

## RESEARCH ARTICLE

## Inactivation of the tick-borne encephalitis virus by RNA-cleaving compounds

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## ABSTRACT

The tick-borne encephalitis virus (TBEV) is an RNA-containing enveloped virus, which poses a major threat to the well-being and health of humans. In this study, we describe an approach to the inactivation of TBEV, which involves the degradation of viral RNA by artificial ribonucleases (aRNases, small organic compounds that exhibit ribonuclease activity *in vitro*). We demonstrate that the incubation of TBEV with aRNases lead to the total inactivation of the virus as indicated by the plaque formation assay data, but retain the viral immunogenic properties, as shown by the ELISA data. We propose that a possible mechanism of TBEV inactivation with aRNase, which includes: i) formation of local breaks in the lipid membrane of the virus caused by aRNase, ii) penetration of aRNase into the viral capsid, iii) degradation of genomic RNA by aRNase. These data suggest that the proposed approach can be used in the production of killed-virus vaccine.

**KEYWORDS:** Artificial ribonucleases, RNA-containing viruses, tick-borne encephalitis, virus inactivation

## INTRODUCTION

Viral diseases represent a global problem for public health worldwide. Among the most dangerous infectious agents for humans, are RNA viruses (Bray, 2008). These RNA-containing viruses are also the causal factor of a significant number of serious diseases within the animal (both domestic and wild) population. Highly-pathogenic RNA viruses such as aggressive strains of corona viruses (SARS), influenza, tick-borne encephalitis virus (TBEV) and dengue virus, represent a constant threat to the humanity (Gould and Solomon, 2008), and demand urgent diagnostics and treatment of these viruses.

TBEV (*Flavivirus* genus) is a single-stranded RNA virus with a spherical nucleocapsid surrounded by a host-derived

lipid bilayer that contains 2 glycoproteins; membrane protein (M protein) and envelope protein (E protein). Exhibiting hemagglutination activity, E protein contains the major antigenic sites, which are responsible for inducing both the formation of neutralizing antibodies and the protective immune response within the infected organism (Maier et al, 2007).

Numerous studies have been carried out in order to explore various approaches concerning the development of antiviral compounds against pathogenic flaviviruses (Sampath and Padmanabhan, 2009). These include both the design of chemical compounds, which interfere with virus replication (Puig-Basagoiti et al, 2009), and compounds affecting specific pathways, crucial for the virus replication (Goodell et al, 2009). Although some of the previously

developed antivirals demonstrated potential, none of them were approved for use in humans. Presently, there are no approved antiviral therapeutics against TBEV.

Viral RNA is a potential target for the inactivation of TBEV, as its damage could abolish the virus replication. Herein, a new approach for the inactivation of TBEV by artificial ribonucleases (aRNases, low molecular weight compounds, capable of catalyzing the cleavage of RNA *in vitro*) was studied. Recently a number of aRNases were designed in the Institute of Chemical Biology and Fundamental Medicine (Novosibirsk, Russia) via the conjugation of RNA cleaving groups to nucleic acids binding molecules (Figure 1) (Konevets et al, 1999; Zenkova et al, 2001; Kovalev et al, 2008; Koroleva et al, 2008). These compounds display a pattern of RNA-cleavage similar to that of natural ribonuclease A (cleave RNA at 5'-C-A-3' and 5'-U-A-3' linkages) and do not interfere with biopolymers other than RNA. For instance, aRNase ABL3C3 built of imidazole-conjugated bis-quaternary salt of substituted 1,4-diazabicyclo[2.2.2]octane (DABCO) (Figure 1A) cleaves RNA under physiological conditions at Pyr-A motifs (Konevets et al, 1999; Zenkova et al, 2001). Another group contains the conjugates of peptide-like molecules with DABCO (R-D-2, K-D-1, Figure 1B and C, respectively) (Koroleva et al, 2005). The aRNase L2-3 is a peptide-mimicking compound, which is comprised of amino acids that are connected via the hydrophobic linker (Figure 1D). Finally, aRNase Dtr12 is a conjugate of two DABCO residues substituted with the aliphatic groups at bridge positions connected by a rigid linker, (Figure 1E) (Koroleva et al, 2008).

In order to produce safe and effective vaccines, it is essential to develop techniques that provide the complete inactivation of the virus and the preservation of its immunogenic activity. Since the modification of surface proteins during the inactivation strongly affects the immunogenic activity of the produced preparations, we investigated the ability of aRNases to penetrate via the viral envelope and to cleave the viral RNA resulting in the production of non-infectious virus preparation, characterized by the unaltered structure of the immunogenic sites.

The aRNases screening revealed compounds that are capable of inactivating the TBEV via the viral RNA cleavage; the study of the structure of the surface epitopes following inactivation was performed using monoclonal antibodies to surface E protein responsible for the immune response *in vivo*.

## MATERIALS AND METHODS

### Cell culture and the virus

Porcine kidney cells (PK) were maintained in Dulbecco minimum essential Medium (DMEM) (Sigma, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco BRL, Germany) at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> (v/v). The TBE virus strain Sofjin was prepared by infecting PK cell culture; the supernatant was collected 2-3 days post infection and then stored at -70°C in aliquots.

### Plaque assay

Virus titers were determined by plaque assay in PK cells growing in 24-well plates (Venturi et al, 2006). Briefly, the

cells were plated in the 24-well culture plates (2×10<sup>5</sup> cells per well) and allow to adhere overnight. Serial tenfold dilutions of the viral suspension were added to the cell monolayer; the cells were then incubated for 1hr at 37°C. After removal of the virus suspension the cells were washed with PBS and DMEM supplemented with 2% (v/v) FCS, 1% (v/v) carboxymethyl-cellulose (Sigma) was added to the well, and the cells were incubated for 72hr at 37°C. The plaques were visualized after 10% (v/v) formaldehyde fixation (1hr at room temperature) by staining with a 1% (v/v) crystal violet solution. The virus titer was estimated by counting the number of plaques observed in each well, and was expressed as plaque-formation unit per milliliter (PFU/ml).

### Cytotoxicity

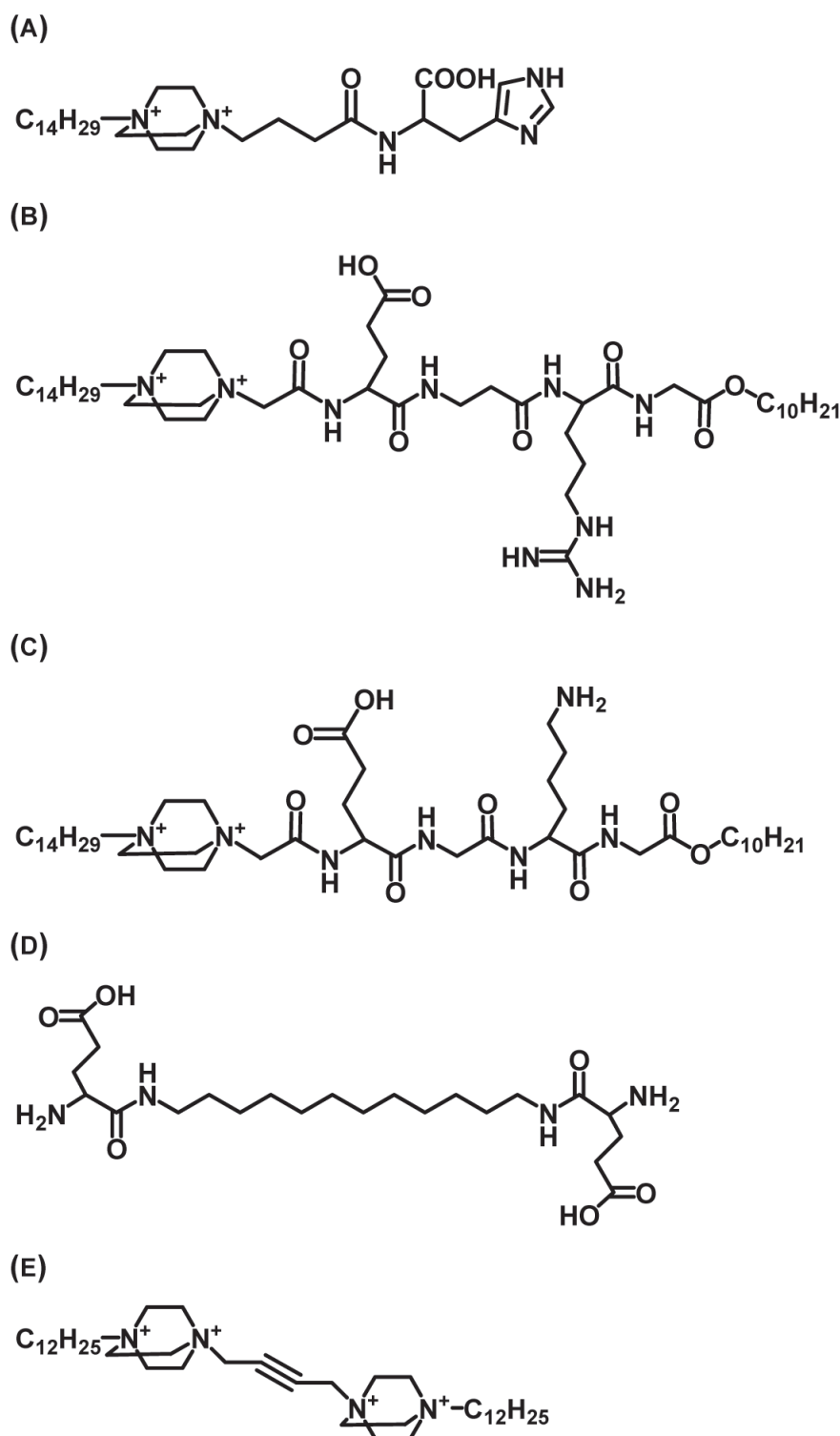
The cell viability was measured on the basis of the mitochondria-dependent reduction of MTT to formazan (Mosmann, 1983). PK cells were plated in the 96-well culture plates in 100µl DMEM with 10% (v/v) FBS as described above. Cells were incubated at 37°C in a humidified atmosphere with 5% (v/v) CO<sub>2</sub> for 24hr to adhere. The medium in wells was then replaced by 100µl DMEM medium without FBS, containing different concentrations of aRNases (from 10<sup>-7</sup> to 10<sup>-3</sup> M) and cells were incubated in the presence of aRNases for 24hr at 37°C and 5% (v/v) CO<sub>2</sub>. MTT solution (10µl, 5mg/ml, Sigma) was then added to each well and the cells were incubated for 4hr at 37°C and 5% (v/v) CO<sub>2</sub>. Following incubation, the medium was removed and DMSO (100µl) was added to each well in order to dissolve the formazan crystals. The extent of MTT reduction to formazan was quantified by measurement of absorbance at 570nm, using Multiscan RC (Labsystems). All experimental points were run in four parallels. Results were represented as a percentage of living cells as compared to the control (untreated cells). The cell-inhibitory concentration (CC<sub>50</sub>) was determined for each compound. Results are expressed as mean percentage values. The S.D. was below 5%.

### Inactivation of TBEV by aRNases *in vitro*

Direct inactivation of TBEV by aRNases was examined by use of a plaques forming assay. The virus (1.5×10<sup>6</sup> PFU) was incubated with various aRNases at concentrations ranging from 0.01µM to 1mM for 18hr at 37°C in 50mM Tris-HCl, pH 7.0, containing 0.2M KCl and 0.2mM EDTA. The aRNases-treated virus was then used to infect monolayers of PK cell grown in 24-well plates for 1hr at 37°C. Following infection, the cells were treated as described above for the plaque assay. Standard placebo-treated virus controls, toxicity controls and normal medium controls were included in the assays.

### Inhibition of virus replication in the cell culture

The aRNases-mediated inhibition of TBEV replication was evaluated in PK cells. Confluent monolayers of PK cells grown in 24-well plates were incubated with different concentrations of aRNases for 2hr at 37°C and 5% (v/v) CO<sub>2</sub>. Following this, the cells were infected with TBEV at a multiplicity of infection (MOI) of 0.1 PFU. After 1hr of incubation at 37°C and 5% (v/v) CO<sub>2</sub>, the medium containing both the non- absorbed virus and aRNase solution was either replaced with a new portion of culture medium, or not. The cells were then incubated for 48hr at 37°C and 5% (v/v) CO<sub>2</sub>. Following incubation, the culture medium was



**Figure 1.** Structures of artificial ribonucleas. ABL3C3 (A), R-D-2 (B), K-D-1 (C), L-2-3 (D), and Dtr12 (E).

collected, cleared by centrifugation at 400xg and 4°C and then subjected to the plaque assays.

#### Reverse transcription and PCR

Total RNA was isolated from virus suspension using RIBOzol-A (FGUN CNIIA Rospotrebnadzor). 1-2µg of total RNA was used for the preparation of cDNA with 2-2.5U of MMLV reverse transcriptase. The reaction was performed for 1hr at 37°C with hexanucleotide random primers and

1mM of each dNTP in a buffer containing 10mM Tris-HCl (pH 8.0), 75mM KCl, 5mM MgCl<sub>2</sub>, 10mM DTT in a total reaction volume of 20µl. Thereafter, cDNA was subjected to PCR amplification in 50µl reaction mixture containing 0.2mM dNTP mixture, 4mM MgSO<sub>4</sub>, 0.15µM forward primer (5'-CGTGAACGTGTTGAGRAAAAGACAGC-3'), 0.15µM reverse primer (5'-TCAACACNAGYCCATTTGGCAT-3'), and 0.15µl of labelled probe (FAM-TCTTTCGACACTCGTCWAGGRGGACCGCCC-BHQ1)

and 1.5U/30 $\mu$ l Hot Start Taq DNA polymerase (Medigen, Russia). The PCR reaction temperature profile was set at 95°C for 3min for the initial denaturation, followed by 45 cycles of 94°C for 10sec and 60°C for 40sec. The fluorescence signal was detected by “Rotor Gene 3000” (Corbett Research Pty Ltd.). The test Ct value was extrapolated against the standard curve (derived from the sample of the virus incubated under the same conditions but without aRNases), in order to evaluate the amount of viral RNA present in each sample. The control sample, which was used for the construction of the standard curve, contained 10<sup>6</sup> virus particles. The number of virus particles was defined by electron microscopy observations. The non-treated PK cells were used as a negative control.

### Enzyme-linked immunosorbent assay (ELISA)

A 96-well ELISA plate was filled with 100 $\mu$ l of viral antigen diluted in 0.01M PBS pH 7.2 per well. Plates were incubated at 4°C overnight. On the following day plates were washed three times with a wash buffer (PBS with 0.05%, v/v, Tween-20; 300 $\mu$ l per well). All subsequent reagents added to the plates were diluted in the wash buffer containing 2% (v/v) FBS. After the addition of each reagent, the plates were incubated in humid air at 37°C for 1hr and subsequently washed three times. Monoclonal antibodies (mAbs) were serially diluted twofold from 1:100 to 1:12800 during assay. After incubation with serially diluted mAbs, plates were washed and incubated for 1hr at 37°C with peroxidase-conjugated goat anti-mouse antiserum. The wells were then washed again and the substrate of horseradish peroxidase-o-phenylenediamine dihydrochloride was added. The colour reaction was stopped after 30min and the plates were examined at 492nm with Multiscan RC. MABs specific to the TBE serocomplex were kindly provided by Dr E Protopopova (State Research Centre of Virology and Biotechnology VECTOR). The titration curves of each mAb were established against the treated virus and compared to those obtained with intact virus.

## RESULTS

### Inactivation of TBEV with aRNases

The aRNases belonging to different aRNases series were tested in terms of their ability to inactivate TBEV *in vitro* and to prevent the viral infection development in cell culture.

Figure 1 displays the structures of aRNases ABL3C3, R-D-2, K-D-1, Dtr12 and L2-3. All these compounds efficiently cleave the RNA substrate *in vitro* under physiological conditions (Konevets et al, 1999; Zenkova et al, 2001; Koroleva et al, 2005; Kovalev et al, 2008). The compounds are water soluble at millimolar concentrations.

The direct inactivation of TBEV by selected aRNases was investigated using the plaque assay method. The virus (1.5 $\times$ 10<sup>6</sup> PFU) was incubated in the presence of chosen concentrations of aRNases for 18hr at 37°C in a buffer solution. After incubation, the treated TBEV was used to infect the monolayer of PK cells grown in 24-well plates, followed by the plaque assay. The results of these experiments are summarized in Table 1. It is clearly visible that incubation in the presence of aRNases significantly reduces the TBEV-induced plaque formation (less than 1 lg PFU/ml), thereby indicating a significant reduction of the number of viable viral particles in the aRNases-treated TBEV samples. It is worthy of note that aRNases exhibited antiviral activity at concentrations close to those at which efficient RNA cleavage occurred.

The cytotoxicity of aRNases in respect to the PK cells was evaluated using the MTT-test. Cells were incubated for 24hr in the presence of aRNases taken in concentrations from 0.1 $\mu$ M to 1mM and then the MTT assay was performed. CC<sub>50</sub> values (*i.e.*, concentrations of the compounds at which 50% of the cells remained viable) were determined from the concentration dependencies (Table 2). The aRNases of different series display different cytotoxicity: CC<sub>50</sub> for L2-3 (2mM) and ABL3C3 (0.5mM) were found to be significantly higher than the corresponding values for other aRNases; thereby indicating the considerably low cytotoxicity of these compounds. aRNases R-D-2 and K-D-2 having a similar structure display moderate cytotoxicity: CC<sub>50</sub> is 0.15mM; aRNase Dtr12 was the most toxic (CC<sub>50</sub> 0.07mM). These CC<sub>50</sub> values for aRNases tested were significantly higher than the optimal concentrations of aRNases for *in vitro* cleavage of RNA substrate (see Table 1).

In another set of experiments the effect of the aRNases on TBEV propagation in the PK cell culture was studied. In these experiments PK cells were incubated in the presence of varying concentrations of aRNases for 2hr. Following this, the cells were infected with the virus at MOI 0.1 PFU. The number of infectious virus particles released from the cells

**Table 1.** Effect of aRNases on TBEV plaque formation.

aRNase	Optimal concentration of aRNases for <i>in vitro</i> RNA cleavage, mM	Concentrations of aRNases used for inactivation of TBEV, mM	TBEV titer, lg(PFU/ml)**
ABL3C3	0.1	0.5	<1
R-D-2	0.1	0.06	<1
K-D-1	0.1	0.06	<1
Dtr12	0.01	0.02	<1
L2-3	1	2	<1
Negative control*	-	-	4.2

\*Titer of TBEV incubated for 18hr at 37°C in the absence of aRNases. Initial titer of TBEV preparation was 6.1 lg (PFU/ml)

\*\*Viral titer was measured following TBEV incubation with aRNases for 18hr at 37°C.

**Table 2.** Effect of aRNases on TBEV propagation in PK cells.

aRNase	CC <sub>50</sub> <sup>*</sup> , mM	Concentrations of aRNases used for inactivation of TBEV, mM	Titer of TBEV <sup>**</sup> , lg(PFU/ml)
R-D-2	0.15	0.06	7.4
K-D-1	0.15	0.06	5.8
		0.03	6.7
Dtr12	0.07	0.02	6.7
		0.01	7.4
ABL3C3	0.5	0.25	6.0
		0.1	7.6
L2-3	2	1	6.7
		0.2	7.4
Negative control <sup>***</sup>		-	7.5

\*CC<sub>50</sub>: Concentration of the aRNases at which 50% PK cells remains viable (MTT assay data)

\*\*Titer of TBEV 48hr post infection of PK cells incubated for 2hr in the presence of aRNase prior to infection with TBEV at a MOI of 0.1 PFU.

\*\*\*TBEV incubated for 48hr at 37°C in absence of aRNases, lg(PFU/ml)

into the culture medium was evaluated 48hr post infection (Table 2). Among the five aRNases tested, four compounds K-D-1, Dtr12, L-2-3, ABL3C3 inhibited the replication of TBEV in PK cells in a concentration-dependent manner. The maximum reduction of progeny virus titer of 1.7 lg (PFU/ml) was observed in the case of aRNase K-D-1 (0.06mM), for other aRNases these differences in the virus titer were 1.5 lg (PFU/ml) and 0.7 lg (PFU/ml) in the case of ABL3C3 and L2-3, respectively, which points to the strong inhibition of the TBEV propagation in PK cell culture. No antiviral activity of R-D-2 was detected at the concentration of the compound that was used. It is worth mentioning that the antiviral effect of the aRNases was observed at a concentration far below their CC<sub>50</sub> values.

Interestingly, the inhibitory effect of aRNases was higher in the presence of aRNases in the culture medium during the development of TBEV infection than when the cell medium containing aRNase and virus was replaced 1hr post infection (see Experimental section for details) by fresh portion of the medium. Under these conditions (aRNases containing cell medium was replaced by fresh portion) aRNase K-D-1 reduces the progeny virus titer by only -0.5 lg (PFU/ml) in comparison to the control, and other aRNases display no antiviral activity under this conditions (primary data not shown). The enhancement of the inhibitory effect in the presence of aRNases in the cell medium might result from the direct inactivation of the virus released from infected cells. Consequently, the assay used did not permit the precise discrimination between antiviral and virucidal effects of aRNases because we could not exclude the possibility that the aRNases could penetrate into the cells and interfere with the intracellular steps of the viral cycle.

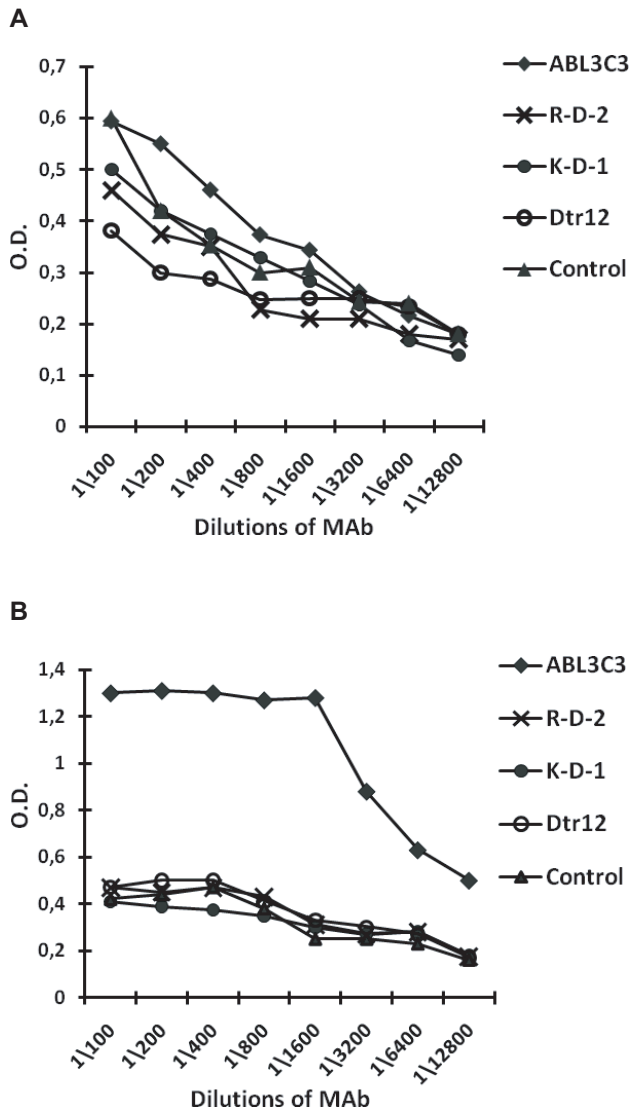
#### Antigen specificity of the envelope protein E of the aRNases-inactivated TBEV

We explored whether aRNases affect the structure of the surface epitope of envelope virus protein. ELISAs were performed using two types of monoclonal antibodies: (mAbs) 4F6 and 10H10 specific to the region of the E protein 19-273

and mAbs 13F6 and E6B specific to the region 273-429 (Figure 2), in order to detect possible changes in the antigen specificity of the envelope. The obtained results (in Figure 2 the data are shown for mAbs 4F6 and 13F6, similar results were obtained for mAbs 10H10 and E6B) indicate that monoclonal antibodies exhibit a similar affinity to the specific regions of the E protein of both the control (no treatment) and aRNase-inactivated TBEV. Closely adjacent curves point to the undamaged state of the antigenic determinants (Karavanov et al, 1990). These experiments confirm the preservation of the structural integrity of envelope protein E upon TBEV inactivation with aRNases. It is interesting to note that the incubation of TBEV with aRNase ABL3C3 even improves the binding of the inactivated viral particles with the monoclonal antibodies 13F6 (Figure 1B).

#### Effect of aRNAases on genomic RNA of TBEV

The main idea of applying aRNase as antiviral agents was to destroy specifically viral RNA. To confirm the mechanism of aRNases-mediated TBEV inactivation the integrity and amount of viral RNA in aRNase-inactivated virus preparations was ascertained by real-time PCR. TBEV was inactivated by aRNases (concentration of the compounds used for TBEV inactivation are shown in Table 1). Viral RNA was isolated from the virus preparations and was used as a template in reverse-transcription/real-time PCR analysis. Figure 3 displays the relative amounts of viral RNAs in the preparation of aRNase-inactivated virus; the level of viral RNA in the control (untreated virus) was set at 100%. It is clearly apparent that the significant reduction of viral RNA amount is observed in TBEV samples treated with aRNase Dtr12. In the case of treatment with ABL3C3, a reliable reduction of viral RNA amount is observed in comparison with the control virus. In the virus preparations inactivated with aRNases R-D-2 and K-D-1 no noticeable changes in the level of viral RNA were detected. However, TBEV titer in these preparations was less than 1 lg PFU/ml – in other words, the virus was entirely inactivated. It is possible that in the case of aRNases R-D-2 and K-D-1,

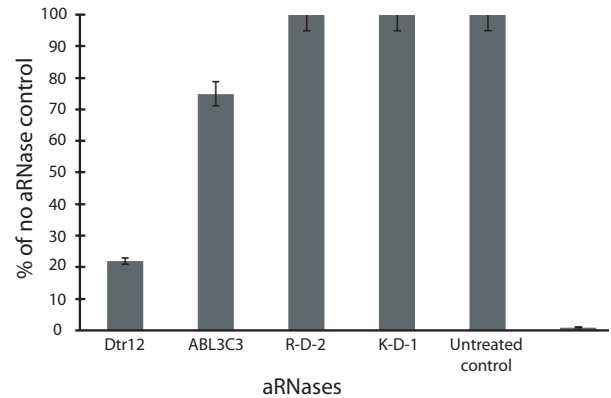


**Figure 2.** The affinity of envelope protein E of TBEV to the monoclonal antibody 4F6 (A) and to 13F6 (B). Data of ELISA assay. Control - TBEV incubated in the absence of aRNases. ABL3C3, R-D-, K-D-1, Dtr12 - TBEV incubated for 18hr in the presence of respective aRNases (concentration of aRNases as in Table 1).

the cleavage of viral RNA occurred in other regions, which were not amplified with the set of primers used in the experimental protocol.

## DISCUSSION

High toxicity and reduced immunogenic activity of the inactivated virus preparation are among the main disadvantages of the agents currently used for the production of the inactivated vaccines. Currently, extensive investigations are performed in order to develop new approaches for virus inactivation, which would avoid the modification of main viral antigens (Sebastian et al, 2008; Giampieri et al, 2009). In this respect, we propose the application of a new class of compounds - artificial ribonucleases (aRNases), which are capable of viral RNA cleaving, but are not affecting the structure of the surface viral antigens. The relative simplicity



**Figure 3.** Levels of TBEV viral RNA in the aRNases inactivated virus preparations. Data of real-time PCR. Viral RNA level is defined as a ratio of the viral RNA level in the aRNase-treated virus preparation in respect to the viral RNA level in the untreated control virus. The indicated values represent the averages for two independent experiments. The test Ct value was extrapolated against the standard curve derived from the virus incubated under the same conditions, but in the absence of aRNases (Untreated control). There are  $10^6$  virus particles in the control sample, which were used for the construction of the standard curve. Negative control - no virus added in the reaction mixture at the RNA isolation step.

and low cost of the synthesis of aRNases combined with low toxicity of the compounds make aRNases attractive agents for virus inactivation.

aRNases can be used for the inactivation of different RNA-containing viruses, including those from *Flaviviridae* family, the propagation of which poses an apparent threat to human health. At present, the general procedure for flavivirus inactivation is used on formaldehyde solution application (Ehrlich et al, 2003). However, the search for new non-toxic drugs is in progress (Meneses et al, 2009; Zhang et al, 2009).

Recently, we reported on the efficient inactivation of the influenza virus by aRNase ABL3C3 (Goncharova et al, 2009) and Acute Bee Paralysis Virus (ABPV) by D3-12, Dp12F6 and K-D-1 (Fedorova et al, 2011). In the present study, the effect of five aRNases on the tick-borne encephalitis virus (TBEV) was investigated. It was found that aRNases having different chemical structures can act as effective inhibitors of TBEV: Incubation in the presence of aRNases for 18hr at 37°C results in the complete inactivation of the virus (Table 1). Along with the ability of all tested aRNases to inactivate TBEV particles, the inhibiting action of aRNases on virus propagation in cell culture *in vitro* (Table 2) was also observed. The maximal inhibiting activity was displayed by aRNase K-D-1 (in the presence of this compound the virus titer was decreased by 1.7 lg PFU/ml). Other aRNases tested displayed lower virus inhibiting effects. Thus, aRNases exhibited both virucidal and antiviral activities.

The integrity of genomic RNA in the inactivated virus preparations using real-time PCR (the length of amplified fragment was 137 nucleotides) was analyzed, in order to validate the putative mechanism of the virus inactivation by aRNases which was shown to be viral RNA cleavage in the

case of Influenza virus and ABPV (Goncharova et al, 2009; Fedorova et al, 2011) (Figure 3). The obtained results reveal that the degradation of viral RNA was observed in TBEV preparations, which were inactivated by Dtr12, while in the ABL3C3-, R-D-2- and K-D-1-inactivated TBEV preparations the levels of viral RNA remained unaltered. It is likely that in the latter case, other regions of viral RNA than those amplified were cleaved. It is worth of note that even a single cut of TBEV genomic RNA (10480 nucleotides) could lead to virus inactivation. Conversely, aRNases used in this study are amphiphiles with the exception of L-2-3 and have the ability to disrupt the viral membrane that could also cause the virus inactivation.

An essential requirement for the effective preparation of inactivated vaccines is the conservation of the ability of viral proteins to cause the immune response initiation. We evaluated the influence of aRNases treatment on the immunogenic properties of TBEV envelope E antigen using a set of monoclonal antibodies against separate epitopes of this protein. Our results provide evidence that the virus - antibodies interactions were not altered upon incubation with aRNases. Interestingly, in the presence of aRNase ABL3C3 we observed an even higher level of virus binding with the 13F6 antibody that can be explained by the possible partial release of the protein from the viral membrane due to the ability of ABL3C3 to cause disruption of the viral membrane (Goncharova et al, 2009).

In conclusion, our experiments with different aRNases revealed their ability to inactivate the TBEV *in vitro*. The virus inhibition effect correlated with the destruction of viral RNA for two aRNases from the test list. Inactivation of TBEV upon aRNases treatment was not accompanied by antigenic determinants degradation of the viral envelope protein E. The data obtained earlier in experiments with the influenza virus evidence the ability of the tested aRNases to inactivate various RNA-containing viruses. Our results confirm that aRNases can be considered as potential agents for inactivated vaccine preparation from viruses with the RNA genome. Furthermore, due to the observed low toxicity and ability to inactivate the TBEV for the aRNase K-D-1, more detailed studies are needed to evaluate the potential of this compound as anti-TBEV therapeutic agent.

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## STATEMENT OF COMPETING INTERESTS

None declared.

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