Review

RNAi –Based Treatment for Rabies: Current Achievements and Insights Towards a Cure

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Abstract | Due to the lack of success of antivirals in inhibiting rabies virus intracellular cycle both in vitro and in vivo, and the high failure rate of this treatment in human patients, the use of RNA interference (RNAi) for Post-transcriptional Gene Silencing (PTGS) has witnessed an increasing interest from those concerned with a cure for rabies, inspired on the success obtained for other viral diseases. This review is intended to present the current situation of RNAi against rabies, as well as insights into the future on the field.

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Introduction

Rabies is an acute and lethal viral encephalitis transmitted by mammals to other mammals, with most of the estimated 61,000 yearly human deaths coming from Asia and Africa transmitted by wild and domestic carnivores. Dogs-transmitted rabies is also endemic in many countries in Latin America and the Caribbean, and sporadic cases still continue to occur in South America and the USA, respectively, with a diverse range of bats as reservoirs, whereas in Western Europe cases are related to travelers returning from endemic countries (WHO, 2013).

The disease is caused by species/genotypes in the *Lyssavirus* genus (*Mononegavirales*: *Rhabdoviridae*), chiefly by the virus *Rabies virus* (RABV). Lyssaviruses present an enveloped, bullet-shaped spiked virion made of the five structural proteins; Glycoprotein (G) that binds to cell receptors, Matrix (M) protein that gives structural stability to the virion, the RNA-dependent RNA-polymerase Large (L) protein, the Phosphoprotein (P), a co-factor for L also involved in trans-axonal virion transport and the Nucleoprotein (N) that binds to the circa 12kb negative-sense, single-stranded genomic RNA (Wunner, 2005).

A treatment for rabies has been applied to tenths of human patients, with only two cases of success, which core is the association of ribavirin, amantadine and ketamine. Ribavirin increases the mutation rate of RABV leading to a lower viral fitness, but a suppression on the production of antibodies has been associated to this compound; amantadine increases the pH of the endosome after virus entry into the cytoplasm, avoiding the low pH-dependent virus uncoating, but no effectiveness has been definitely shown in mice; ketamine, a dissociative drug, has also been shown to inhibit RABV transcription, but again no definitive evidence comes from *in vitro* and *in vivo* trials (Appolinario and Jackson, 2014).

Due to the lack of success of these antivirals in inhibiting RABV intracellular cycle in *vitro* and *in*

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vivo and the high failure rate of this treatment in human patients, the use of RNA interference (RNAi) for Post-transcriptional Gene Silencing (PTGS) has witnessed an increasing interest from those concerned with a cure for rabies, inspired on the success obtained for other viral diseases.

This review is intended to present the current situation of RNAi against rabies, as well as insights into the future on the field.

RNAi is a good strategy against rabies

The first study reporting the use of RNAi to suppress RABV cycle was published in 2007 (Brandão et al., 2007). The authors used a pool of three different 21bp double-stranded RNA molecules (short-interfering siRNAs) targeted to the mRNA of the N protein to unleash the RNAi-mediated degradation of these mRNAs, transfected with liposomes into the cytoplasm of BHK-21 cells previously infected with the PV strain of RABV (*i.e.*, a post-exposure strategy). As revealed by direct fluorescent antibody test (DFAT), a 5-fold decrease in RABV titer was achieved, providing evidence that RABV can be knocked down using RNAi.

Micro RNAs (miRNAs), a precursor form of siRNAs, have also been used to knockdown RABV replication targeting mRNA of N protein in neuroblastoma (N2A) cells infected with fixed and wild type RABV strains (Israsena et al., 2009), with an effective decrease in genomic RNA and mRNA of the N protein, the main achievement being the demonstration that RNAi is not only effective against high virulent wild type strains but also that it works on both pre (prevention) and post (treatment)-exposition applications.

A major achievement was reported by Gupta et al. (2012) when the intracerebral inoculation of mice with an adenovirus vector carrying siRNA coding-sequences to mRNA of N and L proteins, an approach also shown effective *in vitro* (Sonwane et al., 2012), and next challenged with fixed RABV led to a significant protection, providing evidence that RNAi is effective against rabies *in vivo*.

The fact that RNAi does inhibits RABV protein synthesis was shown by Yang et al. (2012) using Western-blotting in brain samples of mice intracerebrally injected with plasmids coding for siRNAs against mRNA of N and then challenged with the CVS fixed strain of RABV, resulting in a reduced N protein synthesis and fewer clinical signs with a lower morbidity.

Targeting more than one mRNA of RABV than can increase the inhibition of virus cycle, improving the applicably of RNAi against rabies, as shown by the pre-treatment of BHK-21 cells transfected with a single plasmid coding for siRNAs against N and G proteins mRNAs simultaneously, leading to a higher decrease of virus titre as measured by DFAT when compared to plasmids coding for each siRNA individually (Meshram et al., 2013).

The effectivity of RNAi against RABV in the presence of high virus titres has also been demonstrated in a post-exposure assay using three individually tested siRNAs transfected with liposomes via injection into mice brain (Durymanova Ono et al., 2013).

On the view of the critical role of siRNA-delivery for a consistent virus knockdown using RNAi, it was also shown that lentivirus and adenovirus deliver systems carrying siRNA coding sequences against mRNA of N inoculated by the intracerebral and the intramuscular routes, receptively, can significantly reduce mortality in mice challenged with fixed RABV strains (Wu et al., 2013; Singh et al., 2014).

In summary, RNAi has been shown to be effective both *in vivo* and *in vitro* against a range of strains and titres of RABV, leading to reduced mortality, morbidity and intensity of signs in mice, with delivery systems being developed to extend the time of action and the potency of the inhibitory effect, with no side-effect being reported. This should make RNAi the therapy-of-choice against rabies, but this is not yet the case.

RNAi is not (yet) a good strategy against rabies: insights into the future

Early diagnosis is paramount for any treatment against rabies. Due to the rapid course of the disease, measured in a few days, no treatment would be effective would the signs be fully manifested. This means that, regardless how effective a therapy against rabies is *in vitro* or *in vivo*, it's of no use if not administered on time to naturally infected patients.

Thus, increasing the awareness of health professionals, including physicians, about the early symptoms and

the epidemiology of rabies can greatly reduce the false negative clinical detection of the disease. Besides, once a patient is clinically or epidemiologically diagnosed as suspected of rabies, techniques such as qPCR in samples of hair follicles and saliva (Wacharapluesadee et al., 2012) serially collected can allow for the classification of such patient as eligible for antiviral treatment in a matter of hours.

Another important issue for RNAi-based therapies is the transient effect of transfected or viral vector-delivered RNAi-mediator molecules such as siRNA and miRNAs, as the RNAi effect on RABV cycle might not last enough time to significantly overcome the fast and continuous viral replication in the central nervous system. Serial, instead of a single delivery, should be considered thus for a broader action of these molecules

For any drug systemically administered and which is intended to reach the central nervous system, the blood-brain barrier is a major obstacle to be overcome, as this structure blocks the access of a range of molecules to this site. It´s thus not unexpected that the use of the current RNAi-mediators delivery strategies alone will not be able to reach RABV in its preferential replication site in a sufficient amount, but the association with drugs that open the blood-brain barrier could improve the delivery of RNAi-based treatment to such site.

The mediation of RNAi by RNA molecules is highly sequence-dependent, meaning that, for a universal effect to be reached, a stable targeted must be found. But, in the case of RABV, a high nucleotide sequence variation exists amongst different variants of the virus (Kuzmin et al, 2005), making universal siRNAs highly improbable. Nevertheless, the fast sequencing of a very short fragment of RABV genome from samples obtained as mentioned above could allow for a fast selection of variant-specific RNAi mediator molecules from a pre-available bank to be promptly administered to a patient.

Another core issue still not taken into account in any rabies treatment proposed so far is the fact that, despite the central nervous system is a preferential site for RABV replication, the virus is pantropic, replicating in organs such as kidneys and liver (Centers for Disease Control and Prevention, 2004). So even if a central nervous system clearance is reached, the virus still present in other organs could theoretically follow the centripetal trans-axonal route again and recolonize the central nervous system, reactivating the disease. In the view of this possibility, though transcranial administration of RNAi-mediator molecules is an adequate approach to proof-of-concept assays, the clinical application should always have the systemic administration as a preferred.

Finally, RNAi leads to knockdown and not knockout of gene expression, meaning that a full suppression of RABV cycle using RNAi alone probably will not be reached, making clear, as for any other anti-rabies treatment, that the suppression of the viral cycle in different routes with the association of different drugs is needed.

Much bench work has thus to be done before RNAi comes to be seriously considered (or fully discarded) as a clinical application against rabies, but it's now up to scientists on the field to carry on with this task, testing new targets for RABV knockdown, new delivery systems, the association with other drugs and further checking for side effects. The effects of RNAi on RABV must still be better elucidated in terms of their effects on viral protein synthesis balance taking into account the interaction amongst the five viral proteins and its consequences on viral RNA synthesis. Finally, the improvement of experimental designs for anti-rabies RNAi to make then compatible and comparable to trials in which other antiviral principles are used, using proper controls and statistical approaches, is still needed in order to bring more reliability to RNAi as an antiviral approach for this disease.

Efforts during the last decades by some Animal and Public Health Authorities, NGOs and research groups have prevented a vast number of new human cases of rabies mainly based on vaccination and population control of reservoirs, pre and post- immunization of humans and education.

Still, thousands of human cases occur worldwide. The treatment of these cases faces nowadays a dilemma: should physicians submit the patient to a highly toxic and dubious treatment with a low chance of cure or should they not to try any antiviral treatment, just keep the patient in a coma waiting for the certain death? Though anti-rabies treatment nowadays faces distrust from some involved in the field, rabies patients are worth the try and RNAi may be a part of a

cure.

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