1 ml of the solution of desirable concentration, 0.10 ml $Fe(II)/H_2O_2$/EDTA/ascorbate, and PBS to 1 ml; the control measurement was done in absence of the solution of the compound investigated; for the background measurement the serum was omitted.

To estimate the effect of Pr(III) FRA of the serum was measured in presence of $NaNO_3$ in concentrations adjusted to have the same NO_3^- content, as these in the corresponding $Pr(NO_3)_3.6H_2O$ solutions.

The $OH^\bullet$ initiated free radicals accumulation in the blood serum alone, and in the presence of the

compounds investigated, was assessed using the formula:

$$FRA = \frac{\Delta A_{sample} - \Delta A_{control}}{\Delta A_{background}} * 100,$$

where FRA - free radicals accumulation in %; ΔA - the relative change of the absorption at 576 nm for 10 min for the control, sample and background.

Data management: Average values and standard deviations of FRA at each concentration of the compounds investigated were calculated. Concentration effect was statistically verified using One Way ANOVA. To evaluate the Pr(III) effect, samples of nitrates containing and not containing Pr(III) were compared. The effects of Pr(III) and PrAOA on the FRA of the blood serum were statistically verified by considering different standard deviations and two-tailed P distribution (non-parametric *t*-test with Welch's correction). The Pr(III) effect was verified by comparing data for praseodymium and sodium nitrates solutions with the same amounts of NO_3^-. The effect of PrAOA was verified by comparing FRA of the complex with this of HAOA for each concentration.

RESULTS

The complex was synthesized by reaction of Pr(III) salt and the ligand, in amounts equal to metal: ligand molar ratio of 1:3. The synthesis was made in different ratios (1:1, 1:2, 1:3) but in all the cases the final product was with the composition 1:3. The complex was prepared by adding an aqueous solution of Pr(III) salt to an aqueous solution of the ligand, subsequently raising the pH of the mixture gradually to ca. 5.0 by adding dilute solution of sodium hydroxide. The reaction mixture was stirred with an electromagnetic stirrer at 25 °C for one hour. At the moment of mixing of the solutions, precipitate was obtained. The precipitate was filtered (pH of the filtrate was 5.0), washed several times with water and dried in a desiccator to constant weight. The obtained complex was insoluble in water, methanol and ethanol, but well soluble in DMSO.

Reaction of Pr(III) and 5-aminoorotic acid afforded a complex which was found to be quite stable both in solid state and in solution. The new Pr(III) complex was characterized by elemental analysis. The content of the metal ion was determined after mineralization. The used spectral analyses confirmed the nature of the complex.

The data of the elemental analysis of the Pr(III) complex serve as a basis for the determination of its empirical formula and the results are presented below. The elemental content of the Pr(III) complex of HAOA ($Pr(AOA)_3.H_2O$) is shown as % calculated/found: C= 26.90/26.64; H= 2.09/2.29; N= 18.83/19.00; H_2O= 2.69/2.25; Pr= 21.07/20.76, where HAOA= $C_5N_3O_4H_5$ and AOA= $C_5N_3O_4H_4^-$.

In our previous work the geometry of 5-aminoorotic acid was computed and optimized with the Gaussian 03 program employing the B3PW91 and B3LYP methods with the 6-311++G** and LANL2DZ basis sets [29]. In the present study the binding mode of the HAOA ligand to Pr(III) ions was elucidated by recording the IR and Raman spectra.

The UV spectra of 10^{-4} M solutions in water and PBS (pH 7.45) of the compounds investigated are seen in Fig.1.

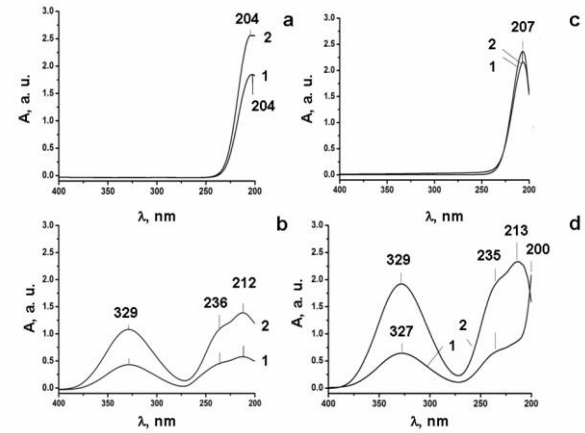

Fig. 1. UV spectra of aqueous (a,b) and PBS (c,d) solutions of: 3×10^{-4} M $NaNO_3$ (a,c-1), and 10^{-4} M solutions of $Pr(NO_3)_3.6H_2O$ (a,c-2), HAOA (b,d-1) and PrAOA (b,d-2).

Spectra were resolved based on literature data [4-9]. The solvent effects on UV spectra of the compounds were observed by comparing aqueous (Fig. 1a,b) with PBS (Fig. 1c,d) solutions. Spectra of nitrates (Fig.1a,c) indicated PBS as a solvent diminishing the relative differences within effects of Na(I) and Pr(III) on NO_3^-. Therefore, in PBS (pH 7.45) it might be expected similar reactivity of NO_3^- of both praseodymium and sodium nitrates toward free radicals produced in blood serum. As Na(I) does not affect the free radicals accumulation, any difference within FRA of sodium compared to praseodymium nitrate might be associated with Pr(III) ions. The intensities of UV spectra of PBS solutions of HAOA and PrAOA (Fig.1d) were significantly higher than these of the corresponding aqueous solutions (Fig.2c), this effect being stronger for the PrAOA spectrum (spectra 2) than for the HAOA spectrum (spectra 1), with no significant shift in positions of λ. The UV spectra of HAOA in water and PBS differed in structure too (Fig.1c,d, spectra 1). A new very intensive and sharp band at about 200 nm was observed in the

PBS solution. This, along with the smaller relative increase in intensities of the rest of the characteristic bands for 5-aminoorotic acid, might be an indication of dissociation of some HAOA molecules in PBS.

The Gauss deconvolution of the experimental spectra is presented in Fig.2.

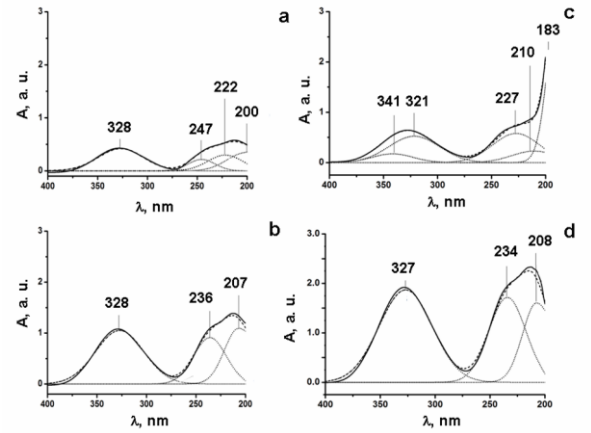

Fig. 2. Gauss deconvolution of the UV spectra of 10^{-4} M aqueous (a, b) and PBS (c, d) solutions of: a- 5-aminootoric acid (HAOA, a, c), and b- Pr complex (PrAOA, b, d). The Gauss components of the spectra are drawn as dotted lines; spectra reproduced the real spectrum with R^2>0.996.

The UV spectrum of 0.1 mM aqueous solution of HAOA (Fig. 2a) was presented as superposition of 4 components, associated with the characteristic bands of 5-aminoorotic acid [4-9]. The Gauss deconvolution of the spectrum seen in Fig. 2c revealed two new components (183 nm and 341 nm). The strong and sharp component at 183 nm was typical for the C=O vibration in the ionized COO^- group, while the component at 341 nm might result from a shift in the $\pi \rightarrow \pi^*$ transition in the aryl ring, due to the ionization:

$$HAOA + H_2PO_4^- \rightarrow AOA^- + H_3PO_4$$

Yet, bands typical for non-dissociated molecules are present in the spectrum (321, 227 and 210 nm).

The effects of $Pr(NO_3)_3$, $NaNO_3$, HAOA and PrAOA on the $OH^\bullet$ induced free radicals accumulation in rat blood serum are illustrated in Fig.3.

In Fig. 3a, curve 1 shows that in the presence of NO_3^- FRA slowly but gradually increases with increasing of the concentration. The effect of Pr(III) is observed in the relatively higher FRA than this in the presence of Na(I) at any given concentration (p=0.0019, 0.0006, 0.0002 and <0.0001 for 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M, respectively). In presence of HAOA and PrAOA FRA in the serum decreased with concentration (Fig. 3b). The relative differences between HAOA and PrAOA were significant at concentrations above 10^{-5} M (p= 0.5000 and 0.3081 for 10^{-7} and 10^{-6} M, and <0.0001 for 10^{-5} M and 10^{-4} M, respectively).

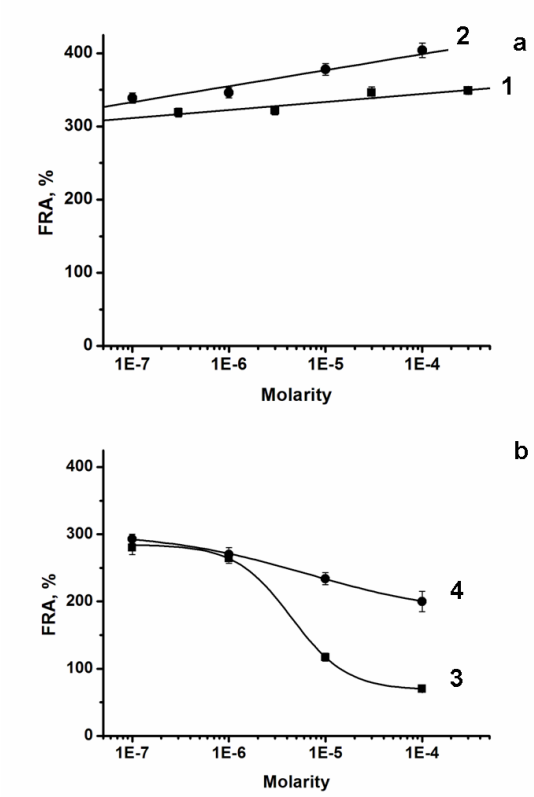

Fig. 3. Effect of nitrates (a) and HAOA containing solutions (b) on the $OH^\bullet$-induced free radicals accumulation (FRA) in rat blood serum: 1- $NaNO_3$ solutions, 2- $Pr(NO_3)_3.6H_2O$ solutions, 3- HAOA solutions, 4- PrAOA solutions

DISCUSSION

Pr(III) accelerated the $OH^\bullet$ induced free radicals accumulation in rat blood serum, while the 5-aminoorotic acid and its Pr(III) complex exhibited antioxidant effect in the same model system, this effect being stronger in presence of HAOA than in presence of PrAOA. The diminishing FRA in the blood serum in the presence of a compound is an indication of its antioxidant activity. From this viewpoint, the NO_3^- and Pr(III) acted as prooxidants, while HAOA and PrAOA showed antioxidant properties.

The UV spectra of the compounds indicated that the medium influenced the state of the dissolved molecules. HAOA partially dissociated, and the energy of some electron transitions in the rest of the undissociated molecules increased. This could increase the chance for interaction with free radicals, resulting in antioxidant activity of the molecule in homeostatic conditions. The Pr(AOA) complex exhibited weaker antioxidant activity than HAOA. The UV spectra of the compound indicated that this complex is stable in homeostatic medium. As no dissociation of the complex was observed in

the UV spectra of PrAOA, the lower antioxidant activity of the complex compared with this of HAOA alone, may be related with the effect of Pr(III) on the partial charges distribution and dipole moment in the ligands.

CONCLUSIONS

1. The complex of Pr(III) with 5-aminoorotic acid has been synthesized and characterized by elemental, UV-VIS and vibrational spectral analyses, including IR and Raman spectra.

2. $Pr(NO_3)_3.6H_2O$ and $NaNO_3$ with same content of nitrate ions increased *in vitro* the free radicals accumulation in rat blood serum, in which the oxidative stress was induced by Fe(II)/H_2O_2/EDTA/ascorbate model system. The stronger effect of Pr(III) than this of the Na(I) nitrate was related with prooxidant effect of Pr(III).

3. 5-aminoorotic acid and its complex with Pr(III) decreased *in vitro* the free radicals accumulation in rat blood serum, in which the oxidative stress was induced by the Fe(II)/H_2O_2/EDTA/ascorbate model system.

3. Different solvent effects of water and PBS (pH 7.45) on the UV spectra of the compounds investigated, were detected, and related with their *in vitro* antioxidant properties.

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ЕФЕКТИ НА PR((III) НИТРАТ И PR(III) КОМПЛЕКС ВЪРХУ НАТРУПВАНЕТО НА СВОБОДНИ РАДИКАЛИ В КРЪВЕН СЕРУМ НА ПЛЪХ

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Постъпила на коригирана на

(Резюме)

Успехът при терапия на рака зависи от качеството на контрола върху оксидативния стрес (ОС). Поради прооксидантната активност на лантанидните йони, лантанидите и техните съединения са перспективни антиракови агенти. Синтезът на La(III) комплекси с антиоксидантни лиганди е атрактивна стратегия при търсенето на антиракови лекарства. 5-Аминооротовата киселина (HAOA) е доказан антиоксидант при хомеостатично pH. В настоящата работа е показано влиянието на HAOA, Pr(III) и техния комплекс, PrAOA, върху натрупването на свободни радикали в кръвен серум на плъх. Pr(III) проявява прооксидантни свойства. В концентрации под 10^{-6} M, HAOA и PrAOA не променят нивото на $OH^{\bullet}$-индуцирания ОС в серума. Над тази концентрация двете съединения са антиоксиданти, с по-слаб ефект при комплекса в сравнение с този на лиганда. Предположено е, че антиоксидантният ефект на PrAOA е кумулативен резултат на прооксидантните свойства на Pr(III) и антиоксидантните свойства на HAOA.