Research Paper

In vitro Effects of a Novel Silver-based Complex on Influenza Virus

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Abstract

Objective: To study the mechanism of antiviral activity of a new silver-based compound of general formula C6H19Ag2N4 LiO6S2 on influenza virus. **Materials and Methods:** Antiviral properties were investigated on test culture of Madin–Darby canine kidney (MDCK) cells with serial tenfold dilutions of the influenza virus. The completeness of viral inactivation was confirmed by determining the virus titer after a double passage of virus-containing suspension in MDCK cell culture. Reverse transcription polymerase chain reaction analysis of viral RNA, enzyme immunoassay, and transmission electron microscopy for morphological analysis of influenza virus particles were applied for analysis of viral RNA and effect of substance influence. **Results:** The results indicate a significant change in the antigenic structure of viral proteins of influenza virus by the silver-containing complex. The main inactivation mechanism of influenza virus by the action of the silver-containing complex is associated primarily with the effect on virion protein structure rather than cleavage of the viral RNA. It was revealed that the mechanism for the inactivation of influenza virus is not associated with cleavage of viral RNA corresponding genes M, NS, or nucleoprotein but more probable with changes of the antigen determinants of virion. It was found a significant damaging effect on influenza virus under action of tested substance at concentration higher than 0.4 mM. **Conclusion:** The novel silver-based complex possesses antiviral properties and could be considered as a prospective antiviral drug.

Keywords: Antiviral activity, influenza virus, monoclonal antibodies, polymerase chain reaction, silver-based complex

Introduction

Influenza is a widely spread and serious disease and still big problem for epidemiology. In this connection, the development of new antiflu therapies and prevention of other airborne viral infections is an urgent task. It is well known that silver is used in medicine for a long time. Nowadays, interest in silver drug growth significant because of problem of infectious diseases and drug resistance. Multidrug resistance becomes one of the main challenges for future medical science. Silver has been studied for its antibacterial potential. However, some authors have also proven the effect of silver against several types of viruses including HIV, respiratory syncytial virus, hepatitis B virus, herpes simplex virus, and monkeypox virus.[1-3] Recently, a lot of reports was published on antiviral and antibacterial properties of silver nanoparticles, including antiviral activity against H1N1 influenza A virus.[4-6] These work showed that metal nanoparticles could be used for novel antiviral therapies. Metal ions may attack multiple targets in the virus. It decreases the development of drug resistance as compared to conventional antivirals. It is especially important for intravenous injections or other routes of administration. Application of nanoparticles in medicine is significantly shorted due to the physical properties. It is especially important for intravenous injections or other routes of administration. Nanoparticles have significant antiviral properties but also possess a toxic effect, particularly, genotoxic.[7] Ionic silver have anti-infectious properties.[8] Thus, the development of novel soluble and low-toxic silver drugs looks promising. A previous study has shown silver as more effective antiretroviral

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substance with non-severe toxic effects in comparison with the nevirapine as standard drug.[9] Recently, novel soluble silver-cystine lithium complex of general formula $C_6H_{19}Ag_2N_4LiO_6S_2$ with low toxicity was investigated.[10] This compound possesses an antiviral activity *in vitro* and *in vivo*. Previously, we established that total viral isolated RNA were completely cleaved for 1 h at 37ºC and RNA within influenza A virions for 2 h in the presence of high concentrations (2 mM) of the silver complex.[11]

The aim is to study the antiviral activity against influenza virus of silver-based compound ($C_6H_{19}Ag_2N_4LiO_6S_2$) and main "targets" identification. To determine and evaluate antiviral activity of the substance, we used following techniques, including reverse transcription polymerase chain reaction (RT-PCR) analysis of viral RNA, enzyme immunoassay, and morphological analysis of influenza virus particles.

Materials and Methods

Tested antiviral compound

The water-soluble silver compound of general formula $C_6H_{19}Ag_2N_4LiO_6S_2$ was prepared in laboratory according to the protocol described in patent.[12] The synthesis procedure includes mixing cystine, silver nitrate, lithium hydroxide, and ammonia in a molar ratio of 1:2:2:8. The reaction mixture was stirred at room temperature for 6 h. Then, the solution was evaporated up to quarter of the initial volume at 40°C in vacuum. Then, we added ethanol. The obtained mixture was incubated at around 4 to 6°C for 12 h. Then, yellow precipitate was filtered, washed, dried, and used for experiment.

Analysis of the mechanism of inactivation of the influenza virus

Determination of the influenza virus titer in the cells

The titer of the influenza virus was determined by counting the number of foci-forming units in the 96-well culture plate using Madin–Darby canine kidney (MDCK) cells. For this, serial tenfold dilutions of influenza virus in Dulbecco's Modified Eagle Medium was added to a monolayer of MDCK cells and then cultured for 24 h. Following incubation, the medium was removed; cells were washed twice with a solution of polyvinyl alcohol. Cells were fixed for 15 min at room temperature in 80% cold acetone (100 µl per well). The wells were washed twice with phosphate-buffered saline (PBS) solution and were added 50 µl of a solution of monoclonal antibody in PBS (using antibodies specific for the nucleoprotein (NP) of influenza virus Type A in a dilution of 1:4000) and was incubated for 1 h at 37°C, at the end of incubation wells were washed 4 times with PBS. The wells were added with secondary antibody of 50-µl conjugate solution in PBS, biotinylated anti-mouse immunoglobulin G (IgG) monoclonal antibodies (MAbs) at a dilution of 1:3000, was incubated for 1 h at 37°C and then washed with PBS. Cells were stained with 100 µl of streptavidin-peroxidase complex diluted to 1:2000 in PBS for 30 min at 37°C, then the cells were washed 4 times with PBS.

Immediately before staining, a solution in dimethylformamide chromogen 3-amino-9-ethylcarbazole (AEC) with a concentration of 8 mg/ml was prepared. About 500 µl of AEC was mixed with 10 ml H_2O, 200 µl sodium acetate (pH 5.8), and 50 µl of 3% H_2O_2. The wells were added with 100 µL of the prepared solution and was heated for 30 min in the dark place at 25°C and then washed with PBS.

Counting of painted infectious foci was performed using an optical microscope. The colored cell or group of cells that are separated from the other stained foci by two unstained cells was counted as one fixed flexion contracture (FFC) (focus-forming units). Each data point was reproduced in four replicates.

Inactivation of influenza virus

Reaction mixtures in volume of 140 µl, containing influenza virus at a concentration of 10^6 FFC/ml, 200-mM KCl, 50-mM Tris-HCl, and 1-mM ethylenediaminetetraacetic acid, were incubated in the presence and the absence of test substance at a concentration of 0.8 mM, at 37°C for 18 h. The completeness of viral inactivation was confirmed by determining the virus titer after a double passage of virus-containing suspension on MDCK cell culture.

Reverse transcription polymerase chain reaction analysis of virus RNA

After incubation, the viral suspension in the presence or absence of a silver-containing complex, viral RNA was isolated using the QIAamp Viral RNA Extraction Kit (QiaGen, Germany) according to the protocol from a suspension containing the influenza virus at a concentration of 10^6 FFC/ml. Suspension was added 560 µl of lysis buffer and 5.6 µg of RNA carrier, and further all procedures were carried out according to the manufacturer's protocol. RNA was washed from the column using 80-µl buffer AVE. The resulting RNA samples were used for RT-PCR or stored at − 20°C until further analysis. For reverse transcription, 10 µl of viral RNA was used to prepare the cDNA in the presence of 100-units reverse transcriptase Moloney Murine Leukemia Virus. Reverse transcription was performed for 1 h at 37°C in the presence of Uni12 (AGCAAAAGCAGG): 10 µl solution of the viral RNA and 1.25 mM of each dNTP in a buffer containing 10 mM Tris-HCl pH 8.0, 75 mM KCl, 5 mM $MgCl_2$, and 0.01 M DTT, in a total volume of 30 µl. Upon completion of the reverse transcription reaction, the reaction mixture was heated to 90°C for 5 min. For the amplification of full-length fragments of the genome of influenza virus we used primers [Table 1] described previously.[13] PCR was performed in a buffer containing 100 mM Tris-HCl (pH 8.3), in the presence of 500 mM KCl, 1.5 mM $MgCl_2$, 0.01% Tween 20 in the presence of 4 units Taq DNA polymerase, 0.25 mM of each dNTP, 10 µM primers indicated in Table 1, and using 2 µl of cDNA template.

PCR was conducted under the conditions of initial denaturation of DNA at 90°C for 2 min, followed by 10 cycles of denaturation at 94°C for 30 s, primer annealing for 30 s at

Table 1: Sequences of the oligonucleotides used in this work

n	Primer[a]	Length of PCR product	Sequence 5'→3'
1	NP_f	1565+29	TATTCGTCTCAGGGAGCAAAAGCAGGGTA
	NP_r		ATATCGTCTCGTATTAGTAGAAACAAGGGTATTTTT
2	M_f	1027	TATTCGTCTCAGGGAGCAAAAGCAGGTAG
	M_r		ATATCGTCTCGTATTAGTAGAAACAAGGTAGTTTTT
3	Ns_f	890+29	TATTCGTCTCAGGGAGCAAAAGCAGGGTG
	Ns_r		ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT

[a]f corresponds to the forward primer. r: Reverse; PCR: Polymerase chain reaction

a temperature decreased by 1°C with 64°C–54°C and 70°C for 4 min and 15–19 cycles of denaturation at 94°C for 30 s, primer annealing at 56°C for 30 s, and 68°C for 6 min. The PCR products were separated on a 1% agarose gel using TBE buffer, the product was visualized by ethidium bromide staining using a gel documentation system "Infinity"1500/36M (Vilber Lormat).

The influence of the silver-containing complex on morphology of influenza virus

Influenza virus suspension was treated with a silver-containing preparation for several times at 37°C. Influenza virus suspension, incubated at 37°C for 1 and 20 h were used as a control. Changes in the morphology of influenza virus particles when exposed to the test compounds was studied by negative staining. After incubating with the complex, samples of influenza virus were adsorbed on coated copper grids for 1 min and then contrasted 2% uranyl acetate solution for 5 s. Excess fluid was removed with filter paper. Samples were examined under the electron microscope JEM 1400 (Jeol, Japan) at an accelerating voltage of 80 kV. Images of the virus particles were obtained using a digital camera Veleta (SIS, Germany).

Study of the influence of silver-containing complex on antigenic determinants of influenza virus by binding monoclonal antibodies to influenza virus

Influenza virus, incubated for 20 h at 37°C with the silver-containing complex in PBS buffer were adsorbed onto plates for immunological reactions ("Medpolimer" Saint Petersburg) for 24 h at 4°C. Virus samples, incubated in the same conditions as the test sample, were used as a control. To increase binding of influenza virus to plates, fetuin was previously adsorbed at 1 μg per well. In order to suppress nonspecific binding to the wells was added a solution of 1% BSA and 0.05% Tween-20 for 2 h at room temperature.

Next, we prepared two-fold dilutions of MAbs to the hemagglutinin (HA) of influenza virus (CDC Atlanta); and MAbs to the NP of influenza virusMAb 8258 (Chemicon International), and added 100 μl to the wells for 1 h at 37°C. Unbound antibodies were removed. Antispecies biotinylated antibodies (Chemicon international) IgG at a dilution of 1:4000 and streptavidin-peroxidase complex (MP Biomedicals) in a dilution of 1:12000 were added sequentially and incubated for 1 h at 37°C. Then, 100 μg per well of o-phenylenediamine was added and kept in the dark for 15 min. Thereafter, the reaction was stopped by adding 50 μl of 0.18 M sulfuric acid. The optical density of the solutions was determined on a multichannel spectrophotometer Multiscan RC at a wavelength of 492 nm. To compensate for the random errors, results were determined according to average value of 3–4 data points.

Statistics

Statistical analysis was performed with Statistica (Statsoft, Oklahoma, USA). We used nonparametric Mann–Whitney U-test for comparing independent samples. Statistical differences were considered as significant at $P < 0.05$.

Results

Analysis of the RNA destruction role in mechanism of inactivation of the influenza virus

Analysis of the role of the RNA cleavage in the inactivation mechanism of influenza virus by silver-containing complex was carried out using the method of RT-PCR. As a control, the amplification of viral RNA incubated in the absence of compound was used. Results of the study of viral RNA isolated from the virus, incubated in the presence of the complex, are presented in Figure 1.

Thus, on the basis of obtained data, it can be concluded that the mechanism of inactivation of the influenza virus at tasted concentration is not associated with cleavage of viral RNA.

The influence of the silver-containing complex on morphology of influenza virus

Preparations of influenza virus which were incubated for 1 and 20 h at 37°C, contained round-shaped virions mainly sized of 80–100 nm with distinct spines on the surface. The structures of the viral particles were as usual. The preparations also included membrane fragments and "fragments" of cellular structures, structureless substance. No significant differences between preparations, which incubated for 1 and 20 h was observed.

On the next step, influenza virus suspension was incubated with the silver-containing complex for 20 h at 37°C. Incubation did not result in significant changes in the number and morphology of the virions compared with the control.

Study of the effect of silver-containing complex on antigenic determinants of influenza virus

In experiments, a set of MAbs was used. The virus was treated with a test complex in concentrations ranging from 0.2 mM to

0.8 mM. Preservation of antigenic determinants was evaluated using ELISA based on the protein binding efficiency with MAbs.

It was shown that incubation of influenza virus with the test silver complex destroys antigenic determinants of the surface protein of influenza virus, which reduces the efficiency of MAb binding to a viral HA [Figure 2a] and did not significantly affect the binding efficiency of MAb to the antigenic determinants of the viral NP [Figure 2b] at low dosage.

Discussion

In recent years, a large number of new antiviral substances of chemical and plant origin have been actively developed and the mechanism of their antiviral action is being investigated.[14-17] Despite this, silver remains a very effective anti-infective agent with great potential. Silver in different form inhibits viruses and can overcome the resistance of the influenza virus to antiviral drugs.[18] However, little is known about the exact mechanism of antiviral activity. Previously, it has been suggested that silver in the form of nanoparticles binds with a viral envelope glycoprotein and inhibits the virus by binding to the disulfide bond regions of the CD4 binding domain within the HIV-1 viral envelope glycoprotein gp120.[19,20] Recently, it has been demonstrated that silver inhibits vaccinia virus infection by preventing viral entry through a micropinocytosis-dependent mechanism. It can be assumed that Ag^+ inactivates viruses by denaturing thiol-containing enzymes by analogy with the antibacterial activity.[21] However, there are no data to support this hypothesis. It was also shown that silver ions might form stable complexes with nucleic acids.[22] It has been previously shown that the influenza virus genomic RNA can serve as suitable targets for antiviral drugs.[23,24] It was found that the analyzed complex in high concentrations (2 mM) is also capable of degrading viral RNA.[11] Results of this work provide more detailed study of some hypothetical mechanisms of influenza virus inactivation under the influence of ionic silver in the composition of the metal complex with cystine. All results were obtained *in vitro* conditions and required further *in vivo* testing. Capability of cleaving RNA at a concentration of 2 mM is a pivotal factor for antiviral activity *in vivo*. However, this action is strongly dose dependent. Incubation of virus in the presence of a test compound in a concentration of 0.8 mM did not lead to decrease efficiency of amplification products corresponding to matrix M, nonstructural NS, or NP genes [Figure 1a-c] compared with the level of efficiency of the amplification of the gene fragment NS after inactivation of the virus in the control. Thus, the absence of cleavage of the viral RNA under the influence of silver complex in low concentrations could not explain antiviral effect of silver complex. Moreover, the influence of the silver-containing complex on morphology of influenza virus was not clearly detected. Incubation of influenza virus for 20 h at 37°C with and without silver complex reveals no significant

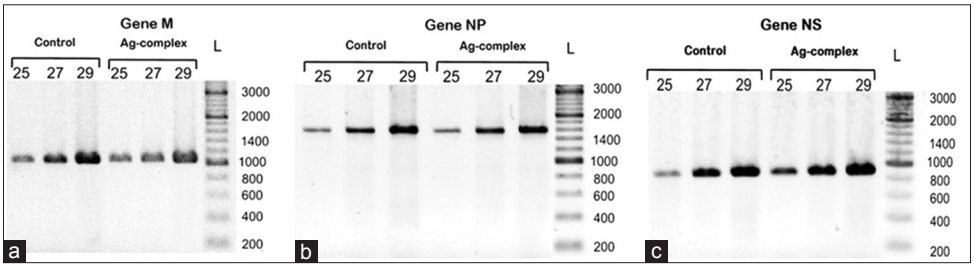

Figure 1: Reverse transcription polymerase chain reaction analysis of genomic DNA isolated from influenza virus, inactivated with silver-containing complex. Reverse transcription polymerase chain reaction analysis of the gene encoding the M (a) gene, nucleoprotein (b), and NS gene (c) RNA isolated from the virus suspension after treatment and from control virus in the absence of the complex. The concentration used for inactivation was 0.8 mM of tested complex. Polymerase chain reaction products were separated in 1% agarose gel and stained with ethidium bromide. The numbers indicate the amount of amplification cycles. Lane "L"-molecular marker of product length

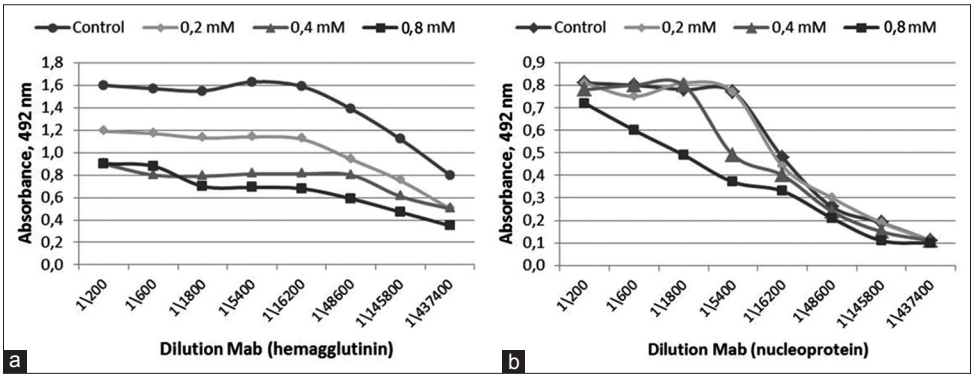

Figure 2: Binding of MAbs specific for influenza virus proteins: hemagglutinin (a) and the nucleoprotein (b) with influenza virus after incubation with the test compound at a concentration 0.2 mM, 0.4 mM, and 0.8 mM. Control samples were incubated in the same conditions without test compound

changes. Viral particles had a size of 80–100 nm, as in the control and distinct spines on the surface. Silver nanoparticles have a more pronounced effect on the morphology of the virus.[25] The studied silver organic substance has a milder effect on the morphology of the influenza virus in comparison with metallic silver. However, we found that test silver complex destroys antigenic determinants of the surface protein of influenza virus, which provides of MAbs binding to a viral HA, that is, viral antigenic determinants damage was revealed. Increasing concentration of the silver compound increases deformation of the viral protein that could prevent viral HA binding to receptor on host cell. It plays an important role in antiviral activity of silver substance. Another way of anti-influenza effect is decreasing enzymatic activity of viral neuraminidase through the breakage of disulfide bonds by silver.[26] However, incubation of the virus with the silver-containing complex in a concentration of 0.2 mM and 0.4 mM did not significantly affect the binding efficiency of MAb to the viral NP antigenic determinants that are consistent with previous data.[11] The noticeable effect on NP appears at a concentration of 0.6 mM and higher. Viral HA is more susceptible to the damaging effect of silver and is an important target. This reduces the binding of the virus to the sialic acid of the cell membranes. These results indicate changes in structure of influenza virus proteins under the influence of tested silver- cysteine lithium complex.

Conclusion

Thus, based on the results of this work, we conclude that inactivation action of the silver-containing complex on influenza virus is associated primarily with the effect on virion protein structure. Cleavage of the viral RNA occurred only at high concentrations of complex. Notably, silver compound does not cause significant changes in the morphology of influenza virus particles, but it has dose-dependent proteins damaging effect. The tested silver-containing complex could be a prospect drug and required further research.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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