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Short interfering RNAs targeting a vampire-bat related rabies virus phosphoprotein mRNA

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ABSTRACT

The aim of this study was to assess the *in vitro* and *in vivo* effects of short-interfering RNAs (siRNAs) against rabies virus phosphoprotein (P) mRNA in a post-infection treatment for rabies as an extension of a previous report (Braz J Microbiol. 2013 Nov 15;44(3):879–82). To this end, rabies virus strain RABV-4005 (related to the *Desmodus rotundus* vampire bat) were used to inoculate BHK-21 cells and mice, and the transfection with each of the siRNAs was made with Lipofectamine-2000^{TM}. *In vitro* results showed that siRNA 360 was able to inhibit the replication of strain RABV-4005 with a 1 log decrease in virus titter and 5.16-fold reduction in P mRNA, 24 h post-inoculation when compared to non-treated cells. *In vivo*, siRNA 360 was able to induce partial protection, but with no significant difference when compared to non-treated mice. These results indicate that, despite the need for improvement for in vivo applications, P mRNA might be a target for an RNAi-based treatment for rabies.

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Introduction

Though efficiently preventable with vaccination, rabies is still present in 150 countries and territories[1] and is a significant burden to public health worldwide.[2] Rabies virus (*Mononegavirales*: *Rhabdoviridae*: *Lyssavirus*: RABV) is an enveloped negative-sense ssRNA virus (c. 12 kb) coding for five structural proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and the large protein RNA-dependent RNA-polymerase (L).[3]

In 2003, the Milwaukee protocol, that included the use of thiopenthal, midazolam, rabies vaccination, anti-rabies serum, ribavirin, alfa-IFN and ketamine, was proposed for the treatment of human rabies. However, this protocol was successful in only two cases and failed in at least 26 other, with strong side effects.[4–6]

RNA interference (RNAi) has been shown to be effective both *in vivo* and *in vitro* against a range of strains of RABV, leading to reduced mortality, with no side effect being reported.[7]

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Table 1 – siRNAs (short-interfering RNAs) for RABV P protein mRNA used in this study.

siRNA	Sense/antisense sequences (5′–3′)	Position[a]
siRNA 360	GACCGUCGAGGAAAUCAUAUCCUAU/AUAGGAUAUGAUUUCCUCGACGGUC	1873–1897
siRNA 649	UCCCGAUCAUCAGGGAUAUUCUUGU/ACAAGAAUAUCCCUGAUGAUCGGGA	2162–2186
siRNA 652	CGAUCAUCAGGGAUAUUCUUGUACA/UGUACAAGAAUAUCCCUGAUGAUCG	2165–2189

[a] Regarding GenBank accession # AB519642.

Table 2 – Primers and probes used in this study.

Primer/probe	Sequence (5′–3′)	Position[a]
actβ_for[b]	ATTGGCAACGAGCGGTT	857–873
actβ_rev	ACGTCACACTTCATGATGGA	950–969
actβ_probe[b]	FAM/ATTCCATAC/ZEN/CCAGGAAGGAACGCTGG/IBFQ	895–920
RABV_for	GAGATGGCTGAAGARACTGTWG	1568–1589
RABV_rev	GGAGAYTGTCCACYTCTATGG	1641–1661
RABV_probe	HEX/CCTTGGAGA/ZEN/TGAGCCTGATTGTCYTCG/IBFQ	1609–1635

[a] GenBank accession numbers: NM_007393.5 (actin-β) and M13215/AB519642 (RABV P protein).
[b] PrimeTime Pre-designed qPCR Assay Mm.PT.56a.33257376.gs (IDT, USA).

This study was designed to assay the effects of short-interfering RNAs (siRNAs) against the mRNA of the P protein of a RABV strain related to *Desmodus rotundus in vitro* and *in vivo* in a post-exposure protocol as an expansion of a previous report.[8]

Material and methods

RABV strain, cells and mice

A field strain of RABV (RABV-4005) isolated from cattle and related to *D. rotundus* vampire bat antigenic variant 3, with titers of $10^{5.1}$ LD50%/mL in mice and $10^{6.1}$ TCID50%/mL in cells, was kindly provided by Pasteur Institute, Brazil, and used for the assays. BHK-21 cells (Baby Hamster Kidney, C-13; ATCC CCL-10, Instituto Adolfo Lutz, São Paulo, Brazil) and Swiss-albino mice (21-day old, 11–14 g) were used for the *in vitro* and *in vivo* assays, respectively. This experiment was approved by the USP School of Veterinary Medicine Bioethics Committee under the protocol # CEUA 2176/2011.

siRNAs

Three siRNAs (Table 1) were designed targeting P protein mRNA using BLOCK-iT™ RNAi Designer (www.invitrogen.com/rnai) based on an antigenic variant 3 RABV sequence (GenBank accession number AB519642) and synthesized in the Stealth™ (Life Technologies, Carlsbad, USA) format.

In vitro P mRNA knockdown assay

BHK-21 cells were grown in 96-well plates (1.5×10^4 cells/well) with MEM (Life Technologies, Carlsbad, USA) plus 10% Calf fetal serum CFS (Life Technologies, Carlsbad, USA) at 37 °C with 5% CO_2 for 24 h. After growth medium was discarded, 100 μL of RABV-4005 at $10^{4.1}$ to $10^{-0.9}$ TCID50%/mL in serum-free MEM were added and incubated at a 37 °C/5% CO_2 for 2 h for RABV entry.[9]

Next, the inocula were discarded and a combination of 1:50 Lipofectamine 2000™ (Life Technologies, Carlsbad, USA)/siRNA were added to each well to a final siRNA concentration of 2 μM with MEM plus 2% CFS for the treated plates. In control plates, siRNA was replaced by MEM plus 2% CFS and Lipofectamine 2000™. In one column of the test plates, MEM plus 2% CFS without RABV were added as a mock-infection and another column was added 1:50 Lipofectamine 2000™ with MEM plus 2% CFS (1:1, v:v) to check for cytotoxic effects. After 24 h of incubation at 37 °C/5% CO_2 and thus at least two RABV replication cycles,[9] control and test plates were tested by direct fluorescence antibody test (DFAT) with an anti-RABV N protein polyclonal fluorescein isothiocyanate conjugate kindly provided by the Pasteur Institute, Brazil, as described by Castilho et al.[10]

qPCR for relative P protein mRNA quantification

For the qPCR assay, only the more efficient siRNA in DFAT test was assayed. The procedure of cell culture, inoculation of RABV-4005 and treatment with siRNA were the same as described in the previous session, with the exception of the dilution of the virus ($10^{2.1}$ to $10^{-0.9}$ TCID50%/mL) and the use of Stealth RNAi Negative Control Duplexes™ (Life Technologies, Carlsbad, USA) at 2 μM for control plates. Each viral dilution was made in triplicate or duplicate in treated and control wells, respectively.

For this study, RABV primers and probe were designed based on sequences of the P mRNA of PV (M13215) and AgV3 (AB519642) strains, and actin-β reverse primer was designed based on sequences of *Mus musculus* (NM_007393.5) and *Mesocricetus auratus* (NM_001281595) (Table 2).

Total RNA was extracted from the monolayers using Cells-to-Ct™ Lysis Buffer (Life Technologies, Carlsbad, USA) as per manufacturer's instructions and cDNA was synthesized with M-MLV Reverse Transcriptase™ (Life Technologies, Brazil) as per manufacturer's instructions, with both actin-β and RABV

P protein reverse primers in the same tube. The qPCR assays were carried out in 20 μL reactions with TaqMan Gene Express Master Mix™ (Life Technologies, Brazil), 400 nM of sense and antisense primers and 150 nM of probe in separate reactions for P mRNA and β-actin, and 2 μL of cDNA in triplicate. The amplification was performed in a 7500© Real Time PCR System (Applied Biosystems, Foster City, CA, USA) at UDG 50 °C/2 min, activation 95 °C/10 min followed by 40 cycles at 95 °C/15 s and 60 °C/1 min.

A lysate of BHK-21 cells inoculated with RABV-4005 was used to construct a tenfold dilution standard curve (10^0 to 10^{-7} dilutions) of each target. Relative RABV P mRNA expression was calculated as described by Pfaffl[11] and standard deviation according to the error propagation method.[12] The evaluation of influence of siRNA treatment in expression of internal control (actin-β) was tested by 2^{-Ct} method.[13]

In vivo P mRNA knockdown assay

For the *in vivo* assay, only the siRNA found as more efficient after the *in vitro* assay was used. To this end, 20 mice were inoculated with 30 μL of RABV-4005 at 10 LD50% by intracerebral route. Two hours after inoculation, 10 mice were injected with 30 μL of 1:1 (v/v) siRNA/Lipofectamine 2000™ (1:50) in a total of 0.3 nmol siRNA/mice (treated group), and the remaining 10 were injected with 30 μL of 1:1 (v/v) Stealth RNAi Negative Control Duplexes™ (Invitrogen)/Lipofectamine 2000™ (1:50) in a total of 0.06 nmol siRNA/mice (control group).

Mice were kept at 21–25 °C, 12 h/day light period and feed/water *ad libitum* and observed daily for up to 30 days for rabies signs including ruffled fur, tremors, ataxia, paralysis, hyperesthesia and death beginning at the 5th day after inoculation. After 30 days, surviving mice were euthanized.

All mice had the central nervous system (CNS) tested by DFAT with the same conjugate used for the *in vitro* assay and as described by Dean et al.[14] Mice were considered as positive for rabies if the rabies signs were observed and if at least one fluorescent focus was found.

The significance of the difference between the groups regarding the survival rate was tested with the chi-square method ($\alpha = 0.05$) using Minitab 16.2.2 (© 2010 Minitab Inc.).

Results

In vitro P mRNA knockdown assay

Based on the N protein-targeted DFAT, regarding the three siRNAs used to knockdown P protein mRNA, the more intense drop in RABV-4005 titer was observed for the cells treated with siRNA 360, with a 1.0 log difference (Table 3) when compared to the control plate, while for the cells treated with siRNA 649 and siRNA 625 the drop in titers were lower.

Table 3 – siRNAs targeting P mRNA of RABV and the respective titers of strain RABV-4005 and log differences in comparison to control.

siRNA	RABV titer (log/mL)	Log drop in titer
siRNA 360	5.0	1.0
siRNA 649	5.75	0.25
siRNA 652	5.87	0.125
Control	6.0	0

qPCR for relative P protein mRNA quantification

The qPCR showed an efficiency of 99.7% ($E = 2$) and 92.5% ($E = 1.93$) and a correlation coefficient *f* value (r^2) of 0.999 and 0.994, respectively, to RABV P mRNA and β-actin standard curves. Using the Pfaffl method,[11] the cells infected with RABV-4005 (10 TCID50%) and treated with siRNA360 demonstrated a 5.16-fold (ranging from 2.30 to 11.56) lower expression level of the RABV P protein mRNA when compared to the cells treated with negative siRNA control (Fig. 1).

Using 2^{-Ct} method,[13] the fold change in the internal control (ratio of the mean of treated and untreated sample) was 1.20 and Student's *t*-test showed no significant difference ($p = 0.70$). Therefore, transfection of siRNA 360 and Stealth RNAi Negative Control Duplexes with Lipofectamine 2000™ did not affect the expression of actin-β gene of BHK-21 cells.

In vivo P mRNA knockdown assay

The survival rates in the groups of mice inoculated with RABV-4005 and treated with siRNA 360 or with the negative siRNA (control group) were 30% and 10%, respectively, though no significant difference was found ($p = 0.5820$).

CNSs of all dead and symptomatic mice were positive, while those euthanized after 30 days of inoculation with RABV-4005 were negative by DFAT.

Discussion

During this investigation, siRNAs targeting rabies virus phosphoprotein mRNA efficiently inhibited the replication of a strain related to *D. rotundus* vampire bat in BHK-21 cells after infection, while, in mice, a significant difference between treated and control groups was not observed.

P protein plays a multifunctional role on RABV replication, including the inhibition of N protein self-polymerization and a co-factor to the L protein during RABV transcription and

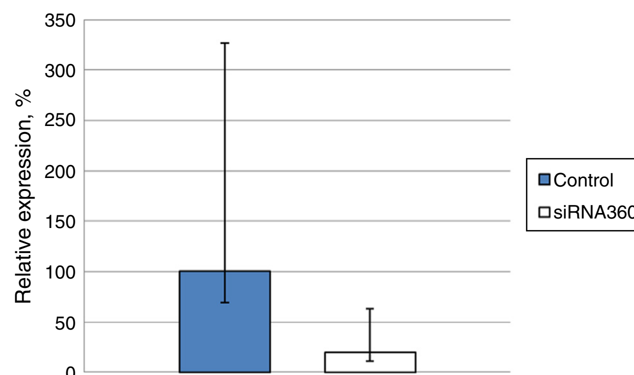

Fig. 1 – RABV phosphoprotein mRNA relative expression in BHK-21 cells infected with RABV-4005 and treated with siRNA360 and treated with Stealth RNAi Negative Control Duplexes™.

replication by stabilizing L and positioning it on the template RNA[3] and this diverse set of functions could help explain why P mRNA knockdown was successful on lowering RABV titers as shown herein.

Though all three siRNAs (Table 1) resulted in drops of RABV titers after DFAT *in vitro*, a more intense effect was observed to siRNA 360 (1 log drop), in agreement with the drop found after the relative qPCR for P mRNA of 5.16 times when compared to control cells, representing a 80.6% decrease in P mRNA.

Based on the *in vitro* results, siRNA 360 was elected to the mice post-inoculation treatment assay and, though the survival rate found for the treated group (30%) was the triple of that for the control (10%) group, a significant difference was not found, probably due to the low number of animals. In a study using an adenoviral vector-delivered siRNA targeting L protein mRNA in mice inoculated with the fixed CVS strain of RABV, a similar (33.3%) survival rate was found.[15]

This lower *in vivo* efficiency of siRNA 360 could be attributed not only to a low half-life of naked siRNAs and its degradation by CNS RNAses when compared to plasmid or vector-based deliveries,[16] but also to the association of this fact to the longer (5 days) minimum incubation period in mice when compared to cell (22 h), what could lead siRNAs to a more intense degradation before they could reach their targets.

A second dose of siRNA must also be considered as possibly improving the effects of siRNAs in mice inoculated with RABV. For instance, mice infected with 10 LD50% of Chandipura virus (Rhabdoviridae) and treated with two dosis of anti-P mRNA siRNAs (0 and 24 h post-infection) showed a 4 log drop in virus titer when compared to control mice.[17]

As shown by others[18] siRNA doses ranging from 0.5 nmol to 3.2 nmol have been more effective in mice against Japanese Encephalitis Virus and West Nile virus, respectively, and indication that an increase in the doses used herein in mice (0.3 nmol) could lead to a more intense P mRNA knockdown and survival rate.

In a previous report by some of the authors of the present article,[8] the use of N mRNA targeted siRNAs was shown to reduce the titers of the PV RABV strain in at least 0.72 log and a 30% survival rate was obtained in mice inoculated with PV and then administered a pool of these siRNAs. Nonetheless, as the PV is a fixed RABV strain and might not reflect the infection dynamics of a natural RABV infection, the use of a non-fixed strain as RABV-4005 can bring RNAi closer to a clinical situation of rabies.

The results presented in this paper are a further support to the use of RNAi as a contribution to rabies treatment protocols and evidence P mRNA as an eligible target to siRNAs development.

Conflicts of interest

The author, Ekaterina Alexandrovna Durymanova Ono, has no conflicts of interest to declare.

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