Investigation of the cytotoxicity of silver nitrate and silver-cysteine nanocomplexes

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Abstract: Currently, a large number of studies are devoted to the investigation of the antitumor activity of silver nanoparticles and compounds, one of which is silver nitrate. However, silver nitrate has systemic and local toxic effects. In this work, a method was proposed for the synthesis of non-metallic complexes that do not contain toxic nitrate ions, and the cytotoxicity of silver nitrate and silver-amino acid nanocomplexes was investigated.

1. Introduction
The study of the antitumor activity of silver nanoparticles and compounds in vitro and in vivo, as well as the study of the mechanisms underlying these effects, is relevant today. There is a theory that the main active agent is not the nanoparticles themselves, but silver ions Ag+, dissociating from the surface [1-4]. However, the molecular mechanisms underlying the antitumor activity of silver ions and the molecular triggers responsible for the activation of these mechanisms have not yet been identified [5]. In addition, there are no studies in which the in vivo effect of silver is studied in, presumably, the most active, ionic form, where the most soluble salt, silver nitrate, acts as an ion donor. This can be explained by the high general and local toxicity of silver nitrate when administered into the bloodstream, in particular, due to the formation of nitric acid during the interaction of silver ions with sulfide groups of proteins and amino acids, toxicity of nitrate ion, as well as the formation of insoluble particles of silver chloride during the interaction of Ag+ with Cl- ions present in the blood. One of the problems of silver nanoparticles that prevent their systemic administration is the presence of a metal core that prevents their biodegradation and excretion from the body.

The aim of this work is to study the toxicity of silver nitrate and silver-cysteine nanocomplexes, as well as to develop a method for the synthesis of these nanocomplexes.
2. Materials and methods

In this work, we used cell cultures: K-562, human chronic myelogenous leukemia, human cervical carcinoma cell line - HeLa, Capan-2 - human pancreatic adenocarcinoma, CT-26 - mouse colon carcinoma, Mouse embryonic fibroblasts 3T3b and transformed embryonic 3T3 fibroblasts -SV40 / All cell cultures were obtained from the Russian collection of vertebrate cell cultures, Institute of Cytology, Russian Academy of Sciences. HeLa, 3T3b, and 3T3-SV40 cells were cultured in DMEM medium (HyClone, USA), and K-562, CT-26, Capan-2 cells were cultured in RPMI-1640 medium (HyClone) supplemented with 10% fetal bovine serum (HyClone, USA), in the presence of 40 μg ml-1 gentamicin (Sigma Aldrich, USA) at 37 ° C in an atmosphere of 5% CO2 Human peripheral blood mononuclear cells (PBMC) of peripheral blood of healthy donors were obtained by centrifugation in a density gradient Ficoll-Paque PLUS (GE Healthcare , USA) according to the standard method. with the addition of 10% fetal bovine serum in the presence of 100 units / ml penicillin, 100 μg / ml streptomycin (BioLot, Russia) and 2 mM glutamine.

To determine the cytotoxic activity of the studied substances in vitro, flow cytometry and colorimetric MTS test were used. To determine the cell viability by the colorimetric method, the MTS Cell Proliferation Colorimetric Assay Kit (BioVision, USA) was used in accordance with the following protocol: cells were pre-scattered in a 96-well plate in the amount of 10 thousand per well, incubated for 24 hours, exposed to silver nitrate and incubated for another 24 hours. Then, 20 μl of MTS reagent was added to each well and the plate was incubated for another 2 hours. The optical absorption was measured at a wavelength of 490 nm using a Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific, USA). Cell survival was determined using the following formula:

\[
\text{Cell viability} = \frac{A_{\text{sample}} - A_{\text{medium}}}{A_{\text{control}} - A_{\text{medium}}} \times 100\%
\]

where \( A_{\text{sample}} \) is the optical absorption value in the well, \( A_{\text{medium}} \) is the optical absorption value of the medium, \( A_{\text{control}} \) is the control optical absorption value.

The study of the size and morphology of the obtained nanoparticles was carried out using dynamic light scattering methods. The hydrodynamic diameter of these complexes for various ratios (R) was measured by dynamic light scattering on a ZetaSizer Nano ZS device, and the electrokinetic potential of the obtained complexes was also determined. The electrokinetic potential of nanoparticles was measured by electrophoretic light scattering.

3. Results

Earlier in the study of the cytotoxic and antitumor activity of silver nitrate, we showed that silver nitrate has a cytotoxic effect, as a result of the study, an IC50 value for the HeLa and K-562 tumor lines was obtained, which is 20 times higher than the IC50 for of human peripheral blood mononuclear cells. It has also been shown that silver nitrate has an antitumor effect against Ehrlich's solid tumor, but the compound also has systemic and local toxic effects [6]. Presumably, one of the mechanisms of local toxicity is the release of nitric acid during the interaction of silver ions with sulfide-containing compounds, such as cysteine, the concentration in the blood of which is normally 166.6-249.9 μM, which, with the introduction of silver nitrate (excluding buffer capacity of blood), can cause a local decrease in blood pH to 4 at a normal value of 7.5. The nitrate ion, in turn, is also a toxic compound. A possible way to reduce the systemic and local toxicity observed with the introduction of pure silver nitrate is the synthesis of non-metallic complexes that do not contain toxic nitrate ions. The simplest, most accessible and physiological complexing substance can be the amino acid cysteine, which can effectively bind Ag + ions. In the course of the work, a general synthesis scheme was developed, and the optimal conditions for the synthesis and purification of such complexes were selected.

When an aqueous solution of cysteine interacts with silver nitrate at different ratios \( R = \text{Cys: Ag} \), nanosized complexes are formed. The hydrodynamic diameter of these complexes for different R was measured by the method of dynamic light scattering, and the values of the electrokinetic potential of
the obtained complexes were also determined. Figure 1 shows the position of the distribution peak in terms of scattering intensity, volume, z-average value, as well as the value of the electrokinetic potential.

![Graphs showing the position of the maximum on the distribution of the hydrodynamic diameter by the intensity of the scattered light, the hydrodynamic diameter over the volume occupied by the scattering dispersed phase, z-average value, and electrokinetic potential values.](image)

**Figure 1.** (a) - the position of the maximum on the distribution of the hydrodynamic diameter by the intensity of the scattered light; (b) - the position of the maximum on the distribution of the hydrodynamic diameter over the volume occupied by the scattering dispersed phase; (c) - z-average value; (d) - electrokinetic potential values. The values are given for the crude complexes.

The complexes under study have a characteristic size of several tens of nanometers up to the ratio \( R = 1: 2 \); with an increase in the mole fraction of silver nitrate in the initial mixture to 2.5, the hydrodynamic diameter increases to \( \sim 100 \) nm, and upon reaching \( R = 1: 4 \) it reaches a micron size. The electrokinetic potential of the investigated particles increases uniformly with an increase in \( R \), when the ratio Ag: Cys = 1: 1, its value is 35 ± 3.78 mV, with an increase in \( R \) to 1: 4, the value of the electrokinetic potential increases to 52 ± 4.4 mV.

When measuring the spectra of ultraviolet and optical absorption of the obtained complexes, it was shown that the absorption values increase with an increase in the ratio \( R \) over the entire investigated wavelength interval. The absence of a broad plasmon resonance peak at \( \lambda = 410 \) nm, characteristic of metallic silver nanoparticles, confirms the non-metallic nature of the synthesized complexes (Figure 2).
Figure 2. UV and optical absorption spectra of AgCys complexes before purification

The measured pH value of the solution after the formation of AgCys complexes was 1.5, which makes the crude complexes unsuitable for both in vivo and in vitro studies.

After the obtained complexes were purified from nitric acid as well as the residues of unreacted starting compounds, the pH value of the resulting solution was 7, which indicates the absence of nitric acid and the potential applicability of the complexes for in vitro and in vivo studies. The purified complexes were analyzed on a ZetaSizer Nano ZS device, the position of the peak of the distribution by scattering intensity, volume, z-average value, as well as the values of the electrokinetic potential are shown in Figure 3.

Figure 3. (a) - the position of the maximum on the distribution of the hydrodynamic diameter by the intensity of the scattered light; (b) - the position of the maximum on the distribution of the hydrodynamic diameter over the volume occupied by the scattering dispersed phase; (c) - z-average value, (d) - electrokinetic potential values. The values are given for the purified complexes.
In contrast to the crude complexes, the size dependence on the Ag: Cys ratio is not directly dependent. At the ratio $R = 2$, the minimum value of the hydrodynamic diameter is observed ($z$-average = 50 nm), there is no increase in size up to hundreds of nanometers and microns at the ratios $R > 2$. Apparently, the initial complexes aggregate and lose their individuality during centrifugation; further reconstitution by ultrasonication leads to the formation of new particles, the size of which depends on both the parameters of the acting ultrasound and the internal physicochemical properties. Figure 4 shows the data of the spectrophotometric study of the obtained complexes.

![Figure 4](image)

**Figure 4.** Ultraviolet and optical absorption spectra of the purified complexes.

As in the case with the initial complexes, there is no broad plasmon resonance peak at a wavelength of $\lambda = 410$ nm. Thus, the larger size of the initial complexes formed at ratios $R > 2$ is an advantage, allowing the isolation of a larger amount of the purified product. This is due to the almost complete sedimentation of particles larger than 100 nm in comparison with small particles formed at low ratios of silver and cysteine ions.

Based on the results of the synthesis and purification of silver-cysteine complexes, we carried out work on the study of the antitumor activity of these complexes in vitro on various tumor and normal, human and mouse cell cultures. As in the case of using silver nitrate, it was shown that cell lines with the highest proliferation rate (Figure 5)

![Figure 5](image)

**Figure 5.** Survival curves of various cell cultures exposed to AgCys complexes with $R = 2.5$. (a) survival curves for human cell lines, (b) survival curves for mice.
As a result of the study, the concentration values of the half-inhibition of the AgCys complexes were obtained for various cell cultures (Table 1).

**Table 1.** Concentration values of the half-inhibition of AgCys complexes for various cell cultures.

| Cell culture                          | IC\(_{50}\), μg / ml |
|---------------------------------------|----------------------|
| K-562                                 | 22.2±1.61            |
| HeLa                                  | 36±2.37              |
| Human peripheral blood mononuclear cells |                      |
| CT-26                                 | 9±2.17               |
| Capan-2                               | 50±1.94              |
| 3T3b                                  | 40±2.46              |
| 3T3-SV40                              | -                    |
| Human fibroblasts                     | 10±0.94              |

In the range of the investigated concentrations, no toxicity for of human peripheral blood mononuclear cells was observed, which indicates the selectivity of the action in relation to tumor cells.

**4. Conclusion**

As a result of the work performed, a method for the synthesis of silver-cysteine nonmetallic nanocomplexes was proposed, and the optimal conditions and concentrations were investigated and selected. The antitumor activity of silver-cysteine complexes was shown in various normal and tumor cell cultures of humans and mice.

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