Short-Interfering RNAs as Antivirals Against Rabies

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This study aimed to test *in vitro* a RNA-interference based antiviral approach for rabies with short-interfering RNAs (siRNAs) against rabies virus nucleoprotein mRNA. BHK-21 cells were infected with serial dilutions of PV rabies virus strain and transfected with a pool of three siRNAs. Direct immunofluorescence staining showed a 5-time decrease in virus titer when compared to a non-treated plate, showing a promising new approach to the development of antivirals for rabies treatment.

Key-Words: siRNA, rabies, antiviral.

Rabies is a fatal encephalitis that affects the *Mammalia* [1] and human cases of the disease are prevalent mainly in undeveloped countries; affected patients experiment extensive physical suffering. Antivirals currently used against rabies include ketamine, midazolam, ribavirin and amantadine, but only one successful case of human treatment happened in 2004 [2]; furthermore, rabies virus still circumvents most actions aimed to treat and control rabies and to create consensual scientific and political efforts on this area and only very few studies focus on antivirals for this disease.

The RNA interference (RNAi) is based on the ability of double-stranded RNAs, *e.g.*, short-interfering RNAs (siRNAs), to specifically trigger mRNA degradation by the cellular RNA-induced silencing complex (RISC) [3], an ubiquitary cytoplasmatic protein complex that harbors dsRNA-binding domains and an exonuclease domain activated by the first after a dsRNA binds to it [4]. This approach has been successful in *in vitro* and *in vivo* assays for Hepatitis C virus [5] and *in vitro* assays for Dengue virus and HIV [6], but no attempt to apply RNAi against rabies virus infection has been reported so far.

The aim of this study was to test the effect of RNA interference on the decrease of rabies virus titer *in vitro*.

Materials and Methods

Three AA(N19)TT siRNAs were designed with antisense strands complementary to rabies virus nucleoprotein (N) mRNA from 221 sequences retrieved from the Genbank (http://www.ncbi.nlm.nih.gov/) aligned by the CLUSTAL/W [7] method with Bioedit 7.0.5.3 [8]: RNA124 (sense 5' GCCUGAGAUUAUCGUGGAG 3'/ antisense 5' AUCCACGAUAAUCUCAGGC 3'), RNA750(sense5'GCACAGUUGUCACUGCUUC3'/ antisense5'UAAGCAGUGACAACUGUGC 3') and RNAB (sense 5' GACAGCUGUUCCUCACUCG 3'/ antisense 5' AGAGUGAGGAACAGCUGUC 3'), targeting the regions starting at positions 123, 749 and 903 of rabies virus nucleoprotein gene, respectively, in an area that codes for a highly functional constrained portion of N protein, which plays a major role in nucleocapsid assembly [9].

All three siRNAs were submitted to BLAST/n at http://www.ncbi.nlm.nih.gov/BLAST/ and no significant non-N gene homology was found. As the secondary structure of a given RNA is a key feature to the effectiveness of RNAi [10], the secondary structure of the target mRNA was evaluated at 35, 37, 39 and 42 ºC with RNADraw v 1.1 (Mazura Multimedia, Dalagatan 9C:320, 11324 Stockholm, Sweden), based on a broad range of body temperatures that a normal person and a rabid patient can experience [11]. No alteration was found to siRNAs 750 and B; regarding siRNA 124, minor secondary structure instability of the target site was found at the described temperatures.

BHK-21 cells grown in MEM/ 5% fetal bovine sera (FBS) in 24-well plates at 37ºC with 5% CO_2 for 48 hours were inoculated with the Pasteur virus (PV) rabies reference strain diluted 10-fold in FBS-free MEM and incubated for two hours at 37º with 5% CO_2 in a volume of 500 µL/dilution. Next, the monolayers were transfected with a pool containing 20 pmols of each of the three siRNA in DEPC-treated water with Lipofectamine 2000 (Invitrogen™, Carlsbad, CA, USA). A control plate was made with the same virus dilution, but no siRNA was added. Further negative controls included wells inoculated with 100 µL Lipofectamine 2000 and DEPC-treated water.

Twenty-two hours post-inoculation, both the control and the siRNA-treated plates were tested by direct fluorescent antibody test (DFA) with anti-rabies virus nucleocapsid antibody conjugated with fluorescein isothiocianate (Biorad Laboratories™, Hercules, CA, USA) and observed with an epifluorescence microscope. Virus titers were calculated by the Reed-Müench method and fluorescence intensity was quantified as +1 (at least one fluorescent focus in the well), +2 (about 50% of the monolayers with fluorescence), +3 (about 75% of the monolayers with fluorescence) and +4 (coalescently fluorescent monolayers).

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Results

The titer of the PV strain in the control plate was $10^{4,375}$ $TCID_{50}$/500 µL while, in the siRNA-treated plate, the titer fell to $10^{3,625}$ $TCID_{50}$, about 5 times lower. Fluorescence intensity in the control plate varied from +3 to +4 in 10^{-2} to +1 in 10^{-4} and fell to +2 in 10^{-2} dilution and to +1 in 10^{-3} dilution on the siRNA-treated plate. No cytotoxic or cytopathic effect was observed in the monolayers inoculated with Lipofectamine 2000 or DEPC-treated water.

Conclusion

We conclude that the pool of siRNAs used herein was able to significantly inhibit rabies virus replication *in vitro*, with no cell damage as depicted from the negative controls and it shows that RNAi can be seen as a promising new approach to the development of antivirals for rabies treatment.

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