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Artificial ribonucleases inactivate a wide range of viruses using their ribonuclease, membranolytic, and chaotropic-like activities

Antonina A. Fedorova [a], Elena P. Goncharova [a], Lyudmila S. Koroleva [a], Ekatherina A. Burakova [a], Elena I. Ryabchikova [a], Elena V. Bichenkova [b], Vladimir N. Silnikov [a], Valentin V. Vlassov [a], Marina A. Zenkova [a, *]

[a] Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of Russian Academy of Sciences, 8, Lavrentiev Ave., Novosibirsk, 630090, Russian Federation
[b] School of Pharmacy, University of Manchester, Oxford Road, M13 9PT, Manchester, United Kingdom

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ABSTRACT

Artificial ribonucleases (aRNases) are small compounds catalysing RNA cleavage. Recently we demonstrated that aRNases readily inactivate various viruses *in vitro*. Here, for three series of aRNases (1,4-diazabicyclo [2.2.2]octane-based and peptide-like compounds) we show that apart from ribonuclease activity the aRNases display chaotropic-like and membranolytic activities. The levels of membranolytic and chaotropic-like activities correlate well with the efficiency of various viruses inactivation (enveloped, non-enveloped, RNA-, DNA-containing). We evaluated the impact of these activities on the efficiency of virus inactivation and found: i) the synergism between membranolytic and chaotropic-like activities is sufficient for the inactivation of enveloped viruses (influenza A, encephalitis, vaccinia viruses) for 1,4-diazabicyclo [2.2.2]octane based aRNases, ii) the inactivation of non-enveloped viruses (encephalomyocarditis, acute bee paralysis viruses) is totally dependent on the synergism of chaotropic-like and ribonuclease activities, iii) ribonuclease activity plays a leading role in the inactivation of RNA viruses by aRNases Dp12F6, Dtr12 and K-D-1, iv) peptide-like aRNases (L2-3, K-2) being effective virus killers have a more specific mode of action. Obtained results clearly demonstrate that aRNases represent a new class of broad-spectrum virus-inactivating agents.

1. Introduction

The emergence of highly pathogenic viruses and re-emergence of previously controlled infections, as well as drug resistance and the speed of the spread of infectious agents due to travelling/transport/trade makes elaboration of new methods for virus inactivation a priority (Pandey et al., 2014). Virus-inactivating agents can be divided into two groups: firstly, highly-specific compounds targeting a specific step of the virus life cycle (i.e., inhibitors of viral enzymes) (Balfour, 1999) secondly, compounds with a broader mode of action that interact with a wide spectrum of viral components to cause virus inactivation (*e.g.*, interaction with lipid membrane (Song and Seong, 2010) or surface proteins/glycoproteids (De Clercq, 2002; Wojcechowskyj and Doms, 2010)). The first type of agent represents the base of modern antiviral therapy, and its definite advantage is the high specificity towards the target viruses (Razonable, 2011). At the same time the use of these agents is hindered by the high probability of mutations arising in the genome of some viruses (influenza virus, HIV, etc.), which results in the resistance of those viruses to this specific reagent (Griffiths, 2009; Nijhuis et al., 2009).

Despite all efforts, effective therapeutic agents have been discovered for only a small number of viruses (Littler and Oberg, 2005), and the small number of universal targets (Merluzzi et al., 1990; Love et al., 2004; Olszewska and Openshaw, 2009) limits the number of universal antiviral agents. The ability of some viruses to exist in latent form (HIV, herpes simplex virus) makes complete virus eradication impossible. To control the global spread of highly pathogenic agents, priority should be given to non-pharmaceutical universal virus inactivation agents, which could stop viruses spreading (Pandey et al., 2014).

Interaction of chemical reagents with surface components of

* Corresponding author.
E-mail address: marzen@niboch.nsc.ru (M.A. Zenkova).

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virus particles (proteins and lipids) may result in virus inactivation due to the destruction of both antigens and the structure of the virus particle itself. Agents capable of interaction with viral genetic material with the preservation of surface antigens are of particular interest, since they can also be used for vaccine production (Singer and Fraenkel-Conrat, 1969).

Due to the aforementioned reasons, we propose artificial ribonucleases (aRNases, Kuznetsova and Silnikov, 2004; Silnikov and Vlassov, 2001; Niittymäki and Lönnberg, 2006) as a novel prospective class of virus-inactivating agents, since: a) they cleave RNA *in vitro* under mild physiological conditions, and b) they are low molecular-weight compounds demonstrating antiviral (virucidal) activity against a number of viruses having different structures (Goncharova et al., 2009, 2011; Fedorova et al., 2011, 2012a, 2012b).

Firstly, we assumed that upon direct incubation of a suspension of RNA-containing viruses with aRNases, these compounds could penetrate into the viral particles and cleave the viral genome, thus inactivating viruses. Indeed, it was proved that aRNases can inactivate enveloped influenza A ((Goncharova et al., 2009; Fedorova et al., 2012a), *Orthomyxoviridae* family, further - IAV) and encephalitis viruses ((Goncharova et al., 2011), *Flaviviridae* family, further - TBEV), as well as non-enveloped acute bee paralysis virus ((Fedorova et al., 2011), ABPV, *Dicistroviridae* family) and encephalomyocarditis virus (EMCV, *Picornaviridae* family) (Fedorova, unpublished data). Moreover, for some compounds the mechanism of viral inactivation was proved to be RNA cleavage: Dp12F6 in the case of ABPV (Fedorova et al., 2011), ABL3C3 and Dtr12 in the case of IAV (Fedorova et al., 2012a), ABL3C3 in the case of TBEV (Goncharova et al., 2011). It was further shown that cleavage of viral genomic RNA within viral particles occurred non-randomly: some genomic regions were more accessible for cleavage with aRNases (Fedorova et al., 2012a). Electron microscopy (EM) revealed no changes in the morphology of ABPV after its treatment with Dp12F6 (Fedorova et al., 2011), but the structure of the lipid membrane of enveloped IAV after its inactivation by ABL3C3 contained a number of breaks, though the surface peplomers were not affected (Goncharova et al., 2009). The third line of evidence demonstrated that some aRNases (ABL3C3 and Dtr12) do effectively inactivate the DNA-containing vaccinia virus ((Fedorova et al., 2012b), *Poxviridae* family, further - VV). EM study revealed significant alterations of the structure of the VV membrane after its treatment with ABL3C3 (Fedorova et al., 2012b).

Herein, we explored how aRNases inactivate viruses of different structures. Structure-functional analysis of the compounds, most of which are amphiphiles, allowed us to line up three possible inactivation-driving principles: 1) ribonuclease activity (interaction and/or cleavage of viral genomic RNA, further - RA), 2) membranolytic activity (interaction with the lipid membranes of membrane-containing viruses allowing the penetration of aRNases into the viral particles and/or causing their destruction, further - MLA), 3) chaotropic-like activity (interaction with surface proteins affording the penetration of aRNases into the viral capsids and/or destabilisation of the surface structures of viral particles, further – CLA). In the present paper we assessed these activities and evaluated their contribution to the inactivation of various viruses (enveloped, non-enveloped, RNA-, DNA-containing) by aRNases.

2. Materials and methods

2.1. Oligonucleotides and reagents

[γ-^{32}P]ATP (specific activity > 3000 Ci/mM) was from Biosan Co (Novosibirsk, Russia). Oligodeoxyribonucleotides T_{26} 5′CCTGGATCCTTCTTCCCACT(T)$_{26}$CGCGCG-AGGTAGAATTCGAA3′ and h2 5′TTCGAATTCTACCTAGACGGCAGCCAGCGTGTTTTCACGCTGGCTGCCGTCTG3′ were purchased from Biosan (Novosibirsk, Russia).

The aRNases were synthesised according to the published protocols (Konevetz et al., 1999, 2002; Kovalev et al., 2004, 2006; Burakova et al., 2014; Tamkovich et al., 2016) and dissolved in DMSO (Sigma, USA) at concentrations 20–50 mM, further dilutions were performed using water or appropriate buffer. The aRNases are **ABL3C3**: 1-tetradecyl-4-γ-[*N*-(1-carboxy-2-(imidazole-4-yl)ethyl) carbamyl]propyl-1,4-diazoniabicyclo [2.2.2]octane dibromide] (Konevetz et al., 1999), **ABL3C1:** 1-tetradecyl-4-[*N*-(2-(imidazole-4-yl)ethyl)carbamyl]propyl-1,4-diazoniabicyclo-[2.2.2]octane dibromide] (Konevetz et al., 1999), **Dp12F6:** 1,4-bis–[(4-{4,4,5,5,6,6,7,7,8,8,9,9,9-tridecafluorononylazonia}-1-azoniabicyclo-[2.2.2]octane-1-methyl)-benzene dichloride dibromide (Burakova et al., 2014), **D3-12:** 1,5-bis-(4-dodecyl-1,4-diazoniabicyclo [2.2.2] octan-1-yl)pentane dichloride dibromide (Burakova et al., 2014), **Dtr12:** 1,4-bis-(4-dodecyl-1,4-diazoniabicyclo [2.2.2]octan-1-ylmethyl) but-2-yne tetrachloride (Burakova et al., 2014), **Dp12:** 1,4-bis-(4-dodecyl-1,4-diazoniabicyclo [2.2.2]octan-1-ylmethyl) benzene dichloride dibromide (Kovalev et al., 2006), **K-2:** glutamyl-glycyl-lysyl-glycine n-decyl ester (Koroleva et al., 2005), **K-D-1:** (1-tetradecyl-1,4-diazoniabicyclo [2.2.2]octan-4-yl)-acetyl-glutamyl-β-alanyl-lysyl-glycine n-decyl ester (Koroleva et al., 2005), **L2-3:** *N,N′*-bisglutamyldodecane-1,12-diamine (Tamkovich et al., 2016).

Human flap endonuclease 1 (hFEN-1) was kindly provided by Prof. S.N. Khodyreva (this institute).

2.2. Cell cultures

Cell line CV-1 was received from the Bank of Cell Cultures (Institute of Cytology, Saint-Petersburg). The cells were grown in DMEM (Sigma, USA) in the presence of 5% FBS (Sigma, USA) and 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin (further - antibiotic mix, Sigma, USA) at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Viruses

Vaccinia virus (*Poxviridae* family) was kindly provided by Prof. S.N. Schelkunov (State Research Centre of Virology and Biotechnology, Koltsovo, Russia). The VV titre was estimated using *in vitro* titration techniques (Kotwal and Abrahams, 2004) and expressed in terms of plaque-forming units per ml of the virus suspension (PFU/ml).

The influenza virus strain A/WSN/33 (H1N1) and ABPV were propagated, treated and titrated as described previously (Goncharova et al., 2009; Fedorova et al., 2012a) and (Fedorova et al., 2011), correspondingly.

EMCV was propagated in Hela cells (centrifugation through 10% sucrose cushion) and titrated in L-929 cells: serial 10-fold dilutions were added to cells, after 48 h cells were fixed with 10% formaldehyde and stained with gentian violet; the number of focus-forming units was calculated by the number of regions of lysed cells. Each virus dilution was added to cells twice, all experimental points were run in triplicate, results are represented as mean and standard deviation.

2.4. Vaccinia virus inactivation

VV at a concentration 10^6 PFU/ml was incubated in 50 mM Tris-HCl, pH 7.0, 200 mM KCl, 1 mM EDTA and one of the aRNases at a concentration range 0.04–400 μM (Dp12F6), 1–500 μM (D3-12), 0.01–1 mM (K-D-1), 0.5–0.7 mM (Dp12), 0.05–0.5 mM (ABL3C3, ABL3C1), 0.01–0.2 mM (K-2) and 1–5 mM (L2-3) at 37 °C for 0–96 h. As a control, a virus suspension incubated in the absence of