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Novel amphiphilic compounds effectively inactivate the vaccinia virus

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ABSTRACT

Recent studies demonstrated the ability of artificial ribonucleases (aRNases, small organic RNA cleaving compounds) to inactivate RNA-viruses via the synergetic effect of viral RNA cleavage and disruption of viral envelope [1,2]. Herein, we describe the antiviral activity of aRNases against DNA-containing vaccinia virus: screening of aRNases of various structures revealed that amphiphilic compounds built of positively charged 1,4-diazabicyclo[2.2.2] octane substituted at the bridge nitrogen atoms with aliphatic residues efficiently inactivate this virus. The first stage was the destruction of viral membrane and structure of surface proteins (electron microscopy data). Thus, 1,4-diazabicyclo[2.2.2] octane-based aRNases are novel universal agents inactivating both RNA- and DNA-containing viruses.
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1. Introduction

Viruses are extremely diverse in life cycles, morphology, replication strategies, target cells, etc. Viral genetic material (DNA or RNA) is covered with protein shell, and this tight RNA(DNA)/protein complex (capsid) for simple non-enveloped viruses represents the virus particle, while the structure of more complex viruses may include lipids and different proteins, covering the capsid. The application of the universal virus inactivation methods, such as treatment with formaldehyde, β-propiolactone, and aziridine [3], or UV-irradiation is limited: the high toxicity of compounds restricts its wide usage as antiviral agents, and occurring in the course of inactivation cross linking reaction, which alter the structure of virus surface epitops [4] restricts their usage upon vaccine production.

The universal virus inactivating agent should display the antiviral activity in respect to viruses of different structures, therefore it should interact with various macromolecules: genetic material of virus [5], proteins and lipids [4]. However, when used for the vaccine production, the compound should not dramatically change the morphology and immunogenic activity of the inactivated virus particle; moreover, it should not be toxic for personnel.

In this respect, artificial ribonucleases (aRNases, small chemical compounds possessing the ribonuclease activity in vitro) represent a new promising class of antiviral agents. Three series of aRNases exhibiting high ribonuclease activity in vitro designed recently were shown to inactivate the RNA-containing viruses: the enveloped influenza A [1] and encephalitis [6] viruses and non-enveloped Acute bee paralysis virus (ABPV) [2]. These aRNases were of ABLkCm series, containing imidazole as catalytic group for the RNA cleavage, and positively charged 1,4-diazabicyclo[2.2.2] octane (DABCO) substituted at the bridge nitrogens with dodecyl residues, affording the affinity to phosphodiester bonds of RNA (ABL3C3, Table 1) [7]; the compounds of the Dxn series, which contain two similarly substituted DABCO residues and no potentially catalytic groups (Dtr12, Table 1); and peptide-like molecules containing amino acids as catalytic groups and hydrophobic substituents (L2–3, Table 1) [8]. It was demonstrated that the viral RNA of ABL3C3- and Dtr12-inactivated influenza virus was cleaved, and the membrane of the inactivated virus particles contained a number of breaks (EM data) [1], but the surface peplomers were not affected [9]. The inactivation of influenza virus with peptide-like compound L2–3 being complete did not result in the prominent cleavage of viral RNA [9].

Here we screen these aRNases on the ability to inactivate DNA-containing vaccinia virus. We found that some aRNases completely inactivate the virus in a short time under physiological conditions. The concentration profile and time course of virus inactivation revealed the main stages of the inactivation process and the electron microscopy (EM) study showed that the virus inactivation occurred via disruption of viral membrane and, possibly, distortion of DNA/protein interactions.

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Table 1
Inactivation of vaccinia virus by aRNases.

aRNase	Structural formula[a]	Vaccinia virus inactivation	
		EC[b] (mM)	Virus titer[c] (PFU/ml)
ABL3C3	CH_3-$(CH_2)_{13}$–N+ +N–$(CH_2)_3$ ... H N ... O ... O OH ... N NH; 2Br⁻	0.4	<10
Dtr12	$C_{12}H_{25}$–N+ N+–CH_2–C≡C–CH_2–N+ N+–$C_{12}H_{25}$; 2 Cl⁻ 2 Br⁻	0.1	<10
L2–3	HO O ... H_2N ... O ... H N–CH_2-$(CH_2)_{10}$-CH_2–N H ... O ... NH_2 ... O ... O H	2	10^5
Control[d]			10^5

[a] The structures of aRNases ABL3C3, Dtr12 and L2–3. The IC_{50} (cell inhibitory concentration) – the concentration of aRNase at which 50% of CV-1 cells survived after incubation at 37 °C for 24 h were 0.05 mM, 35 μM and >1 mM for ABL3C3, Dtr12, L2–3, respectively. The synthesis and RNA-cleaving activity of the compounds were described in [7,8,12–15].
[b] EC (effective concentration) is the concentration of aRNase at which the maximal level of virus inactivation was observed after 24 h of incubation.
[c] The titer of vaccinia virus estimated after the incubation in the presence of aRNase taken at the effective concentration (24 h at 37 °C).
[d] Control – vaccinia virus was incubated under the same conditions in the absence of aRNases.

2. Materials and methods

2.1. Enzymes and reagents

The artificial ribonucleases (aRNases) were synthesized in the Institute of Chemical Biology and Fundamental Medicine (Novosibirsk, Russia) by Dr. Vladimir Silnikov. The aRNases were dissolved in 100% DMSO (Sigma, USA) up to 10–50 mM concentration, and further diluted either with appropriate buffer or MilliQ water.

2.2. Cells

The CV-1 cells were obtained from Russian cell culture collection of Institute of Cytology (St. Petersburg, Russia). The cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Sigma, USA) in the presence of 5% fetal bovine serum (Serva, USA) and 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin (Sigma, USA) at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Virus

The CV-1 cells were grown until confluence in 175 cm^2 culture plates with the initial density 10^4 cells/ml. The confluent monolayer was infected with vaccinia virus at 0.01 multiplicity of infection (m.o.i) and 1 h after the adsorption at 37 °C the cell medium was replaced with a new portion of DMEM, 5% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin. The plates were incubated for 48 h at 37 °C in the humidified atmosphere containing 5% CO_2 until cytopathic effect. The cells were passed through three cycles of freeze-thawing and then disrupted to liberate the virus by ultrasonic treatment with Sonopuls HD 2070 (Badelin, Germany) for 20 s. The resulting suspension was centrifuged for 15 min at 4000 rpm, 4 °C, then the virus-containing fluid was layered onto the 40% sucrose cushion and centrifuged for 90 min at 18000×g, 4 °C using the SV-40 rotor. The resulting pellet was suspended in the buffer containing 50 mM Tris–HCl, pH 9.0, titrated, aliquoted and stored at −20 °C.

2.4. Plaque forming unit (FFU) assay

The virus titer was estimated using the in vitro titration techniques described previously [10] and expressed in terms of plaque forming units per ml (PFU/ml) of the virus suspension.

2.5. Virus inactivation

The vaccinia virus at a concentration 5 × 10^5 PFU/ml was incubated in 50 mM Tris–HCl buffer, pH 7.0, in the presence of 200 mM KCl, 1 mM EDTA and one of the aRNases at a concentration ranging from 0.05 to 0.25 mM for Dtr12, from 0.05 to 0.5 mM for ABL3C3, and from 0.1 to 2 mM for L2–3 at 37 °C for 0–72 h. As a control virus suspension incubated under the same conditions but in the absence of aRNases was used. After 0, 6, 24, 48, 72 h the aliquots (25 μl) were serially diluted in the DMEM and the titer of virus suspension was estimated by titration as described previously and expressed as plaque forming units per ml.

2.6. Cytotoxicity assay

The toxicity of aRNases was determined as described previously [11].

2.7. Electron microscopy

The mixtures containing vaccinia virus (10^8 PFU/ml) were incubated in the presence of 0.1–0.5 mM ABL3C3 as described above for

0–18 h. After 0, 0.5 and 18 h the copper grid covered by formvar film was placed on a drop (~30 μl) of virus preparation for 30 s (the excess of the preparation was removed), and then on a drop of phosphotungstic acid (PTA) for 20 s (the excess of PTA was removed). The dried grids were examined in JEM 1400 transmission electron microscope (Jeol, Japan) at 80 kV and images were obtained by the Jeol digital camera and Veleta (SIS) digital camera integrated in Jem 1400 electron microscope.

3. Results

3.1. Antiviral activity of aRNases

The ability of three different aRNases (Table 1) to inactivate DNA-containing vaccinia virus was tested. The synthesis, biochemical activity of the compounds [7,8,12–15], as well as the antiviral activity against RNA-containing viruses [1,2,6,9] were described previously. The vaccinia virus at a concentration $\sim 5 \cdot 10^5$ PFU/ml was incubated at physiological conditions in the presence of one of the aRNases ABL3C3, Dtr12, L2–3 or in the absence of the compounds for 0–72 h at 37 °C. After the incubation the resulting titer of virus in each reaction mixture was measured using the plaque forming assay. The screening revealed that aRNases ABL3C3 and Dtr12 efficiently inactivate the virus, while peptide-like aRNase L2–3 display no antiviral activity (Table 1). aRNases ABL3C3 and Dtr12 are amphiphilic compounds built of 1,4-diaza-bicyclo[2.2.2] octane residue, bearing two positive charges at quarternized nitrogen atoms substituted with the aliphatic dodecyl residues. The complete inactivation of virus occurred at a concentration 0.4 and 0.1 mM for ABL3C3 and Dtr12, respectively (Table 1).

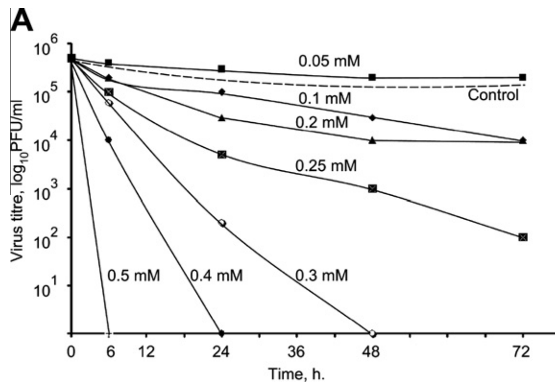
To elucidate the mechanism of virus inactivation, the concentration profile and kinetics of virus inactivation by the aRNases were analyzed. Though for these experiments a very high initial titer of virus suspension was used ($\sim 5 \times 10^5$ PFU/ml), it is seen that virus is rapidly inactivated in the presence of ABL3C3 at a concentration 0.5, 0.4 and 0.3 mM (Fig. 1A): at this concentration the complete inactivation of virus was achieved after 6, 24 and 48 h, respectively. This is also true when the virus was incubated with 0.25 and 0.1 mM Dtr12 (Fig. 1B). With the decrease of concentration of aRNases, the inactivation rate also decreased: for ABL3C3 at concentrations 0.05–0.25 mM and for Dtr12 at 0.05–0.08 mM virus inactivation was not achieved even after 72 h of incubation (Fig. 1). The observed dependence of the inactivation extent on the concentration of aRNase indicates that some interactions between virus particle and aRNase molecules are required, and the decrease of the number of these contacts resulted in the inability of aRNase to inactivate the virus. It should be specially noted, that ABL3C3 completely inactivated influenza virus at a concentration 0.4 mM, and Dtr12 – at concentration 0.02 mM [9]. As mentioned, incubation of virus suspension with both compounds resulted in the cleavage of viral RNA, and the breaks in the membrane of virus particles were detected by EM [1]. Since, the ribonuclease activity of aRNases could not afford the inactivation of DNA-containing vaccinia virus, their ability to disrupt viral membrane can be proposed as the main mechanism of vaccinia virus inactivation.

3.2. The study of the mechanism of virus inactivation

To check this idea we performed the EM study of vaccinia virus upon inactivation by ABL3C3 and monitored the morphology of viral particles incubated for 0–18 h with different concentrations of the compound. Virus incubated under the same conditions but in the absence of aRNase served as a control.

The vaccinia virus reproduction in the cell culture produces various types of virus particles: the major part is the intracellular mature virus, and at a lower extent (less than 3%) – intracellular enveloped virus [16]. The visualized native virus had the typical morphology of the poxviruses (Fig. 2A) [17]: the negative staining revealed virus particles non-transparent for the contrasting agent, possessing randomly arranged prominent and structured surface ridges called “surface tubule elements” (STEs) [18]. The parts of the membrane are visible in the native (Fig. 2A) and control virus (Fig. 2B). The incubation of the virus without aRNase for 0–18 h did not visibly change the morphology of the particles (Fig. 2B) as compared to native virus preparations (Fig. 2A).

The aRNase ABL3C3 at a concentration 0.5 mM caused fast and complete inactivation of vaccinia virus (Fig. 1A): in the virus preparations incubated for 30 min in the presence of 0.5 mM ABL3C3 no virus particles were detected using the EM, since all particles were destroyed (primary data not shown). In order to follow the alterations of virus morphology upon inactivation, the virus was incubated in the presence of 0.1 mM ABL3C3, as under these conditions slow inactivation of virus took place: virus titer decreased ~10-fold after 72 h of incubation (Fig. 1A). The EM study of the virus suspension incubated under these conditions for 30 min revealed the distinct damage of the virus particles: the surface tubules lost the structure and looked as fuzzy globular structures, and “hollows” were visualized on the surface of the viral particles by the penetration of the contrasting agent (marked with red arrows, Fig. 2C and D). After the incubation of virus suspension for 18 h under these conditions (0.1 mM ABL3C3), the particles



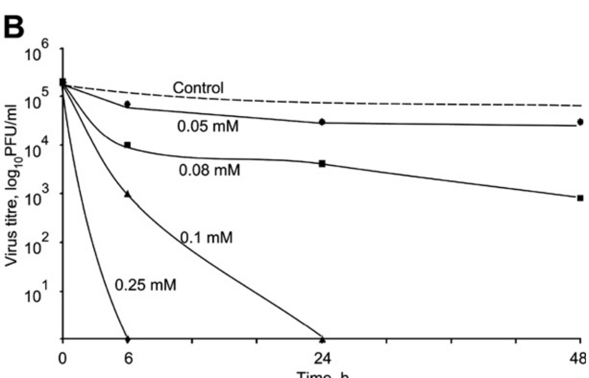


Fig. 1. The kinetics of vaccinia virus inactivation by aRNases ABL3C3 (A) and Dtr12 (B) at different concentration of the compounds. Concentrations of aRNases are indicated at each curve. The vaccinia virus was incubated in 50 mM Tris–HCl buffer, pH 7.0, in the presence of 200 mM KCl, 1 mM EDTA and one of the aRNases at a concentration ranging from 0.05 to 0.25 mM (Dtr12), 0.05–0.5 mM (ABL3C3), and 0.1–2 mM (L2–3) at 37 °C for 0–72 h. As a control virus suspension incubated under the same conditions but in the absence of aRNases was used. The error did not exceed 5% for all experiments.

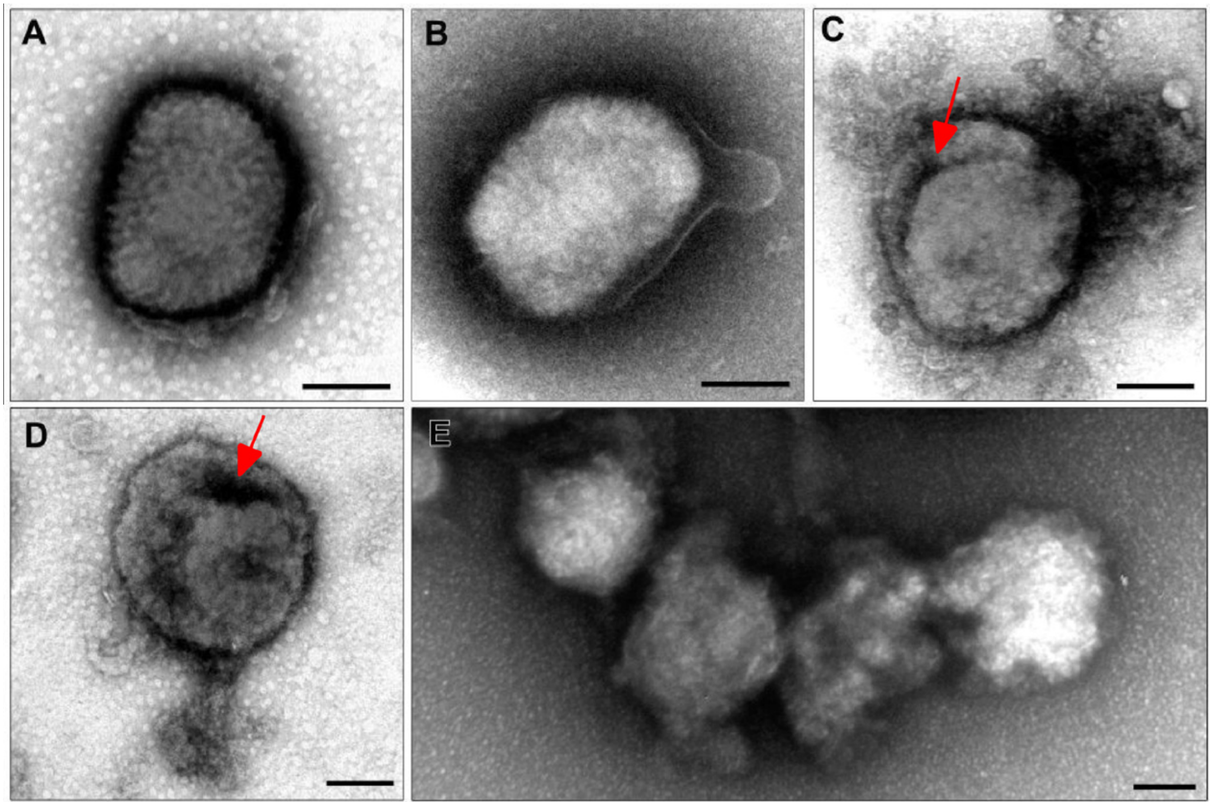


Fig. 2. The electron micrographs of the negatively stained preparations of intact vaccinia virus (A), vaccinia virus incubated under the conditions of inactivation for 18 h in the absence of aRNase (B) and incubated in the presence of 0.1 mM aRNase ABL3C3 for 30 min (C, D) and 18 h (E). Scale bar 100 nm.

completely lost the initial structure and were visualized as the aggregates of non-defined size (Fig. 2E).

4. Discussion

The aRNases were recently shown to display the antiviral activity against RNA-containing viruses: inactivation of non-enveloped ABPV was caused by the viral RNA cleavage [2], and inactivation of the enveloped influenza virus was due to the RNA cleavage and membrane distortion [1,9]. As the role of the membranolytic activity of aRNases in the overall antiviral activity of the compound was evident, in the present study we tested the ability of aRNases to inactivate DNA-containing virus. In this respect vaccinia virus represents a perfect model, as its morphology and life cycle have recently been studied in details [18].

Two aRNases (ABL3C3 and Dtr12) from different series inactivate the vaccinia virus. The structures of these compounds are sharing similarities: they both contain positively-charged DABCO residues – one in ABL3C3 and two in Dtr12, as well as hydrophobic domains – one in ABL3C3 and two in Dtr12. The peptide-like aRNase L2–3, which does not contain DABCO, but contains acid–base groups, did not inactivate the vaccinia virus.

It is known that the mature intracellular virus posses the membrane, which is fully consistent with a lipidic membrane (5–6 nm thick) and is covered with the STEs (a) representing the integral protein extensions; (b) extending ≈3–5 nm from the viral surface; (c) corresponding to the outer receptor-binding viral components [19]. The mature virus enters cells by fusion of the membrane with the plasma membrane of the host cell, releasing the core (and lateral bodies) into the cytoplasm and activating the virus' transcriptional program [20].

Since the protein STEs are protruding from the viral membrane, they might be the first target for amphiphilic compounds in the course of inactivation: the loose of the proper STE spatial organization upon incubation with aRNases observed in the present study lead to the inability of virus to replicate. It seems likely that aRNases work as some kind of chaotropic agent: after the interaction with the surface proteins they may interfere with the lipid membrane. EM study performed at different stages of inactivation revealed that aRNase causes viral membrane damage: the breaks and "hollows" on the surface visible on the EM micrographs of virus particles appeared at the early stages of interaction, and at the latest stages resulted in the loss of the integrity of virus particles required for infectivity. Both, the deterioration of the surface proteins, as well as the introduction of the membrane breaks could lead to the distortion of DNA/protein interactions, which all facilitate the viral inactivation.

The observed chaotropic activity correlates well with the previously received data on the ability of aRNases of Dxn and ABLkCm series to reduce the affinity of aRNase-treated virus to the monoclonal antibodies to the surface epitops of influenza virus [9]. This first step of the interaction of aRNase with influenza virus followed by the disruption of the viral envelope (membranolytic activity) as well as the ribonuclease activity in respect to viral RNA afforded the effective viral inactivation [9]. In regard to non-enveloped virus (ABPV) we can propose that acting as a molecular wedge aRNases Dp12F6 and D3–12 (compounds from the Dxn series) were able to destabilize the tight nucleoprotein and in synergism with ribonuclease cleavage of viral RNA provide the inactivation of the virus [2]. Therefore, the inactivation of RNA-containing viruses is a result of all three activities of the compounds (chaotropic, membranolytic and ribonuclease activities). On the other hand, obtained results clearly show that chaotropic and membranolytic activities are enough to cause the effective inactivation of DNA-containing virus. Moreover, the chaotropic and ribonuclease activities are also sufficient for the inactivation of non-enveloped RNA-virus [2]. The

nature of high antiviral activity of aRNase L2–3 against influenza virus [9] and lack of antiviral activity against ABPV [2] or vaccinia virus requires special attention.

5. Conclusions

In the present work we demonstrated the ability of two aRNases to inactivate the DNA-containing virus. The compounds containing positively charged DABCO residue, substituted at the bridge nitrogen with aliphatic groups demonstrated the concentration-dependent profile of virus inactivation. The electron microscopy study of the inactivated virus preparation revealed that the virus inactivation occurred via significant alteration of the viral morphology: the surface proteins disorganization and membrane destruction. Based on the obtained results, we can conclude, that these aRNases are universal agents for the inactivation of both RNA- and DNA-containing viruses.

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