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

*Ekaterina Alexandrovna Durymanova Ono**, *Sueli Akemi Taniwaki*, *Paulo Brandão*

University of São Paulo, School of Veterinary Medicine, Department of Preventive Veterinary Medicine and Animal Health, São Paulo, Brazil

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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™. *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.

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]

* *Corresponding author at*: Department of Preventive Veterinary Medicine and Animal Health, School of Veterinary Medicine, University of São Paulo, CEP 05508-270, São Paulo, SP, Brazil.
E-mail: katyaono@gmail.com (E.A. Ono).

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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

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