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Antiviral Properties and Toxicity of Ag-Cystine Complex

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Abstract

Water-soluble complex of Ag (I) with cystine (AC-1) had been designed and synthesized as low toxic nuclease. Both cellular and viral isolated RNA were completely cleaved for 1 hour at 37°C; RNA within influenza A virions - for 2 hours in the presence of 2 mM AC-1. The RNase activity of AC-1 was accompanied by absence of a damage of the viral proteins as shown by semi-quantitative ELISA with specific antibodies, hemagglutination and hemagglutination inhibition titering. To detect binding of living cells and viruses with potential drugs a novel label-free real-time approach based on long range surface waves on one-dimentional photonic crystal surface in micro fluid channel was used. Antiviral properties of AC-1 were shown both *in vitro* and *in vivo*. Protection index of AC-1 after multiple peroral administrations was 77.8 ± 13.9%. Cytotoxicity of AC-1 varied in a range 0.01-0.04 mM for different tissue cultures. Its toxicity for mice appeared to depend on the administration way. The absence of the biosensor-detected binding of AC-1 with blood serum and cellular proteins corresponded to its limited toxicity. Permanent binding of the *Influenza A* virus with AC-1 revealed its antiviral potential. Taking into consideration new molecular target - RNA, a relatively low toxicity after multiple peroral administrations and evident anti-influenza virus properties one could conclude that the novel low-molecular-weight RNase AC-1 without immunogenic and allergenic activities might be used for an inactivated vaccine preparation and for combined therapy of the influenza.

Keywords: Low-molecular-weight artificial RNase; *Influenza A virus*; Permissive tissue cultures; Reverse transcription - real time PCR; Label-free real time optical detection; Protection index

Introduction

High mutation rate of RNA-containing viruses and rapid rearrangements of viral quasi species cause their resistance to available drugs. Innovations and implementations of new antiviral medications remain insufficient. Despite widely used inactivated and subunit vaccines against influenza as well as highly specific and sensitive diagnostics the influenza annual outbreaks remain public health concern [1,2]. Resistance of the *Influenza virus* to known drugs may inspire a search of new molecular targets for recommended combined therapy. Main criteria for anti-viral drugs should include a selective inactivation of certain viruses, a minimal possible influence on host cellular biopolymers, a prolonged action to avoid multiple frequent administrations as well as should take into consideration both innate resistance and immunity status of patients. Nucleoside analogs are competitive inhibitors of both viral and cellular enzymes and consequently are toxic. Non-nucleoside inhibitors of viral proteases, reverse transcriptases, integrases, and neuraminidase of *Influenza virus* are more specific and, therefore, less harmful compared to nucleoside derivatives. High molecular weight proteins such as specific immunoglobulins and RNases are not known to be able to penetrate into both host cells and enveloped viruses and therefore currently excluded from a consideration as potential specific anti-viral medications.

The ubiquitous RNases in body fluids varying in a range from 12.3-13.7 kDa for human urine to 45 kDa for serum RNase and 150 kDa for its aggregates hampered to assign a role for these enzymes. Protective role of RNase in milk against retroviral infection has been envisaged [1]. RNA-containing *Orthomyxoviruses* seem to be sensitive to the elevated levels of RNases in blood [2]. Therefore, a research of artificial RNases with low molecular weights providing possible penetration into *virions* and into infected cells are promising directions of anti-viral drug development during the last 20 years.

Currently known unspecific artificial RNases include both metal-free compounds and complexes of transition metals Cu^{2+} [3], Zn^{2+} [4], Ln^{3+} [5], Eu^{3+} [6] with organic ligands, biogenic amines [7], some peptides [8,9] and other low molecular weight organic compounds [10-12] capable to cleave phosphodiester bonds of certain dinucleotides (CpA and UpA motifs). Site-specific artificial ribonucleases are based on antisense oligonucleotides [13] and ribozymes and, therefore, capable to cleave specific sequences [14-16]. Despite successful inactivation of viruses by using specific [17] or unspecific [18] artificial RNases *in vitro* their penetration into eukaryotic cells and stability remain essential problems hampering their implementation.

Our aim was to study antiviral properties and toxicity of a novel complex of Ag (I) with cystine.

Materials and Methods

Tissue cultures

Madin-Darby Canine Kidney (MDCK) epithelial cells, mouse subcutaneous connective tissue L929, green monkey kidney Vero cells and porcine embryo kidney PS cells were obtained from the Russian State Tissue Culture Collection (D.I. Ivanovsky Institute of Virology of the Federal Research Center of Epidemiology and Microbiology of N.F.

Gamaleya of the Russian Ministry of Health, Moscow, Russia) and grown in Eagle minimal essential medium (EMEM) supplemented with 10% fetal bovine serum (HyClone, "Thermo Scientific", USA) in the presence of 100 U/ml penicillin and 100 U/ml streptomycin.

Viruses

Influeza A virus of subtypes *H1N1* (strain A/swine Iowa 15/30) and H3N2 (strain A/Aichi/1/68) were from the Russian State Collection of Viruses (D.I. Ivanovsky Institute of Virology of the Federal Research Center of Epidemiology and Microbiology of N.F. Gamaleya of the Russian Ministry of Health, Moscow, Russia).

Antiviral drugs

Water-soluble silver (I) complex with cystine $Li^{+}[Ag^{+}_{2}Cys_{2}^{-}(OH^{-})_{2}(NH_{3})_{2}]$ (AC-1) had been synthesized as earlier described [19]. In brief, the silver-cistine compound was prepared at room temperature by mixing cistine, lithium hydroxide, silver nitrate and ammonia in a molar ratio of 1:2:2:8. The reaction mixture was stirred at room temperature for 6 hours, and then evaporated at 40-50°C in vacuum up to 1/4 of the initial volume. After addition of ethanol the mixture was cooled and incubated at +4-+6°C for 12 hours. The resulting water-soluble yellow fine precipitate was filtered, washed with ethanol and dried.

RNA isolation

Total RNA were isolated from control MDCK cells and those infected with the *Influenza virus A* (H3N2 or H1N1) by lysis in guanidine isothiocyanate, phenol-chloroform deproteinization with subsequent alcohol precipitation [20]. Isolated RNA before and after incubation with AC-1 was analyzed by electrophoresis in SDS-agarose gels with subsequent ethidium bromide staining.

Cleavage of nucleic acids

AC-1 was dissolved in RNase-free sterile tridistillated water. The isolated total RNA (5-10 μg in 5 μl) was incubated with 5 μl AC-1 solutions at 37°C for 1 h. Then reaction products were assayed in reverse transcription with random N6 primers ("AmpliSens", Moscow, Russia) with subsequent real-time PCR with SyberGreen I and primers specific to the *Influenza A virus* (A/Aichi/2/1968(H3N2)) hemagglutinin gene (GenBank (http://www.

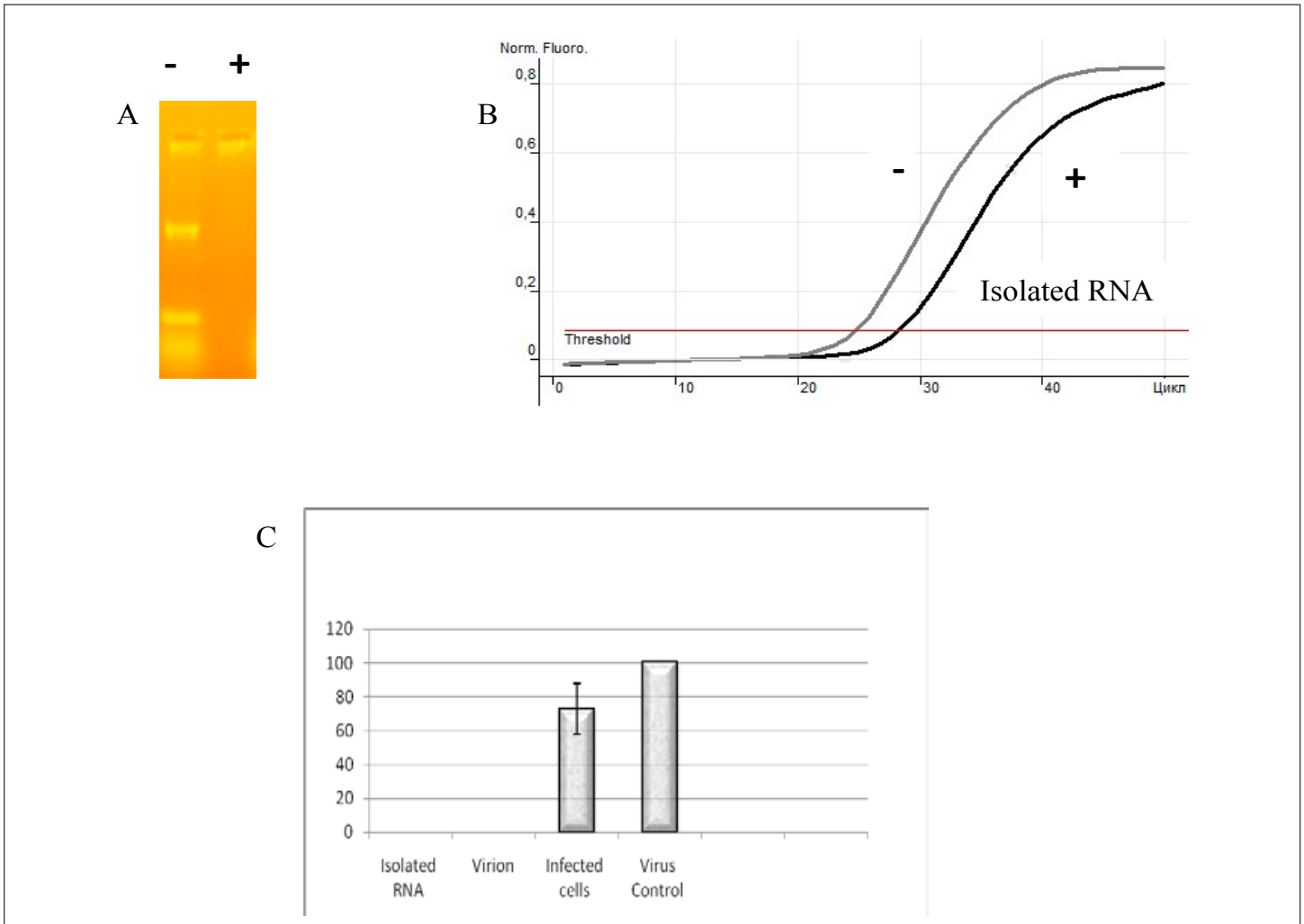

Figure 1: Cleavage of total RNA (A) and the influenza A virus RNA (parts B and C) after incubation with 2 mM AC-1 at 37°C.

A- Electrophoregram of total isolated RNA before (lane "-") and after (lane "**+**") incubation with AC-1 in SDS-2% agarose-TAE gel with subsequent ethidium bromide staining.

B- Results of reverse transcription with subsequent real time PCR with Syber Green I of the influenza virus RNA before (lane "-") and after (lane "**+**") incubation of the influenza virus-infected MDCK cells with AC-1.

C- Diagram of the viral RNA cleavage in virions and infected cells compared to isolated RNA (left, as a positive control of RNase activity of AC-1) and RNA without incubation with AC-1 (right, negative control).

ncbi.nlm.nih.gov) accession number J02090)

InAA-F 5'- TCTGTCTGGCTCTCGGCC -3' and

InAA-R 5'- GATTGTTGCATATTTTCCCCG-3' synthesized in "Syntol", Moscow, Russia.

ELISA

The *Influenza virus* antigens were revealed in hemagglutination and hemagglutination inhibition tests, as well as in semi-quantitative ELISA with immobilized polyclonal antibodies, secondary antibodies against mouse IgG with horseradish peroxidase with subsequent staining with orthophenylendiamine [21]. Immunoascitic fluid was used as a positive control and PBS as a negative control.

Washed human erythrocytes (blood group O) were used at a concentration of 0.75%. The lowest virus (A/Aichi 1/68) concentration that agglutinated erythrocytes was determined after 60 min [22].

Real time optical detection

Photonic crystal (PC) [23-25] (http://pcbiosensors.com) was sonicated in isopropanol and water and treated in plasma cleaner. All further procedures were performed while PC was mounted to the flow cell of the Biosensor EVA 2.0 (http://pcbiosensors.com). The baseline (the thickness and refraction index) was registered in the running buffer at the beginning of all measurements. The clean PC was rinsed by 0.1 mg/ml polyallylamine (pAA) 65. After washing to remove an unbound pAA65 with water 0.1% fresh glutaraldehyde was added for further binding with primary NH_2-groups of proteins. Modification of the PC surface was controlled in real time and continued for less than 1 min at each stage. After the baseline stabilization, a PC was exposed to subsequent solutions of proteins or AC-1 as well as cellular or the *Influenza virus* suspensions as described in results and shown on figures.

Morphology of the eukaryotic cells onto aminated PS was controlled by staining with trepane blue according to [22].

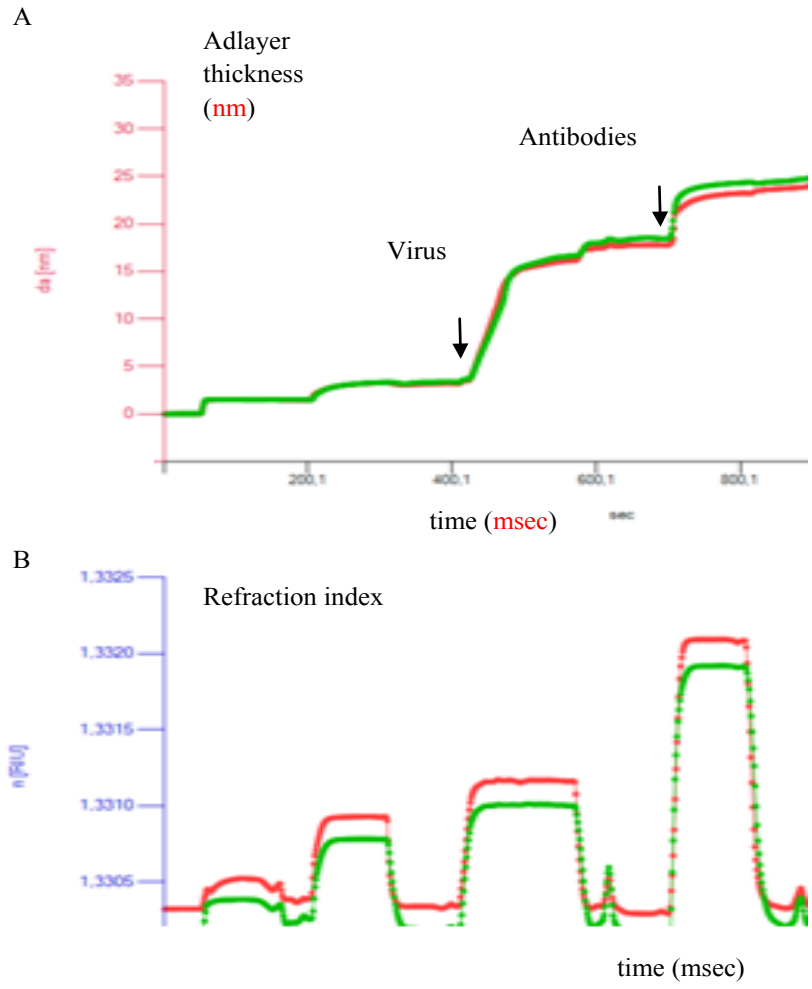

Figure 2: Conformational stability of the influenza virus antigens.
Results of the label-free real time optical detection of binding of the influenza A virus before (green curve) and after incubation with 16 mM AC-1 (red curve) with the specific mouse polyclonal antibodies. Arrows show stages of subsequent addition of the influenza A virus (left arrow) and the virus-specific polyclonal antibodies (right arrow). Coincidence of 2 curves of optical detection of binding of the virus antigen with the corresponding antibodies proves the stable native conformation of the viral proteins after incubation with AC-1.

A- Changes of ad layer thickness after subsequent addition of pAA, glutaraldehyde, the influenza A virus [before (green curve) and after incubation with 16 mM AC-1 (red curve)] and the virus-specific antibodies.
B- Corresponding changes of refraction indexes in the same time range (msec).

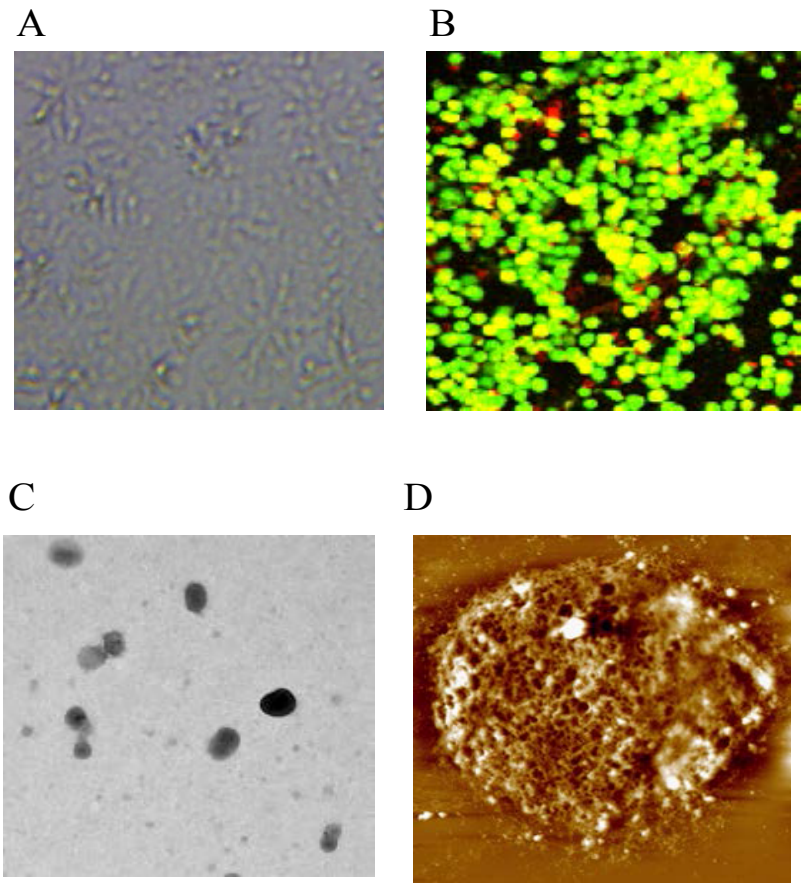

Figure 3: Native conformation and viability of MDCK cells and influenza A/Aichi 1/68 on the surfaces modified with 0.1 mg/ml pAA65 and 0.1% GA.
A- Staining of the immobilized MDCK cells with trepane blue dye.
B- Staining of immobilized MDCK cells with fluorescent dyes "Live and Dead" ("Molecular Probes", "Life technologies", USA).
C- Transmission electron microscopy of the isolated influenza virus A/Aichi 1/68.
D- Atomic force microscopy of the MDCK cell infected with influenza A/Aichi 1/68.

Atomic force microscopy (AFM)

Samples were analyzed by using atomic force microscope "Integra Prima" (NT-MDT, Russia). All the AFM observations were performed with high-resolution silicon cantilevers (Nanotuning, Russia) with resonance frequencies from 190 to 325 kHz. Free amplitude of the cantilever in the air was in the range of 1-10 nm.

Transmission electron microscopy (TEM)

Morphology of the *Influenza A* virions were examined using transmission electron microscope JEM-2100 (JEOL, Japan) at magnifications varying from 8,000x to 20,000x.

Cytotoxicity

Subsequent 10 times dilutions of 20 mM solution AC-1 was added onto confluent monolayers of MDCK, L929,Vero and PS cells in duplicate or quadruplicate in sterile 48- or 96-well tissue culture plates. Last readings for cytopathic effect (CPE) formation were made when no further progression of cellular degradation was evident in the titration for at least two subsequent days. To estimate toxicity two methods were used:

1) MTT and 2) biosensor-based optical detection in real time.

MTT test: Tissue cultures PS, Vero and L929 were grown in 48-well or 96-well plates in culture mediums with or without different concentrations of the tested substance AC-1 in the presence of 10% fetal bovine serum up to confluent monolayers in control wells without any additions. After washing three times with the sterile PBS MTT solution was added until final concentration 0.5 mg/ml and the plates were incubated for 3 hours at 37°C. Then MTT solution was removed from wells and 100 µl dimethylsulfoxide (DMSO) to dissolve MTT crystal was added. Optical densities were measured at 570 nm and background - at 630 nm by using the plate immunoreader "Uniscan" ("Picon", Russia).

The biosensor-based optical detection in real time was performed as described above (section 2.7).

Toxicity for mice

AC-1 was diluted in sterile tridistilled water at 5 concentrations: 160 mM, 20 mM, 2 mM, 0.2 mM and 0.02 mM immediately before experiments. The fresh solutions were administered into adult ICR mice 16-18 g in 3 different ways:

1. Peroral administrations of 25 µl 160 mM solution AC-1 5 times each day with intervals 1 day between administrations;
2. Intranasal administration of 50 µl per mouse of 20 mM, 2 mM, 0.2 mM and 0.02 mM solutions of AC 1;
3. Subcutaneous injections of 100 µl per mouse of 20 mM, 2 mM, 0.2 mM and 0.02 mM solutions of AC 1.

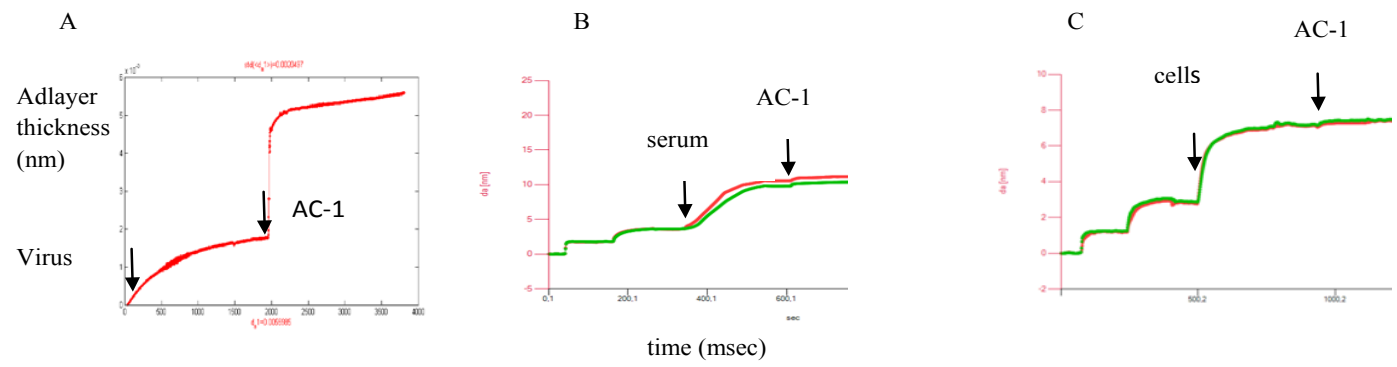

Figure 4: Real time optical detection of binding of the influenza A virus, mouse serum and MDCK cells immobilized onto one-dimensional photonic crystal surface with 160 mM AC-1.
A- The influenza virus;
B- Mouse serum (1:100);
C- MDCK cells.

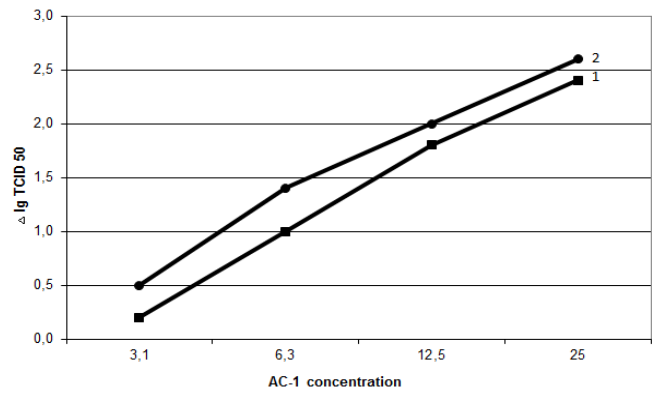

Figure 5: Differences in lg $TCID_{50}$ between the influenza virus control titers and those in the presence of various concentrations of AC-1 added before (2) or after (1) infection of MDCK cells.

Toxicity of AC-1 for mice was estimated on base of lethality as well as external signs such as body mass reduction, inflammation, physical activity, adequate behavior, absence of tremor, paresis, spasms and convulsions compared to control animals during 20 days after AC-1 administrations. Maximal nontoxic doses corresponded to ½ dose of AC-1 which did not cause noticeable consequences on organism and behavior of mice.

Infection of MDCK cells with the influenza virus A

Monolayers of MDCK cells were infected with the influenza virus A/Aichi/1/68 in serum-free medium in the presence of 5 μg/ml trypsine according to [26]. To determine the infectious titers of the influenza A virus the subsequent 10-times dilutions of the virus suspensions from the mouse lungs were added to monolayers of the washed cells (4 wells per each dilution). After incubation of the infected cells for 24 hours at 37^{0}C the virus was detected using hemagglutination test with human erythrocytes (blood group 0) [22], the viral antigens – in ELISA [21] and the *Influenza A* virus RNA - by means of reverse transcription with real time PCR. Half maximal tissue culture infectious doses ($TCID_{50}$) were determined from quadruplicate repeats.

Multiplicity of infection (MOI) used for further *in vitro* experiments was 0.1.

Infection of ICR mice

Mice ICR (males, 10-12 g) were intranasally infected under light ether anaesthesia with 50 μl of the influenza A/Aichi 1/68 virus adapted to the mouse lungs (10 lethal dose LD_{50}) [27]. The infected mice were observed for 20 days postinfection. Protection coefficient (k) and protection indexes (PI) were calculated according to following formula.

$$k = \frac{\text{\% died in a control group}}{\text{\% died in an experimental group}}$$

$$PI = \frac{k-1}{k} \times 100\%$$

Average life span was counted as the following

$$1/\tau = \frac{X_1/t_1 + X_2/t_2\ X_3/t_3 + X_4/t_4 + \ldots + Xa/ta}{n}$$

Where t_1, t_2,……t_a - day of death of a mouse after infection with the *Influenza virus*;

X – Amount of lethal outcomes of the virus-infected mice in that day;

n - Total amount of experimental animals per a group.

Antiviral properties *in vitro*

MDCK cells were propogared in EMEM with 10% fetal bovine serum until confluent monolayer. Freshly prepared AC-1 solutions of various concentrations were added to wells in 2 hours before or after the infection of the MDCK with the *Influenza A virus* H3N2 (strain A/Aichi/1/68). Microscopic observations of CPE caused by the viral infection continued during 48 hours.

Antiviral properties *in vivo*

To study antiviral properties in adult laboratory mice the freshly prepared AC-1 solutions of different concentrations were administered

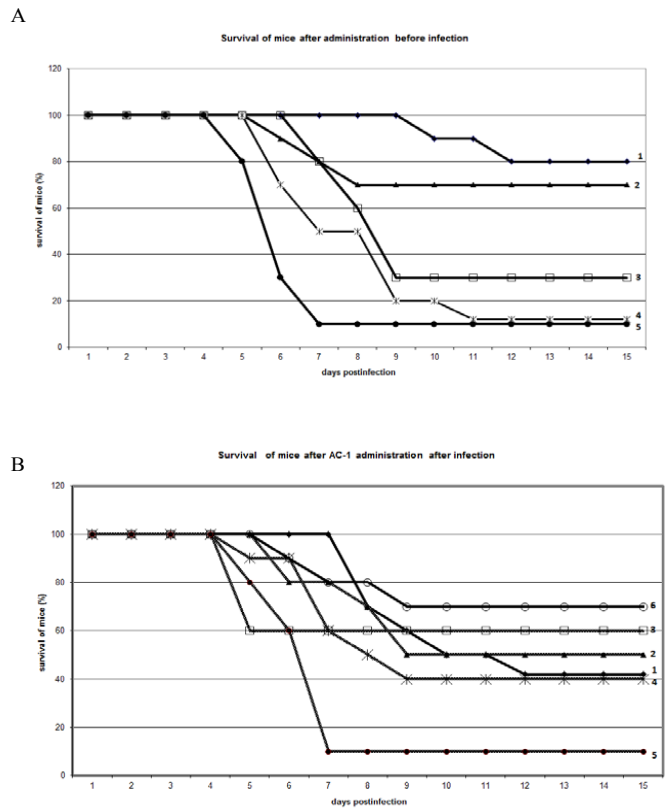

Figure 6: Survival of mice after AC-1 peroral administrations 5 times before (part **A**) and after (part **B**) the intranasal infection with the influenza A virus A/Aichi/1/68.

1- 160 mM AC-1;
2- 16 mM AC-1;
3- 1.6 mM AC-1;
4- 0.16 mM AC-1;
5- The virus control;
6- Tami flu.

perorally. Both single and multiple administrations according to prophylactic and therapeutic schemes were performed. The treated and infected with the influenza virus mice were observed for 21 days post infection.

Statistical analysis

Continuous variables were compared using Student's t-test [28]. P <0.05 was assumed to be significant.

Results and Discussion

Synthesis of AC-1

$Li^{+}[Ag^{+}_{2}Cys_{2}^{-}(OH^{-})_{2}(NH_{3})_{2}]$ (short name AC-1) was designed as a low toxic silver complex and a potential nuclease and synthesized as previously described [19]. Structure of AC-1 was confirmed by means of IR, UV, NMR (including ^{109}Ag NMR) spectroscopy shown in Supplementary materials and data of element analysis [19].

RNA cleavage

Complete cleavage of any isolated RNA was revealed after its incubation with 2 mM AC-1 for 1 hour at 37°C (Figure 1). For full degradation of the *Influenza virus* RNA within extracellular virions 2 hours were required (Figure 1). However, the AC-1-mediated RNA cleavage inside infected cells was not exhaustive (Figure 1). Neither cellular DNA nor viral antigens (Figure 2) were damaged after incubation with AC-1. Discrepancy between complete degradation of the viral RNA in virions and partial cleavage inside infected cells might be caused by the AC-1 penetration into virions but only partly in cells and stoichiometric ratios. Taken together, the data suggested a possible inactivation of the influenza virus without its antigen damages for an inactivated vaccine preparation or for preventive treatment at the early stage of infection.

Optical detection of binding of AC-1 with *Influenza virus*, cellular and blood serum proteins

Conformational stability of the viral antigens was controlled by semi-quantitative ELISA, hemagglutination tests and optical detection of their binding with the virus-specific antibodies before and after treatment with AC-1 (Figure 2). Stable morphology and viability of both host cells and virions on aminated surfaces were confirmed by the staining with trepane blue and fluorescent dyes, as well as by using the atomic force, the transmission electron and the fluorescent microscopy (Figure 3).

Permanent binding of AC-1 with the *Influenza virus* (Figure 4A), a weak binding with mouse sera (Figure 4B) and the absence of any detectable interaction with immobilized MDCK cells (Figure 4C) suggested its potential antiviral properties and a limited toxicity.

Toxicity

Cytotoxicity of AC-1 for different tissue cultures was shown to vary (Table 1). Its toxicity for mice appeared to depend on the administration routes. Peroral administrations were less harmful compared to other ways and didn't cause any observed disturbances. Intranasal administration of both 20 and 2 mM AC-1 caused lethal outcomes. A single subcutaneous injection of 160 mM or 20 mM AC-1 caused tremor, refusal to eat, adynamia and fatal outcomes whereas lower doses beginning from 16 mM did not result in any damages. The discrepancies might be explained by the accumulation of AC-1 in lungs and stomach. The absence of binding of AC-1 with the cellular and serum proteins (Figure 4) fitted with its limited toxicity both *in vitro* and *in vivo* (Table 1).

Antiviral properties of AC-1

RNA is known to be the genetic material of many pathogenic viruses so artificial RNases might serve as antiviral drugs as was shown for non-enveloped RNA-containing viruses [2, 18]. However, penetration of RNases into enveloped RNA-containing viruses is hampered by both lipids and viral encapsidation proteins. All our attempts to inhibit *Flaviviruses* with AC-1 were unsuccessful (data not shown) despite available data about other artificial RNases [29].

The Influenza virus is known to be the most sensitive to RNases [2]. Antiviral properties of AC-1 *in vitro* (Figure 5) and *in vivo* (Figure 6 and Table 2) essentially coincided and proved incomplete inactivation of the influenza virus. The highest PI and an average life span were observed

Tissue cultures				Mice		
MDCK	L929	Vero	PS	Peroral	intranasal	subcutaneous
0.03[a]	0.03	0.01	0.04	160 [b]	0.2	2

Table 1: Toxicity of AC-1 for tissue cultures of different origin and mice after different administration routes

[a]CC_{50}: concentration of AC-1 (mM) that caused 50% cyto toxicity of eukaryotic cells
[b]Concentration of AC-1 (mM) that did not result in detectable damage of mice.

Peroral administration of AC-1	Concentration of AC-1, мМ	Lethality (% ± error), n=10 mice per a group	Average life span after infection (days)		Protection Index (PI) (%)
			relative*	In comparison with control	
Before infection	160	20 ± 13.3	54.7	+48.1	77.8 ± 13.9
	16	30 ± 15.3	23.0	+16.4	66.7 ± 14.9
	1.6	70 ± 15.3	11.5	+4.9	22.3 ± 13.1
	0.16	90 ± 10	8.1	+1.5	
	Water	90 ± 10	6.6		
After infection	160	60 ± 16.3	13.0	+6.0	33.3 ± 14.9
	16	50 ± 16.7	14.8	+7.8	44.4 ± 15.7
	1.6	40 ± 16.3	12.5	+5.5	55.6 ± 15.7
	0.16	60 ± 16.3	11.6	+4.6	33.3 ± 14.9
	TAMIFLU 7.5 mg/kg	30 ± 15.3	23.8	+16.8	66.7 ± 14.9
	Water	90 ± 10	6.9		

Table 2: Antiviral properties of AC-1 against the influenza virus A/Aichi/1/68 in vivo after its multiple peroral administrations

Peroral administration of AC-1	Concentration	Infectious titers in lungs (lg $TCID_{50}$)	Δlg $TCID_{50}$ in comparison with control
Before infection	160 mM	1.3 ± 0.2	3.45
	16 mM	2.5 ± 0.3	2.25
	1.6 mM	2.9 ± 0.4	1.85
	0.16 mM	4.3 ± 0.3	0.45
After infection	160 mM	2.8 ± 0.2	1.95
	16 mM	2.7 ± 0.2	2.05
	1.6 mM	2.75 ± 0.3	2.0
	0.16 mM	3.0 ± 0.2	1.75
	TAMIFLU (7.5 mg/kg)	1.75 ± 0.4	3.0
Control		4.75 ± 0.2	0

Table 3: Infectious titers of the influenza A virus in mouse lungs.

after daily peroral administration of 160 mM AC-1 5 times before the virus infection (Table 2 and Figure 6).

Infectious titers of the Influenza virus A (in lg $TCID_{50}$) in lungs of mice after the multiple peroral administrations of the AC-1 were lower than in control group (Table 3) proving only partial inactivation of the virus inside infected cells in accordance with Figure 1.

Taking into consideration new molecular target, low toxicity after peroral administrations and evident anti-*Influenza virus* properties the novel low-molecular-weight AC-1 without immunogenic and allergenic activities might be used for an inactivated vaccine preparation and for combined therapy of the influenza.

Conflict of interest: None

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