siRNA intervention inhibiting viral replication and delivery strategies for treating herpes simplex viral infection

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Abstract The effective treatment of herpes simplex virus (HSV) infections generally involves the use of antiviral nucleoside drugs, but with increasing reports of antiviral resistance, the use of these drugs is challenged. Hence, a need arises to explore alternate treatment options. In this review we have discussed various targets that have been explored to control the HSV replication using siRNA therapeutics. We have also discussed the advantages of targeting a less explored UL10 gene to develop an alternate therapeutic intervention. Gene silencing can induce an inhibitory activity to virus spread and infection. The capacity and suitability of UL10 gene as siRNA induced silencing target in eliciting the desired antiviral effect in patients is identified and particularly discussed. The major challenge associated with the siRNA therapeutics is their delivery. The various viable delivery options, that are being explored in the recent times is summarized and different delivery pathways and strategies are reviewed as a part of the study.

Keywords Herpes simplex virus · Gene silencing · siRNA · siRNA delivery · UL10 gene

Introduction

The herpes simplex virus (HSV) belonging to human Herpesviridae family exists as two subtypes, HSV 1 and HSV 2, which are responsible for a multitude of diseases like primary and recurrent infections of mucous membranes (e.g., gingivostomatitis, herpes labialis, and genital HSV infections), keratoconjunctivitis, neonatal HSV infection, visceral HSV infections in immunocompromised hosts, HSV encephalitis, Kaposi's varicella-like eruption, and an association with erythema multiforme [32]. According to WHO, approximately 3.7 billion people (67%) under 50 years of age are affected by HSV 1 and 417 million people (11%) in the age of 15–49 are infected with HSV 2 globally. Antiviral nucleoside drugs like acyclovir, famciclovir, valacyclovir are the most effective medications available for the treatment of these infections. However, they only alleviate the symptoms of the infections but not completely cure them. Effective vaccines against these infections are yet to be developed.

The epidemiological studies of HSV have become a bridge to reorient novel and effective methods for prevention and treatment. There are some significant statistics conducted across various parts of India providing an insight of HSV's prevalence. One such study was carried out in the demography of Chennai, Tamilnadu, India [24]. Data from samples collected monthly in the year 2013, revealed that males, particularly in the age group of 1–5 years (7.6%) and 6–12 years (12.2%), were HSV positive when compared to the female groups as they were highly susceptible than women. A longitudinal low risk demographic analysis conducted in Andhra Pradesh, India, defined the incidence of HSV-2/syphilis and their associated risk factors, in the selected sample during the years 2004–2005 and 2010–2011. Remarkably, the incidence of HSV-2 was

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found to be elevated in female population of rural areas compared to urban residents [10]. Type specific serology (TSS) test in a cross-sectional study, performed during a course of 10 months included 44 patients who were at risk for genital herpes. Compared to primary genital herpes cases, the presence of IgG in recurrent genital HSV2 diagnosis had a higher sensitivity, specificity, and very good positive predictive value [21]. Owing to the high incidence of the HSV infections, and reported increase in cases of drug resistance, several novel techniques are being explored and one of the potential alternate interventions is the small-interfering RNA (siRNA) therapeutics.

siRNA therapeutics

Treatment with siRNA is a very promising technique that can be used to treat many diseases. Silencing certain essential viral genes using siRNA can decrease the disease burden in patients. Several studies showing reduction of viral titer to a good extent upon siRNA treatment in in vitro models have been reported. The major limitation found by most groups is serum instability of naked siRNAs. Here, we have discussed the studies that have shown the application of siRNA therapeutics for inhibiting HSV infection.

A study reported the targeting of VP16 and DNA polymerase genes with siRNAs, effective in inhibiting HSV 1 replication [35]. In plaque reduction assay, these siRNAs showed nearly 85% and 70% reduction respectively, in HSV 1 replication. Few capsid protein coding genes of HSV were inhibited using siRNA technique in an exploratory study by Jin and his group [12] to recognize the gene that is best suited to inhibit its replication. The genes, UL18, UL19, UL26, UL26.5, UL35 and UL38 were tested in vitro with siRNAs with promising results. The proteins VP23 and VP5 encoded by UL 18 and 19 genes, proved efficient in reducing the viral replication both individually or in combination. The intracellular genome load of the virus was significantly reduced with UL18; UL19 gene knockouts.

UL29 and UL28 genes, which encoded proteins that are essential for replication and packaging of the virus, were targeted with short-hairpin RNAs (shRNAs) and their subsequent effect on viral replication was studied. The results indicated the significant suppression of HSV 1 replication with UL29shRNA and UL28shRNAs and their combinations showing synergistic effects. This study also highlighted the effectiveness of using adeno virus (AdV5) for delivery of shRNAs [27]. In a similar study, several siRNAs were synthesized enzymatically to target vital HSV-1 genes, UL54, UL29, and UL27. Pools of individual siRNAs in an equimolar mixture, inhibited HSV-1 replication, with UL29 siRNA pool displaying a significant

activity [19]. Further, siRNAs synthesized against UL29 were tested on HSV-1 infected mice. The anti-HSV-UL29 siRNA swarm alleviated the symptoms of the infection and inhibited the viral shedding and replication thereby increasing the survival time of the mice [20].

One study tested three siRNAs synthesized for targeting UL39 gene of HSV-1. One of the sequences was effective in inhibiting 99% replication of the HSV-1 infection [4]. siRNAs were also tested for their potential to control the herpetic encephalitis, induced by HSV-1 in mice model. All siRNAs alone and in combination with drug acyclovir were administered. siRNA treated mice showed reduced clinical signs of infection and prolonged life by inhibiting replication of HSV-1 [5]. One study also reported inhibition of UL36 and UL40 genes by siRNA technique effective in controlling HSV-1 infection [22]. An article studied the effect of inhibition of polypeptide 4 genes in regulating HSV-1 in retina epithelial cells using siRNA treatment [6]. Wang and team reported successful reduction of dermal lesions in mice infected with HSV-1 by siRNA treatment targeting nuclear paraspeckle assembly transcript 1 (NEAT 1), an important component of paraspeckles, which are constituents of nuclear bodies of the host cell [31]. Few reports are available on siRNA for prevention and control of HSV-2, targeting both viral and host genes (*nectin-1* and *UL29*) [33].

Though several reports are available of successful targeting of viral and host genes to inhibit the replication of HSV-1 and HSV-2 in in vitro, limited success was achieved during preclinical study using rodent models. The major concern with naked siRNA therapy is its serum nuclease stability. Most of these viral studies targeted either HSV-1 or HSV-2 inhibition, and a very few have attempted targeting both using same siRNA. HSV-1 and HSV-2 have good similarity in their gene sequence. Hence, it is sensible and beneficial to use a siRNA that targets the same gene of either the viruses with limited or no differences among them. Apparently, there are many siRNA therapeutics under progress using vectors for various diseases. However, there are no reports of siRNA targeted study on HSV-1 or HSV-2 yet.

All these different studies highlight the importance and potential of siRNA as a therapeutic regimen. Establishing this alternate form of treatment can greatly benefit the global population and help create pathways that can potentiate into therapies that combat various infections that keep arising in the modern world.

UL10 gene in HSV infection

The success of siRNA therapy is essentially based on the target selection. Several genes are being explored as targets capable of inducing the desirable outcome, but a complete inhibition has not yet been reached. A gene UL10 was studied for its role in the viral infection, but very limited studies have focused on the effect of silencing this gene on the viral infection. The following study tries to combine the available literature on the gene to establish its role in infection and potential in inhibition. The importance of UL10 in the viral life cycle was studied and its structure and sequences were analyzed. The UL10 encodes the envelope protein called glycoprotein M (gM), which plays a crucial role in secondary envelopment of the viral life cycle [3]. It encodes a membrane protein with 8 potential transmembrane regions.

The herpes virus, like the other DNA viruses, comprises of 16 membrane proteins in its envelope. During viral assembly and envelopment, an orderly sub cellular localization of envelope proteins is necessary. To ensure proper incorporation, the membrane proteins should be localized to appropriate intracellular membranes [16]. Targeting motifs of proteins direct the assembly, but certain other envelope proteins rely upon other proteins to elicit their incorporation. The essential entry proteins gD, and gH/gL complex, needs envelope protein gM and the gK/pUL20 complex, to reach their appropriate intracellular virus assembly compartments [16].

Around 13 different glycoproteins are present in the mature HSV1 virus. Among these glycoproteins, most are involved in viral egress with only five responsible for viral entry. gM along with redirecting the gD and gH/gL complex from surface of the cell to trans-Golgi network, is also involved in modulating the final capsid envelopment [36]. The deletion of gM decreases the viral yield and promotes syncytium formation. The gM protein was observed to be modified with an N-linked glycosylation and is found to associate with membranes from infected cells [18]. The gM is conserved throughout the family of Herpesviridae and using transfection-based assays, the activity of gM in modulating internalization and subcellular targeting of viral proteins was established. In a study with gM deletion virus, the absence of gM expression resulted in an inhibition of proper internalization of glycoprotein H-L (gH-L). This correlated with defects in the entry of virions and their spread across cells, suggesting the important role of gM in efficient targeting to domains involved in secondary envelopment of infected cells [22].

The UL10 deletion mutant was constructed which lacked a major portion of the UL10 open reading frame (UL10-del). This mutant demonstrated a lower virion

release than the wild-type, with a negligible temperature sensitivity or host range phenotype. The study also explored the capacity of this mutant to induce infection in a mouse model and found that the UL10-del did establish a latent infection, however, the growth at the periphery was impaired and the spread of infection in the nervous system was less than the wild or revertant viruses [18]. Deletion studies with UL10 also indicated that its absence made the entry of the viruses into the cells slower [13]. Zhang et al., 2009, reported that gM is specifically targeted to nuclear membranes.

During the later time of disease prognosis, gM reaches the nuclear envelope and it was reported that the gM is quickly targeted to the nuclear membrane [36]. The nuclear gM seems to fulfill a nuclear function in the initial stages, independent of its interaction partners. In one study to determine the functional hierarchy of glycoproteins in viral infection stages of envelopment and egress, comparison of viruses mutated with different number of mutations, engineered to lack expression of certain viral proteins indicated that the absence of either UL20 or gK proteins resulted in the most prominent defaults in cytoplasmic envelopment and contagious virion development, succeeded by double deletion of UL11 and gM [2].

Since, both pUL11, a tegument protein and gM are responsible for a part of secondary envelopment, and the deletion studies with either one of the genes, yielded only limited effects on replication, a simultaneous deletion study was performed. On *Vero* cells, the deletion of either pUL11 or gM resulted in a fivefold decrease in the virus titers and 40–50% decrease in the plaque diameters. On rabbit kidney cell line (RK13) however, the results were much more pronounced with around 50-fold reduction in titer values and 70% reduction in the plaque size [17]. The simultaneous deletion led to an increment in defects associated with growth and in RK13 cell line, large insertions of intracytoplasmic capsids and tegument materials were observed.

Another glycoprotein gN (UL49.5) is also functionally related to the glycoprotein M. The gN resides on the endoplasmic reticulum and then shifts to the trans-Golgi network, in presence of gM, connected covalently by a single disulphide bond. So, a study explored the effects of deletion of these genes on viral activity. The deletion of gN generated a mutant virus with growth behavior similar to the wild-type, while removal of gM led to the formation of an attenuated phenotype. The deletion of gN in union with gM mutants, did not greatly affect the plaque size thus indicating the non-essential nature of gN for the functioning of gM [28].

An active antiviral effector, tetherin, tethers the enveloped virion particles to the host cell membrane and prevents their release, thus restricting the infection. However,

the glycoprotein gM demonstrates a moderate anti-tetherin activity. In one study, gM efficiently removed the tetherin from plasma membrane thus indicating its role in infection establishment [1]. Another study indicated the presence of a functional core within the gM which targets it to TGN, a favourable site of envelopment. The complete deletion of complete gM/UL10 in a study, generated an attenuated phenotype indicating its role in virulence [28]. Studies by El Kasmi and Lippé, suggested that gM encoded by the UL10 is responsible for cell–cell fusion and viral entry [7].

All these studies depict the necessity of UL10 gene product in viral infection establishment. The absence of this gene product can greatly impact the viral infection and its spread. Hence, this gene possesses the potential to induce a desirable effect with siRNA silencing.

siRNA delivery complications and interventions

With the growing siRNA potential as a therapeutic tool in treating various diseases, efficient delivery of the siRNAs is most important to ensure the efficacy of treatment. However, there are certain limitations, when it comes to delivery of siRNAs. New, novel techniques are being explored to develop an efficient technique of delivery, but further research is needed before a technique can be optimized. siRNAs with their specificity, adaptability and capability of inducing gene silencing are being pursued actively in therapy for cancers. However, owing to the instability of naked siRNAs in the blood stream, careful designing of delivery systems is essential.

Nanoparticles are one of the most commonly pursued choices for increasing the naked siRNA stability. Nanoparticles offer unique advantages like a desirable particle size, inert and non-immunogenic nature of the materials, enhanced circulation time, and the ability to be imaged or tracked [29]. Certain limitations like the poor water solubility, hydrophobicity and limited bioaccumulation are also associated with this delivery pathway but the advantages outweigh the disadvantages.

The most logical choice of siRNA delivery pathway is its introduction into the system to ensure that proper dose of siRNA reaches the target tissue. This pathway offers reduced off-target effects and circumvents obstacles associated with systemic delivery [25]. However, topical siRNA delivery is also gaining significant importance and has progressed into many clinical trials. Other delivery systems like sustained-release and local delivery systems are also being explored as alternate delivery methods for siRNA delivery. Certain delivery systems have been fabricated using materials like poly (lactic-*co*-glycolic acid) (PLGA). This material has inherent in vivo degradation properties and hence these hydrolytically degradable

polyesters are used as scaffold and microparticle systems [25]. Another phenomenon called the substrate-mediated uptake is also being engineered as a delivery system for efficient cellular uptake.

Local delivery of siRNA is another method of directing the cellular responses in treating various diseases. Microparticles, scaffolds, electrospun fibers, hydrogels and surface coatings are some of the local delivery methods that can be explored. To enhance the specificity of the RNAi delivery in vivo, certain materials like targeting moieties, cell-penetrating peptides, fusogenic peptides and stimuli-responsive polymers can also be used [26]. RNA aptamers are also popularly used to deliver siRNAs to target tissues and cells. The major advantage associated with the use of RNA aptamers is the approach of in vitro transcription for aptamer production. This facilitates the formation of products which are free from contamination by bacterial or cell products [26].

Increasing ongoing research on a newer class of materials called the polymer-based nanoparticles is also being reported. Bio-engineered materials like lipid based nanoparticles, polymeric nanoparticles, inorganic nanoparticles and RNA based nanoparticles are some of the examples of such particles being used for siRNA delivery [15]. Lipid nanoparticles are widely used biomaterials which facilitate the siRNA delivery. Around 20 siRNA phase I clinical trials reported the nanoparticle usage, mostly lipid based for their delivery [15]. Most widely used, commercially available lipid-based reagents are—lipofectamine, 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), and RNAfect, along with others. Certain virus based delivery strategies are also developed and explored for efficient siRNA delivery. However, problems like the induction of insertional mutagenesis and undesired immune responses, complicate the efficacy of this option [11].

Inorganic nanoparticles are another highly explored areas of delivery systems. The high surface to volume ratio of these particles, and their efficient loading capability make them attractive for use as siRNA delivery systems. Certain examples include, gold nanoparticles, magnetic nanoparticles, mesoporous silica nanoparticles and calcium phosphate nanoparticles. Chemical modifications of siRNAs also significantly impact the stability and uptake of siRNAs [8]. The sugar moiety is the most commonly modified portion with the incorporation of either halogens, amines or deoxy groups. The modifications in siRNA backbones also cause considerable impact on the stability of naked siRNAs.

The termini of the siRNAs are also sometimes altered to improve upon the pharmacokinetic and thermodynamic properties of the siRNAs. However, during modifications, care should be elicited not to induce any changes in the

siRNA specificity or their silencing activity. A novel siRNA delivery system was created with ternary complex of polyethyleneimine (PEI) and γ -polyglutamic acid [14]. This complex displayed a prominent silencing activity almost equal to the lipofectamine complex with siRNA and no cytotoxicity being reported. Thus, techniques are being explored to develop significant delivery options, to improve the viability of using siRNAs as effective therapeutics.

The cationic cell penetrating peptide (CPP) is another substance that can be utilized to guide the siRNA delivery directly into the cells surpassing the cell membrane [30]. Together, these form a non-covalent complex that is positively charged and possess the added advantage of directly combining the siRNAs, without the requirement for a chemical modification. Polymeric and dendrimeric carriers are also used for delivering siRNAs. Along with the use of PLGA and PEI, other polymers like chitosan and gelatin are also being investigated for use. Conjugation of the siRNAs with molecules like cholesterol, bile acids and proteins like antibodies, greatly improve the cellular internalization and targeting and delivery of the siRNAs [30].

Certain other siRNA delivery systems are also often explored as potential siRNA delivery strategies. Exosome-mediated siRNA delivery systems, oligonucleotide nanoparticles and RNAi-microsponges are examples of some of such delivery platforms [34]. Exosomes offer specific delivery with little or no toxicity and immunogenicity in the systems making them very attractive as potential delivery strategy. The high abundance of the exosomes in the bodily fluids further adds to the advantage.

Pharmacological approach also enhances the delivery of siRNAs by using various mechanisms of action on different cell types. A high throughput chemical screen was utilized to identify the compounds that have the potential to improve the siRNA delivery [9]. Small, chemical enhancers were selected and applied to the established delivery models of lipid nanoparticles and cholesterol conjugated siRNAs. These acts either by modifying the endocytic uptake or by improving the siRNA release from the endosomes. Stimuli-sensitive delivery strategies are another novel delivery techniques in the nano-platforms for improving the therapeutic potential of siRNAs. This platform provides better release characteristics for the delivery owing to its response to local stimuli which facilitates the nucleic acid release [23]. These systems are either redox sensitive or pH sensitive or respond to enzymatic environments. By combining this stimulus sensitive nature with the lipid or polymer nanoparticles of siRNA, improved efficacy and targeting can be achieved.

Various delivery strategies have been explored or being explored that show tremendous potential in revolutionizing

the siRNA therapeutics. The challenges associated with the delivery are being addressed continuously and novel techniques are evolving to address these trials. This review aims to offer a consolidated knowledge on the potential of siRNA as a therapeutic regimen in treating various viral infections and identifies UL10 gene as a target capable of eliciting an action with siRNA. The challenges associated with the delivery of siRNA are also addressed with novel technologies that are being pursued to improve efficiency.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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