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Research Article

RNA INTERFERENCE AS ANTIVIRAL THERAPY: DREAM OR REALITY?

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ABSTRACT

Soon after discovery of RNA interference (RNAi), its potential as effective antiviral therapy was recognized. Since then RNAi has been variously exploited for antiviral purposes which could effectively block viral replication *in vitro*. For *in vivo* use, however, delivery issue, toxicity, RNAi suppression and viral escape are still major hurdles. Here, we provide an overview of the RNAi strategy and review the approaches that have been developed to surpass the obstacles and to achieve targeted gene silencing for antiviral and other therapies.

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INTRODUCTION

RNAi is a highly specific, evolutionarily conserved, post transcriptional gene silencing mechanism used by cells for gene regulation, protection of genome against transposable elements and the attack of RNA viruses. While RNAi acts as an important innate antiviral defense mechanism in plants and insects (Zamore et al. 2000; Hamilton & Baulcombe 1999), whether RNAi as antiviral immune mechanism really exists in mammals, remains unclear (Haasnoot & Berkhout 2010; Umbach & Cullen 2009). However, the enzymatic machinery for RNAi is used by the other endogenous non-coding small RNAs like microRNAs (miRNAs), for regulation of gene expression (Pfeifer & Lehman 2010; Hajeri & Singh 2009). It is estimated that RNAi mediated gene regulation controls the expression of about 30% of mammalian genes, many of which are involved in functions like cell fate, proliferation and death (Lewis et al. 2005).

Discovery

The phenomenon of RNAi was first observed by Napoli and colleagues in 1990, when they were trying to over express an enzyme responsible for plant coloration by introduction of the exogenous gene in petunias (Napoli et al. 1990). Surprisingly, the introduced gene resulted in a blockage of pigment synthesis. The phenomenon of gene suppression, however, was not understood at that time. It was Andrew Fire and Craig Mello who finally established the mechanism in 1998, by demonstrating that injection of short stretches of 23mer to 25mer nucleotides sequence of double stranded RNA (dsRNA) into the nematode *Caenorhabditis elegans* resulted in silencing of endogenous genes (Fire et al. 1998). Fire and Mello were awarded the Nobel Prize for Medicine or Physiology in 2006 for their discovery. This mode of gene silencing was different from the already established anti-sense mode of gene silencing in that, although both are based upon sequence complementarity to messenger RNA (mRNA), RNAi has a catalytic component that makes it possible for a single small interfering RNA (siRNA) molecule to bind with and destroy thousands of copies of mRNA molecules and thus is 1000 times more effective than antisense oligonucleotides (ODNs) in silencing target gene (Grunweller et al. 2003; Miyagishi et al. 2003; Bertrand et al. 2002). Subsequently, it was reported that RNAi could take place in numerous other organisms including invertebrates, and vertebrates (Elbashir et al. 2001). Elbashir and colleagues in 2001, showed that transfection of synthetic siRNAs could silence genes in mammalian cells (Elbashir et al. 2001). Since then RNAi has found widespread applications ranging from use in functional genomics and gene knock-down, to treatment of various medical conditions like macular degeneration, cancers and viral infections.

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Mechanism

RNAi machinery in mammals and plants is used intrinsically by microRNAs (miRNAs) for regulation of gene expression. The miRNAs are small non-coding RNA molecules transcribed by polymerase II as long primary transcripts or pri-miRNAs (Lee et al. 2004). The pri-miRNAs undergo a sequential maturation process in which they first bind to a microprocessor, a complex of two proteins in the nucleus which contains the DiGeorge Syndrome Critical Region-8 (DGCR8) protein and a nuclear RNase III called Drosha (Lee et al. 2003; Han et al. 2004; Denlí et al. 2004). Next the Drosha cleaves pri-miRNAs into pre-miRNAs, which are the imperfectly paired stem-loop miRNA precursors having 60 to 80 nucleotides. The pre-miRNAs are then exported to the cytoplasm by Ran GTP-dependent Exporting-5 transporter (Lund et al. 2004; Yi et al. 2003). The Dicer (an RNase III family enzyme) at first, interacts with its double stranded RNA-binding protein partner (TAR RNA binding protein; TRBP) and other partners to cleave pre-miRNAs into mature double-stranded miRNAs of 19-25 bp with 2 nucleotide 3' overhangs (Lee et al. 2002b). Then, Dicer together with R2D2 protein and its partners, couple with miRNAs duplex to form RISC loading complex (RLC), which helps in the loading of the miRNA duplex into another multiprotein complex, having Argonaute protein as its core component. This new complex is called RNA-induced silencing complex (RISC) or miRNA protein complex (miRNP) (Chendrimada et al. 2005; Hajeri & Singh 2009). After attachment to RISC, the passenger strand of miRNA is lost, while antisense strand guides RISC to the target mRNA. The miRNAs pair perfectly or imperfectly with the target mRNAs and thus may either result in endonuclease Argonaute 2 mediated cleavage and destruction of guide strand-mRNA duplex or blockage ribosome movement halting mRNA translation, respectively (Eulalio et al. 2008).

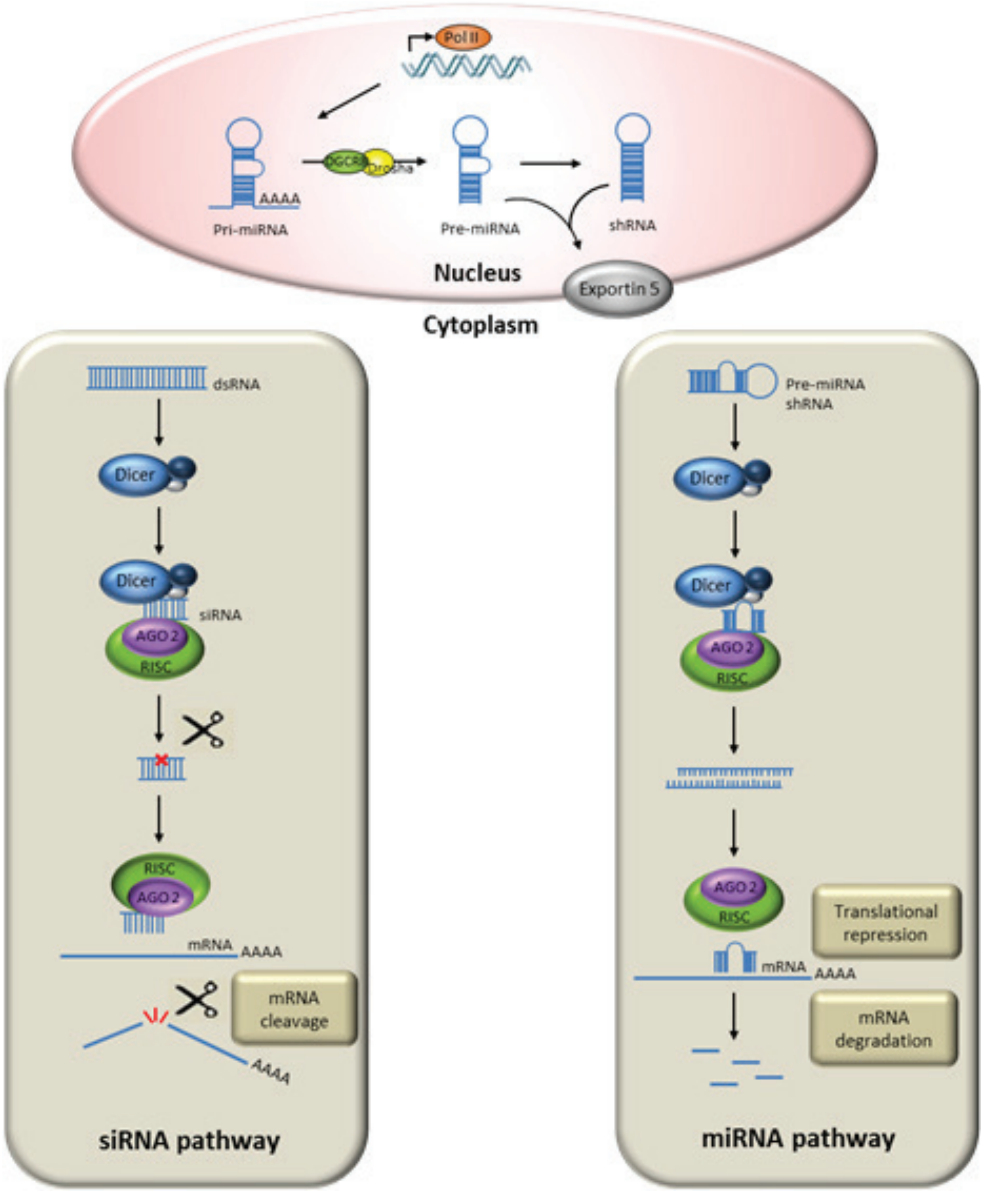

Figure 1. Mechanism of RNA interference.

The degree of miRNA-mRNA complementarity has been considered to be the key determinant of the regulatory mechanism. Thus, in case of a perfect match, mRNA is cleaved by RISC, while central mismatches may result in repression of mRNA translation (Pfeifer & Lehman 2010). Based on recent developments in understanding miRNA biology and mechanisms, at least three main models can be proposed by which miRNAs could modulate gene expression post-transcriptionally: (1) inhibition of translation initiation, (2) postinitiation inhibition of translation, and (3) mRNA degradation (Li & Rana 2012). Two others endogenous small RNA species have been identified, as piwi-interacting RNAs (piRNAs), and endogenous siRNAs (endo-siRNAs). piRNAs are germ cell-specific and have been shown to regulate transposon activities and spermatogenesis in *Drosophila melanogaster* (Klattenhoff & Theurkauf 2008). Endo-siRNAs are also involved in transposon silencing in *Drosophila, Caenorhabditis elegans* and mammals (Czech et al. 2008).

Plasmid or viral vectors expressing short hairpin RNAs (shRNAs) and chemically synthesized siRNAs mimic mammalian pre-miRNAs and miRNAs respectively and thus use cellular machinery for gene silencing. The only difference being that, the antisense strand of siRNA, acting as a guiding strand pairs perfectly with the target mRNA resulting in RISC-mediated cleavage of the target mRNA (Elbashir et al. 2001). Viral/plasmid vector based shRNAs, transcribed from a polymerase III promoter, are produced in the nucleus and exported to the cytoplasm by exportin-5 together with GTP-bound form of its cofactor Ran, and are processed by Dicer like pre-microRNAs to produce siRNAs (Yi et al. 2003). A schematic representation of RNAi mechanism is shown in Figure 1.

Interfering RNAs as an antiviral therapeutics

Targeting viral mRNA with siRNA is an attractive strategy since firstly, one can prevent synthesis of critical viral proteins to disrupt viral life cycle, secondly with siRNAs, being highly specific, there is little chance of side effects, and finally, understanding of gene function is not required: only viral genome sequences are needed (Spurgers et al. 2008). Soon after discovery of RNAi, its potential as effective antiviral therapy was recognized. RNAi antiviral treatment was first used by Bitko and Barik in 2001 against respiratory syncytial virus (RSV) (Bitko & Barik 2001). Since then the approach has been variously exploited for antiviral purposes, not only through the use of chemically synthesized siRNAs but also by expressing the shRNAs from plasmid or viral vectors (McCaffrey et al. 2003; Banerjea et al. 2003; Lee et al. 2002a). RNAi through these various approaches could effectively block viral replication *in vitro* but for *in vivo* use, delivery issue is still a major hurdle.

Difficulties in use of RNAi as an antiviral approach

In addition to delivery issues, the therapeutic use of RNAi *in vivo* also faces other practical difficulties like off-target effects, RNAi suppression and viral escape.

Off-target effects:

Although RNAi is highly specific, in addition to the intended mRNA suppression, it also produces unintended effects on gene expression. For *in vivo* delivery, off-target effects are major concerns since the down-regulation of self-genes could have serious biological consequences. They may result from either a partial sequence complementarity of RNAi construct to non-targeted mRNA or induction of a variety of immune and toxicity related effects emanating from certain motifs or patterns in the RNAi construct itself (Rao et al. 2009).

Specific off-target effects dependent on siRNA sequences

Although initially thought to be highly specific, RNAi was shown by expression profiling to produce off-target effects whereby a particular siRNA will bind to other mRNAs in addition to/instead of the one originally targeted. Partial sequence complementarity in both the passenger or guide strands of RNAi construct can produce off-target gene suppression Jackson et al. 2003). The off-target silencing has been reported for transcripts with as low as 7 nucleotides complementarity with the guide strand (Lin et al. 2005). siRNAs like miRNAs can also bind to sequences with partial complementarity at the 3'-UTR rather than overall homology between the siRNA and targets (Valencia-Sanchez et al. 2006; Birmingham et al. 2006; Anderson et al. 2008a). Furthermore, both the siRNA and shRNA with a complementarity in the "seed region", the 5′-end of the guide strand, can produce similar off-target expression profiles (Jackson et al. 2006a), however shRNA is reported to induce fewer off target effects than siRNA (Rao et al. 2009).

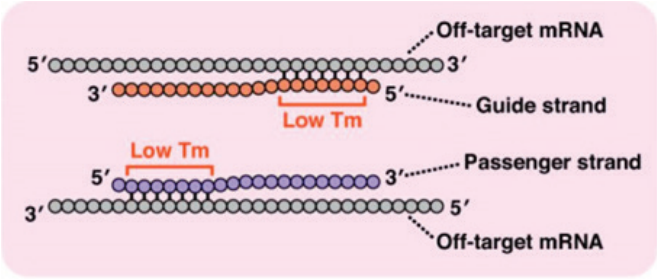

Figure 2. Seed dependant off-target effect. The capability of siRNAs to induce seed-dependent off-target effect is highly correlated to the thermodynamic stability of the duplex formed between the seed region of siRNA guide strand and its target mRNA (Ui-Tei et al. 2008b; Naito et al. 2009).

Non-specific off-target effects - Induction of type 1 interferon (IFN) and other immune responses

The animal immune system can discriminate between self and non-self nucleic acids. The double-stranded RNA (dsRNAs) longer than 30 bases can induce an immune response via protein kinase R

(PKR), resulting in a general degradation of mRNA and inhibition of translation as well as up regulation of interferon (IFN) stimulated gene expression (Bumcrot et al. 2006). Moreover, even the smaller (<30 nucleotides) siRNAs, although initially considered non-immunogenic (Elbashir et al. 2001), have been found to induce a partial cytokine and type-1 IFN response via toll like receptors (TLRs) (Sledz et al. 2003; Kariko et al. 2004; Hornung et al. 2005). It is also reported that although RNA sensing receptors are also found in the cytoplasm, nucleic acids mediate immunoactivation mainly through TLRs 7 and 8 (activated by ssRNA), TLR9 (by unmethylated CpG motifs in bacterial plasmids) and TLR3 (via dsRNA) (Hornung et al. 2005; Judge et al. 2005; Diebold et al. 2004; Heil et al. 2004; Karin et al. 2004). siRNA immune stimulation via TLR7 and TLR8 on endosomes can be sequence dependent and therefore is possibly avoidable (Hornung et al. 2005; Judge et al. 2005; Sioud 2005). Certain RNA sequences have been identified to be particularly immunostimulatory, such as "UGUGU" (Heil et al. 2004) and "GUCCUUCAA" (Hornung et al. 2005). The chemically synthesized siRNAs delivered by transfectants enter cells through endocytosis and are therefore more prone to cause immune stimulation via TLRs 7/8 found on endosomes. Conversely, shRNAs expressed intracellularly by plasmid and viral vectors, follow more closely the endogenous RNAi pathways and thus prevent interferon response (Rao et al. 2009; Schlee et al. 2006).

Unwanted participation in miRNA pathways

The enzymatic machinery involved in RNAi in mammalian cells is naturally used by miRNAs which play an important role in cell physiology. Since miRNA and shRNA share common enzymatic pathway for their processing, the over-expression of shRNA in vivo is known to produce toxicity due to over-saturation of endogenous miRNA pathway involving exportin-5 and RISC component Argonaute-2 (Grimm et al. 2006). However, *in vivo* toxicity can be alleviated by the selection of efficient but safe shRNA expression cassettes, and applying minimal effective vector doses (Grimm et al. 2006).

Despite the high specificity of siRNA activity, some mismatches are often tolerated and can still reduce expression of off-targeted genes (Du et al. 2005), (Dahlgren et al. 2008). Consequently, even carefully screened siRNAs can cause significant changes in expression of unrelated genes (Persengiev et al. 2004; Scacheri et al. 2004). Many of these off-target effects are mediated by the participation of siRNAs in miRNA pathways (Behlke 2008). The miRNA translational suppression pathway is directed by imperfect base pairing between target gene and guide strand of the miRNA and the specificity of this process is defined by 6-7 bases at the 5'-end (seed region) of the latter (Doench & Sharp 2004; Lin & Feng 2005; Jackson et al. 2006a). Consequently, 6-7-base matches between a siRNA and non-targeted gene can mediate an off-target effect by the miRNA translational suppression pathway (Birmingham et al. 2006). Homology screening of the seed region of siRNA candidates with all genes seems to be prudent (Anderson et al. 2008b).

Passenger strand activity

Only functional participation of the guide strand of the double-stranded siRNAs is desired. Nevertheless, some variable amount of the passenger strand can be loaded into RISC and trigger off-target gene-knockdown (Clark et al. 2008). Asymmetric 25-nt/27-nt siRNA can be designed to preferentially load its guide strand decreasing then the potential off-target effects from the passenger strand (Rose et al. 2005). Dual-targeting siRNAs can be also designed targeting different sites within a single mRNA or two separate mRNAs target. These siRNAs may reduce the potential for off-target gene silencing, increase the opportunity to knockdown the desired target gene(s), and potentially provide synergistic effects by both strands (Tiemann et al. 2010).

Viral encoded suppressors of RNAi

Several plants and invertebrates use RNAi as an immune mechanism to counter viral infections (Zamore et al. 2000; Hamilton & Baulcombe 1999). Therefore, viruses infecting these plants and insects have evolved their countermeasures by producing various suppressors of RNAi silencing (SRS) (Voinnet et al. 1999; Li et al. 2002). Some recent studies suggest that RNAi in mammals is also involved in antiviral responses and cellular regulatory miRNAs have a function in restricting virus replication in the cells (Haasnoot & Berkhout 2010). Moreover, several mammalian viruses like hepatitis C virus (HCV), influenza virus, Ebola virus, human immunodeficiency virus (HIV), vaccinia virus, and adenoviruses type 2 and 5 are known to produce SRS factors that inhibit the RNAi mechanism. Many of these factors are multifunctional proteins which are known to inhibit interferon response as well (Haasnoot & Berkhout 2010; Umbach & Cullen 2009). Since the viruses producing SRS can be efficiently inhibited by RNAi, it appears that viral-suppression activity does not pose a serious difficulty for therapeutic RNAi (Haasnoot et al. 2007).

Viral escape

Several applications of a single siRNA can lead to the emergence of viral escape mutants due to mutations in the target sites making viral genomes resistant to RNAi (Boden et al. 2003). Since, unlike eukaryotic DNA polymerase, RNA viruses lack proofreading activity, they tend to have a high error rate during replication which facilitates development of viral mutants (Coffin 1995). After discovery of RNAi, an early study by Jacque and colleagues (Jacque et al. 2002), showed that even a single nucleotide mismatch in the siRNA target region may

be sufficient to reduce the silencing effect of an siRNA on HIV-1. Interestingly, viral escape to RNAi can also occur by a point mutation outside the target sequence of siRNA, if this mutation changes local RNA folding into a structure that reduces the accessibility of the target sequence (Westerhout et al. 2005). Several viruses have been reported to escape suppression by effective siRNAs, which include HIV-1, HCV, HAV, *peste des petits ruminants virus* (PPRV) and Poliovirus (von Eije et al. 2008; Wilson & Richardson 2005; Kusov et al. 2006; Gitlin et al. 2005). Studies of HIV-1 and HBV populations have shown that many resistant mutants may pre-exist before they have been exposed to inhibitors and the siRNAs may also exert selection pressure on these pre-existing resistant mutants (Najera et al. 1995; Wu et al. 2005).

The problem of RNAi escape can be more severe in chronic diseases as these require an extended antiviral therapy but may be less crucial in treatment of acute diseases (Haasnoot et al. 2007). However, design and selection of siRNAs targeting conserved regions of virus and combination of siRNAs targeting multiple genes or regions in viral genome can help avoid problem of resistance to siRNA therapy (Khaliq et al. 2010). In a study using a T cell line expressing three potent shRNAs against HIV-1, it was found that even after an extended culturing for more than 100 days there was no viral replication (von Eije et al. 2009). The emergence of escape mutants of PPRV was prevented after 20 passages in cell culture in presence of three combined siRNAs (Holz et al. 2012). A combination of four shRNAs that target different sequences of HBV genome has entered a phase I clinical trial (Castanotto & Rossi 2009). However, use of multiple siRNA/shRNA doses must be optimized before use as some studies have shown that it is possible to saturate the RNAi pathway with high levels of shRNA expression (Grimm et al. 2006). Another possible alternative to targeting multiple conserved viral genomic regions is to use siRNAs that recognize the mutated target sites through use of siRNAs or shRNAs that target the most likely escape variants (Ter Brake & Berkhout 2005). Since viruses tend to mutate, an alternative means could be to down-regulate cellular factors which are required by the virus to either enter a cell or to replicate.

In vivo delivery of interfering RNAs

Compared to the large number of studies using siRNAs in cell culture, there have been relatively few studies *in vivo*. The progress is slower due to difficulties in delivery, especially by systemic route of administration (Dykxhoorn et al. 2006). The issue of drug delivery is not unique to RNAi based therapeutics, but here it is a major obstacle as the drug cargo (siRNA) is nuclease sensitive, has a net negative charge, and is hydrophilic, thus making difficult to keep it stable in serum and capable of crossing the anionic and hydrophobic plasma membranes (de Fougerolles et al. 2007; Robbins et al. 2008; Czauderna et al. 2003). In order to use RNAi as an antiviral approach, the siRNAs can either be chemically synthesized (Bitko & Barik 2001) or shRNA expression cassettes with Poll III promoters can be inserted into plasmid (McCaffrey et al. 2003) or viral vectors (Banerjea et al. 2003; Uprichard et al. 2005). The viral vectors can efficiently use their own machinery to reach the target cells, transfer their genome into the cells and express shRNA molecules.

In theory, naked siRNAs and plasmid vectors cannot readily cross the cell membrane and therefore need delivery vehicles to help them to enter the cell cytoplasm. However, systemic delivery of naked siRNA into the bloodstream is being tested for siRNA delivery to the kidney, liver, and some solid tumors. Similarly, the naked siRNA can be injected directly into the target tissue, tumor or lesion. Tissues such as eye, lung, skin are suitable for topical delivery of sRNA (Whitehead et al. 2009). Bitko and colleagues successfully delivered naked siRNAs intranasally in a mouse model against respiratory syncytial virus (Bitko et al. 2005). Interestingly, they compared delivery of siRNA with and without a transfection reagent, and noted only a marginal enhancement (20%) in the knockdown of the respiratory syncytial virus (RSV) target gene when lipid agent was used. However, the mechanism by which these cells take up these siRNA molecules remains unknown (Wu & McMillan 2009). The systemic delivery route, however, is more difficult to approach. The unmodified siRNA has a half-life of less than an hour in human plasma and the siRNA molecules circulating in blood, are rapidly excreted by kidneys due to their small size (Layzer et al. 2004). Moreover, recognition of naked siRNAs by the phagocytic system is associated with immunological and cytotoxic stimulation, that could occur right after injection through activation of circulating mononuclear phagocytosis or inside the cytoplasm and endosome compartments of the target cell (Daka & Peer 2012). The vectors are therefore needed not only to improve the bio-availability of siRNAs but also to provide protection from nucleases and help in penetration of anionic plasma membranes (Li et al. 2006; Feng et al. 2008).

Physical methods of siRNA delivery

The physical methods for *in vivo* delivery of siRNA involve mainly electroporation and hydrodynamic injection. The hydrodynamic injection involves a quick injection of siRNA or plasmid DNA (pDNA) in a large volume of physiological buffer, around one-tenth the mass of the animal, within a few seconds in the tail vein of the animal and this results in a delivery mainly to the liver (Budker et al. 2006; Tada et al. 2006). McCaffrey and colleagues in 2002 in their studies on *in vivo* siRNA delivery by hydrodynamic method, demonstrated that co-injection of luciferase expressing plasmid with siRNA targeting luciferase produced efficient silencing of luciferase expression in the mouse liver (McCaffrey et al.

2002). Various studies using this method could deliver naked siRNA to hepatocytes, demonstrating functional knockdown of specific genes in the livers of mice (McCaffrey et al. 2002; Zender & Kubicka 2007; Lewis & Wolff 2007). Most studies are using hydrodynamic method for delivery of siRNA to liver as it can be much more easily transfected by this method as compared to other organs. This procedure is not clinically viable because of potential damage to liver, as well as due to danger of volume overload side effects like right sided heart failure (Weinstein & Peer 2010). Moreover, it would be impossible to scale up this method for use in humans or large animals.

The electroporation involves administration of an electric current for short duration, which temporarily increases the permeability of cell membranes, and thus allowing for passing of nucleic acids through membranes. The electric field polarizes the membrane molecules and temporarily destabilizes the membrane integrity, which results in greater permeability for the exogenous materials (Heller & Heller 2006). This technique has been successfully used to transfer siRNAs locally (Golzio et al. 2007), but it cannot be used for systemic administration. Similarly, ultrasound is also used for nucleic acid delivery (Taniyama et al. 2002). These physical methods of delivery of siRNAs either cannot be used systemically or are too dangerous for clinical use.

Chemical vectors for delivery

The development of chemical vectors for siRNA delivery has been influenced by the studies on intracellular DNA delivery (Lu et al. 2009). However, there are important differences between delivery of siRNA and DNA: i) size and charge of siRNA are lower than those of DNA; ii) cytosol is the place of action for siRNA while DNA has to enter nucleus to be effective (Schroeder et al. 2010).

The chemical methods of siRNA delivery involve formulation of negatively charged siRNA molecules with various polycations, like cationic lipids, peptides, polymers, or inorganic nano-structured materials, either through electrostatic or ionic interactions, with the aim of enhancing pharmacokinetic behavior, nuclease resistance, cellular uptake, target specificity and safety (Misra et al. 2010). The general principle is based on complex formation between anionic siRNA molecules and cationic polymers resulting in complexes with net positive charge for facilitating interaction with anionic cell membranes. Moreover, the physico-chemical properties of the complexes like size, morphology, surface charge and stability, are the major determinants of transfection efficiency which in turn are determined by structure of polycation, the polycation/nucleic acid stochiometry (+/- charge ratio), pH and order of mixing of nucleic acids and the vectors (Issa 2006). Though most delivery vectors are cationic, the net surface charge of the vector-siRNA complex, has to be near neutral to avoid toxicity, excessive uptake by reticulo-endothelial system (RES) and aggregation by serum proteins (Misra et al. 2010; Rao 2010). In order to deliver siRNA cargo into target cell cytoplasm, the formulation has to surpass various physiological barriers (Fig. 3) by shielding siRNA from serum nucleases, avoiding aggregation by anionic serum proteins, escaping from RES, penetrating endothelium, getting internalized through endocytosis, and finally being able to deliver siRNA from the endosomes to the cytoplasm before degradation by lysosomes (Misra et al. 2010; Rao 2010). The in vivo use of siRNA by chemical delivery systems is limited by siRNA packaging efficiency, colloidal stability of the complex, internalization, and endosomal escape (Reischl & Zimmer 2009).

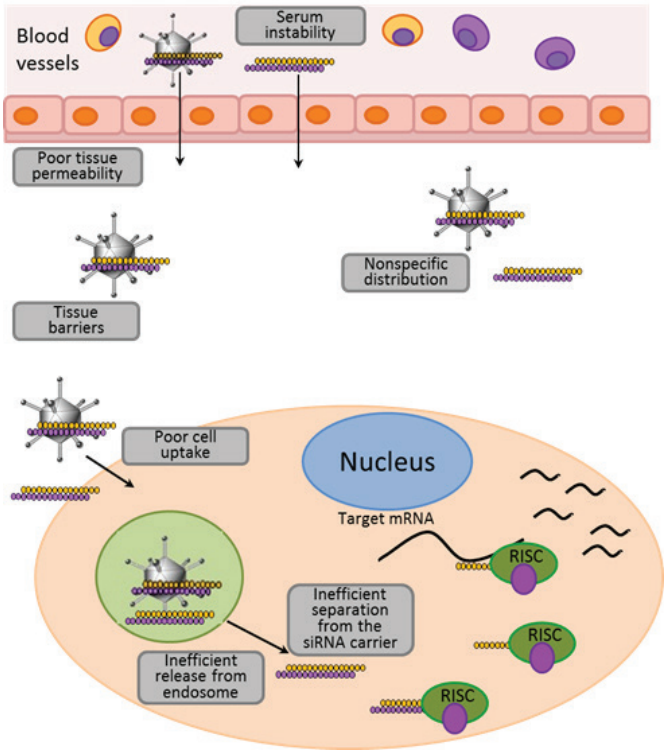

Figure 3. Main physiological barriers to in vivo delivery of siRNA. Once in the bloodstream, siRNA must avoid aggregation by serum proteins, degradation by serum nucleases, filtration and phagocytosis; be transported across the vessel wall; diffuse through the extracellular matrix surpassing tissue barriers; be taken up into the cell; escape the endosome; and to be dissociated from its carrier and released to the cellular machinery.

The chemical vectors used for siRNA delivery can be broadly divided into three major groups: lipids, cationic polymers, and peptides.

Lipids as nucleic acid delivery vectors

Phospholipids which are a major component of cell membranes, tend to form spontaneous spherical structures called liposomes upon contact with water (Bangham et al. 1965). Felgner and colleagues (Felgner et al. 1987) were first to demonstrate the effective use of

liposomes for *in vitro* transfection of DNA. These liposomes were based on a cationic lipid called DOTMA (N-[1-(2,3,-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride) (Felgner et al. 1987). Since then, many natural as well as synthetic cationic lipids have been developed and used for delivery of nucleic acids including DNA, antisense oligonucleotides and siRNAs. The preparation of these new cationic lipids involved either modification of the nature of cationic lipids (Felgner et al. 1994), fatty acid side chains (Rosenzweig et al. 2000), or formulations with some additional lipids (Budker et al. 1996). Despite initial difficulties found in in vivo transfection, the death of a human patient participating in gene therapy trials based on an adenoviral vector in 1999 (Raper et al. 2003) and the resulting temporary ban on use of viral vectors for gene therapy by US Food and Drug Administration (Couzin & Kaiser 2005), lead to a renewed interest in chemical vectors.

Typically, the cationic lipid molecules are amphiphilic or amphipathic in nature and are made up of three parts: a cationic head-group, a hydrophobic anchor, and a linker (Fig. 4a and 4c). The cationic head-group is required for binding to and complexation with nucleic phosphate groups, whereas hydrophobic part probably assists in assembling the lipids into polycationic scaffold and facilitating absorptive endocytosis or fusion with plasma membranes (Eliyahu et al. 2005). The cationic lipids are classified on the bases of number of positive charges, nature of linker bond, and nature of hydrophobic anchor (Eliyahu et al. 2005). Although cationic lipids are the most common lipids used for liposome based transfections, often neutral and/or anionic lipids are mixed with these in variable ratios to neutralize excessive cationic charge and to improve endosomal escape (Zhang et al. 2004). The anionic lipids, when added to nucleic acid-cationic liposome complexes, not only reduce cellular toxicity but also reduce nonspecific interaction with anionic serum proteins like albumin as well as extracellular matrix (Lee & Huang 1996; Mastrobattista et al. 2001). Mastrobattista and colleagues in 2001 were able to improve transfection by preparing positively charged polyplexes coated with an anionic lipid (Mastrobattista et al. 2001).

The neutral lipids play a role of a helper when formulated with cationic lipids for preparation of liposomes. There are three neutral lipids which are often incorporated in the formulations: dioleyl phosphatidylethanolamine (DOPE), cholesterol and dioleoyl phosphatidyl choline (DOPC). The DOPE is known to destabilize lipid bilayers and is believed to be involved in endosomal disruption and thus enabling nucleic acids to escape endosomes before being destroyed by lysosomes (Farhood et al. 1995). Cholesterol is also used as a helper lipid. Although it forms more stable but less efficient complexes with nucleic acids than those containing DOPE *in vitro*, cholesterol containing lipoplexes show higher biological activity due to greater cell uptake and stability in serum, compared to lipoplexes with DOPE when these complexes are utilized *in vivo* (Eliyahu et al. 2005). The liposome mediated transfection has been shown to take place mostly by endocytosis (Lu et al. 2009). However, probably a minor pathway mediated by fusion between siRNA containing lipoplexes and the plasma membrane is responsible for around 5% of siRNA delivery in to cell cytoplasm (Lu et al. 2009).

Cationic liposomes

Liposomes are vesicles composed of a phospholipid bilayers with an aqueous core (Reischl & Zimmer 2009). They are classified according to their size and their number of bilayers: Small Unilamellar Vesicles (SUV), Large Unilamellar Vesicles (LUV), Multilamellar Vesicles (MLV), and Giant Unilamellar Vesicles (GUV) (Fig. 4b) (Lorin et al. 2004). The complex formation between anionic phosphate group from nucleic acids and cationic amine head group of cationic liposomes results in formation of lipoplexes, which are quite different from liposomes in structure as the final charge as well particle size changes and these changes are determined largely by lipid / nucleic acid (+ / -) ratios (Pires et al. 1999).

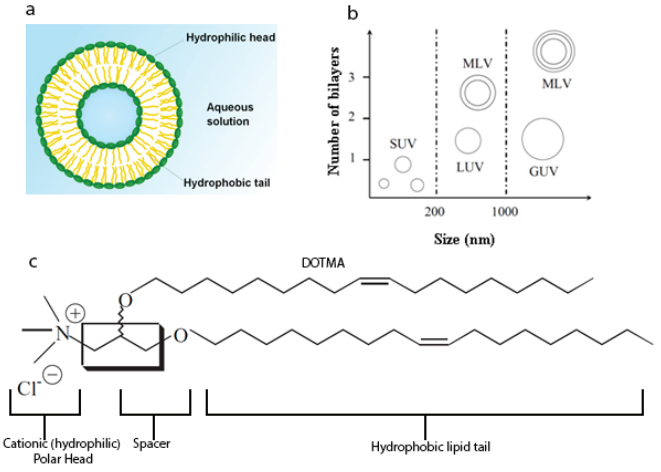

Figure 4. Structure of a cationic liposome. (a) formed by phospholipids in an aqueous medium (Boutet 2007); (b) Classification of liposomes according to size and number of bilayers (Lorin et al. 2004); (c) Representative structure of cationic lipid DOTMA, modified (Karmali & Chaudhuri 2007).

In vivo delivery of siRNA with liposomes

DOTAP (N-[1-(2,3-dioleoyloxy)]-N-N-N trimethyl ammonium propane) and Oligofectamine were some of the first lipid formulations used for *in vivo* delivery of siRNA and effective gene silencing of TNF-alpha and beta-catenin in mice (Verma et al. 2003; Sorensen et al. 2003). Since then siRNAs have been successfully delivered locally but there are not many reports of successful systemic liposome based delivery of siRNAs against systemic viral infections. However, there are some studies showing promising results in terms of successful delivery of siRNAs and effective viral suppression *in vivo*

by formulations based up on neutral lipids. For example, Morrissey and colleagues (Morrissey et al. 2005) using three daily intravenous injections in mice of siRNA (3 mg/kg/day) complexed with the neutral liposomes called "stable nucleic acid-lipid particles" (SNALPs) stabilized with PEG (polyethylene glycol) , could reduce serum DNA of hepatitis B virus by more than 1.0 $\log_{10}$ and the effect lasted for up to 7 days after dosing. In another experiment using intraperitoneal delivery of siRNAs complexed with SNALPs for seven days against Ebola virus has been shown to protect guinea pigs against viremia and death shortly after the virus challenge (Geisbert et al. 2006). Using a lactosylated liposome based upon neutral lipid the phosphatidylcholine, Watanabe and colleagues in 2007 could effectively deliver siRNA targeting hepatitis C virus (HCV) in a transgenic murine model, resulting in suppression of intrahepatic HCV expression without interferon response (Watanabe et al. 2007). SNALPs developed by Protiva Biotherapeutics and Alnaylam, have also been successfully used to deliver siRNA in non-human primates, whereby using a SNALP-formulated siRNA dose of 2.5 mg/kg, could markedly suppress the Apolipoprotein B (APOB) (Zimmermann et al. 2006). Another siRNA-lipoplex referred to as AtuFECT01 (AtuPLEX) was used intravenously in mice (Santel et al. 2006). This designed cationic lipid contains neutral fusogenic and PEG-modified lipid components, for improved pharmacokinetic properties, cellular uptake, and efficient siRNA release from the endosomes after endocytosis. This complex is characterized by a highly charged head group, which allows for more efficient siRNA-binding as compared to other commercially available cationic lipids such as DOTAP or DOTMA. The biodistribution analysis on systemic administered siRNA-AtuPLEX indicated a predominantly specific targeting of endothelial cells in the vasculature of several organs after single dosing. The authors advocate that the potential lipid-associated toxicity of the AtuPLEX is also reduced (Santel et al. 2006). Novobrantseva and colleagues demonstrate the ability of two types of liposomal formulations KC2 (an ionizable lipid) and C12-200 (a cationic lipid) to mediate silencing in nonhuman-primate and rodent myeloid cells (Novobrantseva et al. 2012). The high *in vivo* potency of these formulations and their ability to deliver siRNA to myeloid cells makes them potentially applicable for clinical use to treating multiple human diseases, such as modulating chronic inflammation in autoimmune disease, protecting against myeloid-tropic viral infections, reprogramming tumor-associated macrophages, restoring functionally insufficient cells, or killing malignantly transformed immune cells. Delivering siRNA to myeloid cells could provide novel approaches (Novobrantseva et al. 2012). Lipidoids are non-glycerol-based cationic lipid particles synthesized by conjugation of amines to acrylate or acrylamide (Akinc et al. 2008). Lipidoid-formulated siRNA system upon systemic administration showed potent and specific dose-dependent gene silencing using a pool of siRNA sequences targeting different genes a pool of five siRNA sequences (Akinc et al. 2008; Love et al. 2010).

Despite encouraging results in animal models, there are no known reports of non-viral delivery of siRNAs in farm animals. Systemic application of siRNA molecules by lipid based carriers is still challenging and issues of toxicity and need for targeted delivery still need to be addressed.

Cell penetrating peptides (CPPs)

CPPs or protein transduction domains (PTDs) are short peptides having fewer than 30 residue peptides, derived from natural or unnatural protein or chimeric sequences (Heitz et al. 2009). Most of the CPPs possess a high density basic amino acids (arginines and/or lysines), which are proposed to interact with the anionic surface of the cell membrane and enhance internalization of the peptides (Endoh & Ohtsuki 2009). Among chemical delivery vectors CPPs are unique in that the concentrations of CPPs that are used for molecular delivery, produce very low or undetectable cytotoxicity (Endoh & Ohtsuki 2009).

CPPs were first discovered when Frank and Pabo in 1988 observed that HIV-1 Trans-Activator of Transcription (Tat) protein, which transactivates transcription of the HIV-1 genome, crossed the cell membrane by itself (Frankel & Pabo 1988). Later, a minimal peptide fragment of Tat (49-59 amino acids) involved in cellular uptake was identified by a French group (Vives et al. 1997). The Tat peptide has been shown to successfully deliver siRNAs *in vitro* (Meng et al. 2009). Another major discovery in CPP domain was a 16-mer non-viral peptide derived from the Drosophila Antennapedia homeodomain protein (Derossi et al. 1994). This peptide, named Penetratin^{TM}, is capable of transfecting nucleic acids, antisense oligonucleotides (Allinquant et al. 1995), and peptide nucleic acids (PNAs) (Morris et al. 1997). An MPG peptide was used to perform the first non-covalent delivery of nucleic acids in 1997 (Morris et al. 1997). The MPG peptide is an artificially constructed 27 amino acid amphipathic CPP containing a lysine rich basic region derived from the nuclear localization signal (NLS) of the SV40 large T antigen and a hydrophobic region derived from the HIV-gp41 coat protein (Morris et al. 1997). Both the MPG peptide and a modified MPGΔ^{NLS} (having a single mutation of the second lysine residue in the NLS motif to serine) can transfect siRNA *in vitro* however latter is more efficient as it delivers siRNA into cytoplasm instead of nucleus (Simeoni et al. 2003). MPG peptides have been also used to deliver siRNA into mice by systemic route (Crombez et al. 2009). Another milestone was achieved when a chimeric CPP "Transportan", derived from

the N-terminal fragment of the neuropeptide galanin linked to a wasp venom peptide called mastoparan was successfully used *in vivo* for delivery of peptide nucleic acids (PNAs), in mice (Pooga et al. 1998). The first CPP based transfection of siRNA was performed with a MPG peptide (Simeoni et al. 2003) and since then numerous natural and as well as engineered CPPs have been tested for siRNA delivery (Meade & Dowdy 2007). Polyarginine-based peptides have also been successfully used for siRNA delivery. Majority of the CPPs possess basic amino acids (arginines and/or lysines), through which these interact with cell membranes and help in the internalization of CPPs (Endoh & Ohtsuki 2009). The experiments on Tat and penetratin revealed that the role of positive charges is crucial for translocation (Deshayes et al. 2005). After studying various cationic polypeptides, CPPs with polyarginines were found to be more efficient than other cationic polypeptides like polyhistidines and polylysines and among polyarginine peptides, Arg7 and Arg9 have been the most widely used for *in vitro* and *in vivo* delivery (Deshayes et al. 2005).

Although cellular uptake mechanism for CPPs has been reported to be associated with endosomal pathway (Richard et al. 2003), there is no unified mechanism established for CPP uptake mechanism up to now, and probably numerous factors are involved (Heitz et al. 2009). However, there is a consensus that the initial contact between the CPPs and the cell membrane takes place by electrostatic interactions with negatively charged proteoglycans (Heitz et al. 2009). Moreover, as CPPs are diverse in chemical and physical structures, it is suggested that different properties like CPP molecule length, charge delocalization as well as size and charge of the cargo can have impact upon peptide uptake mechanism (Mueller et al. 2008). Cellular uptake of CPPs, therefore, does not occur by any single mechanism, thus energy independent direct diffusion, macropinocytosis, clathrin-mediated endocytosis, and caveloe/lipid raft mediated endocytosis may all occur (Duchardt et al. 2007).

CPPs can be classified in two ways. These are classified either on the basis of mode of bonding with cargo into, those requiring covalent linkage with siRNAs and others which, being amphipathic, can form stable non-covalent bonds. Secondly, these can also be subdivided structurally into those which polycationic having clusters of polyarginine in the primary sequence or those which are amphipathic (Heitz et al. 2009).

Covalent attachment of CPP to siRNA

Tat conjugated to the modified antisense strand of siRNA was successfully used to inhibit EGFP gene *in vitro* (Chiu et al. 2004). However, most of Tat-siRNA was found localized in endosomes and there was a concern that the attachment of Tat to siRNA may disrupt functionality of siRNA molecules (Juliano et al. 2008). To address this concern, CPPs have also been linked to siRNA through disulfide bond which is cleaved when conjugates reach reducing environment of cytosol, resulting in successful RNAi (Davidson et al. 2004; Muratovska & Eccles 2004). The covalent strategy usually requires complex chemistry for conjugation and there is also risk of alteration of biological activity of siRNA therefore non-covalent strategy of delivery appears more suitable (Heitz et al. 2009).

Non-covalent CPP-siRNA complex formation

Assembling siRNA/CPP complexes through non-covalent interactions is advantageous as it simplifies conjugation protocols but also eliminates the need for optimization of individual syntheses schemes. Additionally, there is also lower likelihood that CPP will interfere with the bioactivity of the cargo (Heitz et al. 2009). The non-covalent delivery of siRNA is based up on electrostatic interactions between siRNA and amphipathic peptides (Endoh & Ohtsuki 2009). The amphipathic peptides possess hydrophobic (polar) and hydrophilic (non-polar) domains. The amphipathic property of CPPs may arise from either primary or secondary structure (Deshayes et al. 2005). Primary amphipathic peptides consists of the sequential assembly of a domain of hydrophobic residues with a domain of hydrophilic residues while secondary amphipathic peptides are produced by conformational state that allows positioning of hydrophobic and hydrophilic residues on opposite sides of the molecule (Deshayes et al. 2005).

Since siRNAs are negatively charged, they can bind with positively charged CPPs through nonspecific electrostatic interactions and provide permeability by covering the siRNA surface with positive charges from the CPP (Endoh & Ohtsuki 2009). In 2003, a non-covalent strategy based on MPG was found to efficiently deliver siRNA into cell lines (Simeoni et al. 2003). Similarly, this non-covalent mode of delivery has also been used for other CPPs like, Tat, polyarginine, and transportan-derived peptides (Heitz et al. 2009). A novel 20-amino acid amphipathic peptide, CADY, has been recently described which combines both cationic arginine and aromatic tryptophan residues into its design (Crombez et al. 2009). It forms stable complexes with siRNA through electrostatic interactions and interestingly uses a non-endocytic mechanism to pass through plasma membrane, thus avoiding endosomal entrapment (Crombez et al. 2009). This CPP can transfect a variety of cell lines, including difficult to transfect primary cell lines (Crombez et al. 2009). Since CADY-siRNA is stable in 50% serum for 24 hours and leads to significant knockdown with sub-nanomolar concentration, it may be effective in vivo as well (Crombez et al. 2009).

In vivo delivery with CPPs

CPPs have been delivered in vivo with some successes and failures. MPG peptide was used for

systemic *in vivo* delivery of siRNA targeting essential cell cycle protein cyclin B1, resulting in efficient blockage of tumor growth (Crombez et al. 2007). In another experiment, siRNA targeting HIV was successfully delivered through a CD7-specific single-chain antibody conjugated to the oligoarginine peptide (scFvCD7-9R) and could effectively suppress HIV infection in a mouse model (Kumar et al. 2008). The CD7 receptor is rapidly internalized after antibody binding, therefore it has been exploited for the targeted delivery of several monoclonal antibody (mAb) (Kumar et al. 2008). Kumar and colleagues (Kumar et al. 2008) using a nonamer arginine at the carboxy terminus of a peptide derived from rabies virus glycoprotein (RVG) were able to transfer siRNA into neuronal cells *in vivo*, resulting in efficient gene silencing after intravenous injection into mice. Furthermore, systemic delivery of RVGR9 conjugated antiviral siRNA complex could protect mice from encephalitis induced by Japanese encephalitis virus (JEV) infection, which is the first report on a nontoxic method of siRNA delivery across the blood brain barrier (Anantpadma et al. 2010). CPP based siRNA delivery has also entered at level of preclinical and clinical trials, with Traversa Inc. testing HIV Tat-based and Panomics Inc., testing secondary amphipathic peptide-based non-covalent delivery of siRNA (Heitz et al. 2009).

Nanoparticles (NPs)

Numerous nanoparticles (NPs) were investigated for siRNA delivery including: dextran–spermine nanoparticles (Jiang et al. 2012), calcium phosphate nanoparticles (Guo et al. 2010), thioketal nanoparticles (Conde et al. 2012), crosslinked iron oxide nanoparticles (Shahzad et al. 2011), liposomal integrin target-stabilized nanoparticles (Wang et al. 2013), galactose-conjugated liposome nanoparticles (Jiang et al. 2012), etc. Among these numerous NPs formulations designed, those based on gold nanoparticles (AuNPs) have been extensively investigated to cancer genes silencing without undesirable immune response or off-target effects (Guo et al. 2010; Conde et al. 2012). Shahzad and colleagues showed that reconstituted high-density lipoprotein nanoparticles (rHDL- nanoparticles) can efficiently deliver siRNA in vivo to silence genes that are critical for cancer growth and progression without toxicity (Shahzad et al. 2011). Inorganic nano-particles as single-walled carbon nanotubes (SWNTs) associated with other chemical molecules or peptides have been used to obtain promising tumor targeting siRNA delivery system (Wang et al. 2013). An increase in phagocytic activity is observed upon using highly charged NPs, prominently cationic, as well as hydrophobic particles. In order to avoid opsonization, strategies that mask the NP charge and hydrophobicity may be applied, such as PEGylation (Daka & Peer 2012).

Exosomes are naturally occurring, membranous nanovesicles of 40-100 nm in diameter. All mammalian cells and cell lines are thought to be competent for exosome production, although the yield varies from cell to cell (Lakhal & Wood 2011). Furthermore, exosomes have consistently been found in most biological fluids including blood, urine, cerebrospinal fluid, breast milk, amniotic fluid, malignant pleural effusions and ascites (Simpson et al. 2008). Exosome function is determined by their cell-type-specific protein and miRNA cargoes as such, modulation and regulation of vascular homeostasis, antigen presentation to T cells, cytokine transport, but also progression of disease processes by transfer of oncogenes, infectious cargo (such as HIV particles) or pathogenic proteins between neurons (Schorey & Bhatnagar 2008). Exosomes are then natural carriers of RNA constituting potential delivery vehicles for exogenous nucleic acid cargoes. Exosomes purified from dendritic cells and targeting neuronal components were used as vehicles of siRNA delivery and were able to deliver them into the brain (Alvarez-Erviti et al. 2011). These results show that exosomes promise to revolutionise the field of drug delivery by enabling drug delivery across otherwise impermeable biological barriers (Lakhal & Wood 2011).

Aptamers

Aptamers are nucleic acid-based molecules that bind to a specific target molecule such as small molecules, proteins, nucleic acids, and even cells and tissues. RNA-aptamers/siRNA chimeras allow better tissue internalization and amenability for modifications, make it an appropriate strategy for induction of targeted RNAi delivery *in vivo* (Daka & Peer 2012). This systems has been shown to specifically inhibit tumor growth in a mouse prostate cancer model (McNamara et al. 2006). An aptamer/siRNA chimera anti-gp120, in which both aptamer and siRNA portions directed against HIV genes, was used to specifically target cells expressing HIV receptor gp120 leading to repression of HIV replication in tissue culture tests (Zhou et al. 2008). These interesting results make this system an attractive approach for systemic RNAi application.

Other chemical vectors

Aptamers are RNA or DNA oligonucleotides that fold by intramolecular interaction into unique three-dimensional conformations capable of binding to target antigens with high affinity and specificity (Muratovska & Eccles 2004). The aptamers have been used as siRNA vectors in a mouse tumor xenograft model, however their systemic use requires addition of nuclease stabilizing agents and endosmolytic functionalities (Golzio et al. 2007). Dendrimer molecules are repeated branched species characterized by structural perfection (Golzio et al. 2007). Dendrimers have been used successfully for in vitro delivery of siRNAs (Simeoni et al. 2003). Polyethyleneimines (PEI) are polycation-containing

block copolymers (Muratovska & Eccles 2004), which have also been used for delivery of siRNA molecules in vivo, although PEI use *in vivo* has some toxicity issues (Crombez et al. 2009). Chitosan is a biodegradable, biocompatible and non-toxic cationic polymer obtained from deacetylation of chitin, which has been proposed as biocompatible alternative to cationic polymers, suitable for non-viral nucleic acid delivery (Crombez et al. 2007). Chitosans have been used for siRNA delivery in vitro as well as in vivo (Kumar et al. 2008). Antibody-protamine fusion carriers have also been shown to be efficient in delivery of siRNA to HIV-infected or envelope-transfected leukocytes (Anantpadma et al. 2010).

Viral vectors

Like chemical vectors, the viral vector based delivery of shRNA also profited from technology that already existed for gene therapy. Soon after delivery of siRNAs *in vitro* (Bitko & Barik 2001), it was demonstrated that siRNAs can also be expressed from plasmid DNA as "short hairpin RNAs" (shRNAs) (Brummelkamp et al. 2002; Paddison et al. 2002). This finding paved the way for viral vector based RNAi therapeutics. U6 (Paddison et al. 2002) and H1 (Brummelkamp et al. 2002) were the first polymerase III promoters described that could express functional siRNAs (González-Rojas et al. 2010). However U6 promoters have been reported to be more efficient *in vivo* (Makinen et al. 2006). Typically, shRNA transcription starts in a position outside the promoter sequence, continues along 19-29 nucleotide long top strand, the 4-19 nucleotide long hairpin loop and finally the bottom strand and terminates after the second or third residue of track of 4-6 thymidines (González-Rojas et al. 2010). The termination is so designed that it results in a 3' two- nucleotide overhang after RNAse III cleavage as is the case with natural pre-miRNAs (González-Rojas et al. 2010). The shRNA sequences are selected and translated to DNA, and these are normally synthesized in the form of two complimentary oligonucleotides that are annealed and cloned downstream of the selected promoter and regulatory sequences (González-Rojas et al. 2010). The constructed shuttle plasmids expressing shRNA are next tested for inhibition of target gene *in vitro*. Moreover, the cassettes may be inserted into expression clones for production of required recombinant virus vectors. In addition to standard shRNA resulting in production of a 21 nt long siRNAs, numerous other variations of shRNA have also been used. Longer shRNAs producing 27- 29 nt long siRNAs (Kim 2005; Siolas et al. 2005), and multiple shRNAs simultaneously expressing different shRNAs against different target regions have been used (Liu et al. 2008; Aagaard et al. 2008). The viral vectors have certain advantages over chemical vectors for delivery of siRNAs like (González-Rojas et al. 2010);

1. Tissue specific delivery of shRNAs.
2. Possibility of inducible or tissue specific promoters.
3. Both transient as well as stable expression of shRNA is possible, according to the viral vector chosen.
4. Better transduction efficiency compared to non-viral transfections even in cells like lymphocytes that are traditionally hard to transfect.
5. Cost effectiveness.
6. Most of the available viral vectors have already been tested clinically in phase I safety trials.

The most widely used viral vectors for shRNA delivery include adenovirus, adeno associated virus (AAV), lentivirus, retrovirus, and baculovirus. In contrast to the gene therapy, shRNA expressing cassettes tend to be of small length and their expression is possible even by the smallest vectors (González-Rojas et al. 2010). However, viral vectors are selected on the basis of tissues required to be transduced and also whether stable or transient transduction is required. For chronic infections like HIV stable transduction is desirable to avoid repeated administration of vectors while for acute infections, transient transductions with shRNA expressing vectors would suffice. The RNA viruses such as retrovirus and lentivirus produce stable transduction as they integrate into host genomes while non-integrating DNA viruses like adenovirus, baculovirus and AAV maintain their genome episomally in the host cells.

For gene therapy as well as for RNAi, replication defective adenoviruses can be used since they are non-pathogenic and can transiently transduce a variety of both dividing and non-dividing cells without integration to the host genome (González-Rojas et al. 2010).

Adenoviral vectors

In the past it has been considered that adenovirus serotypes belonging to species A, C, D, E and F use the coxsackie and adenovirus receptor (CAR) as a docking site providing a high affinity virus-to-host association (Roelvink et al. 1998). CAR was previously thought to be a primary Ad5 receptor, but now there are studies according to which adenovirus type 5 also uses many other receptors for docking like the heparan sulfate proteoglycan (HSPG), vascular cell adhesion molecule 1 (VCAM-1), major histocompatibility complex class I (MHC 1), scavenger receptors (SR), while indirect binding it also uses dipalmitoyl phosphatidylcholine receptors (DPPCRs), coagulation factor X (FXs), and Lactoferrin receptors (LfRs) (Arnberg 2009). Although receptor binding is thought to play a major role in adenoviral tropism, this alone cannot explain all aspects of in vivo host-virus interactions like enhanced transductions of liver cells or adenoviral uptake by Kupffer cells (Sharma et al. 2009). Excessive adenoviral binding to hepatocytes may be partly explained by binding of central depression of adenoviral hexon with Gla domain of coagulation factor X (FX) which has been found to produce efficient transduction of hepatocytes (Kalyuzhniy et al. 2008).

That may explain excessive liver tropism of adenovirus where FX could be the main determinant of in vivo liver transduction (Kalyuzhniy et al. 2008).

The internalization of adenovirus occurs by a secondary interaction between RGD motifs on penton base protein and integrins αvβ3 and αvβ5 (Wickham et al. 1993) through clathrin-coated pits mediated endocytosis (Wang et al. 1998). The virus then escapes by lysing endosomal membrane and with the help of microtubule mediated translocation, enter the nuclear pore complex where viral DNA is released for expression (Barnett et al. 2002). The transcription of wild type virus initiates with expression of early E1 genes, which activate synthesis of viral genes and replication. E1 and the non-essential E3 genes were deleted in first generation adenoviral vectors to render them replication deficient (González-Rojas et al. 2010). But these first-generation adenoviruses express viral genes to low levels, including virus associated (VA) RNAs which have been described to saturate the cellular silencing machinery, leading to toxicity (Andersson et al. 2005). Although functional inhibitory exogenous shRNAs have been expressed for first generation adenoviral vectors without saturation of silencing machinery (Narvaiza et al. 2006). The second generation vectors, which have deletions in the E1-E4 locus are comparatively less immunogenic and show prolonged expression of recombinant genes (Gao et al. 1996). Whereas third generation adenoviruses are produced by deleting all the viral genes, as a result of which expression of viral genes is avoided resulting in lesser immunogenicity (Schiedner et al. 1998).

The recombinant replication deficient adenoviruses have been successfully used *in vitro* as shRNA delivery vectors against numerous viruses like hepatitis B virus (Uprichard et al. 2005), measles virus (Otaki et al. 2007), hepatitis C virus (Sakamoto et al. 2008), dengue virus (Korrapati et al. 2012) and the animal *peste des petits ruminants virus* (PPRV) (Nizamani et al. 2011). The adenoviral vectors expressing shRNA have been successfully used in vivo against several viral infections. After pre-treatment of guinea pigs and swine with adenoviral vectors expressing shRNA against foot and mouth disease virus (FMDV), and challenging 24 hours later with the target virus, Chen and colleagues could protect the animals from major clinical manifestation of the disease (Chen et al. 2006). Similarly adenoviral vectors have also been used to protect mice against porcine circovirus type 2 (Feng et al. 2008).

Baculoviral vectors

Among the numerous baculoviruses, *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) is the most well studied and extensively used virus for foreign gene expression (Hu 2005). AcMNPV possesses a circular double stranded DNA genome which is condensed into a nucleocapsid core by a protamine-like protein. Naturally AcMNPV are occluded in a polyhedron which after being ingested by insects is dissolved in alkaline midgut, releasing infectious virions (Hu 2005). The AcMNPVs only replicate in insect cells and naturally infect insects belonging to the order *Lepidoptera*. Baculoviruses primarily enter insect cells through clathrin-mediated, low-pH dependent endocytic pathway while they may enter mammalian cells through multiple pathways including caveola-dependent mechanism as well (Long et al. 2006). However, AcMNPV can infect insect cells and transduce mammalian cells even in the absence of endocytosis, by direct fusion at cell surface under low pH conditions (Dong et al. 2010).

The baculoviruses can enter the mammalian cells but they are unable to express their genes because the baculovirus promoters are inactive in these cells (Kost & Condreay 2002). One of the interesting consequences of this is the absence of pre-existing antibodies against baculovirus in mammals (Kost & Condreay 2002; Volkman & Goldsmith 1983). For gene transfer and expression in mammalian cells, so-called BacMam viruses have been generated by incorporation of mammalian cell-active expression cassettes (Kost et al. 2010). BacMam baculoviruses are capable of transducing wide variety of cells including non-dividing cells (van Loo et al. 2001) and primary cells (Sarkis et al. 2000). They have been used to reduce viral infections in vitro of numerous viruses like porcine arterivirus (Chen et al. 2006), PPRV (Nizamani et al. 2011), hepatitis C virus (Suzuki et al. 2008), hepatitis B virus (Starkey et al. 2009), and influenza viruses A and B (Suzuki et al. 2009).

The baculovirus can prove to be good shRNA delivery vectors in vivo. Although delivery of siRNA to the appropriate cells or tissues is a major challenge, problem of inactivation by complement is resolved (Hofmann & Strauss 1998). Chemical or genetic modification can also overcome problem of serum inactivation (Yang et al. 2009; Kaname et al. 2010). Furthermore, it is reported that baculoviruses do not have deleterious effects on mammalian cells even when used at very high multiplicity of infections (MOIs) (Andersson et al. 2007). Moreover, not only can the baculoviruses be easy and cheap to produce, they can be grown to high titers in cell cultures as well. Although baculovirus vectors have not been used thus far for shRNA delivery in vivo, but these have been successfully used for gene transfer into mouse brain and rabbit retina (Lehtolainen et al. 2002; Kinnunen et al. 2009).

Other viral vectors

Many other viral vectors have been used for RNAi mediated silencing of viral infections like adeno-associated virus (AAV) (Moore et al. 2005), herpes simplex virus (Lambeth et al. 2009), retroviruses (Brummelkamp et al. 2002), and lentiviruses (Banerjea et al. 2003). Moreover, these viral vectors have also been

used against infectious disease of veterinary importance. Infectious bursal disease virus (IBDV) by an avian adeno associated virus delivered miRNAs targeting VP1 and VP2 genes in vitro (Wang et al. 2009). Recombinant herpesvirus of turkey (HVT) expressing shRNAs against genes gB and UL29 of the Marek's disease virus (MDV) moderately reduced viremia in chicken (Lambeth et al. 2009). In another study, avian leukosis virus-based retroviral vectors expressing shRNA against MDV gB glycoprotein gene and ICP4 transcriptional regulatory gene could significantly reduce MDV viremia in vivo (Chen et al. 2009).

Ex-vivo delivery

si/shRNAs are often delivered to target cell ex-vivo, and these modified cells are then re-injected back into the patients (Abdel-Wahab et al. 2005; DiGiusto et al. 2010).

Self-delivering compounds

Scientists at RXi Pharmaceuticals Corporation have used an alternative approach to delivery RNAi drugs. They have developed chemically modified RNAs that can enter cells without a vehicle. These novel compounds are termed 'self-delivering' RNAi compounds or sd-rxRNA (Plane 2010). sd-rxRNAs are hybrid oligonucleotide compounds that are able to bind cells, uptake and release into them. It has a single-stranded phosphorothioate region, a short duplex region, and contains a variety of nuclease-stabilizing and lipophilic chemical modifications combining the cellular uptake properties and favorable tissue distribution of the single-stranded RNA and the intracellular potency of the double-stranded RNAi compounds. In addition, chemical modifications give it a long-lasting intracellular activity. RXi promises that these next-generation RNAi drugs, designed for therapeutic use, have drug-like properties, such as high potency, target specificity, serum stability, reduced immune response activation and efficient cellular uptake.

Modified siRNA compounds

Most siRNAs used in research today are chemically synthesized as single-stranded oligonucleotides and then annealed into double-stranded form. This approach permits incorporation of a wide variety of modifications that can help solve some problems associated with administration of siRNA into cells or in vivo organisms. Some problems that can be addressed using modification include:

1. Susceptibility to nuclease degradation
2. Activation of off-target effects
3. Cell uptake and pharmacokinetics.

The kind of modifications to employ have to be chosen based on the design of the siRNA used, specific sequence, intended application, and method of delivery. Blunt-ended 27-nt dsRNAs, which are cleaved by Dicer to lead to the release of 21-nt siRNAs, exhibited a higher gene-silencing effect than 21-nt siRNAs (Kim et al. 2005). Dicer-substrate siRNA (DsiRNA), which is an asymmetric duplex RNA composed of a 25-nt sense strand with a blunt-end at the 3′-end and a 27-nt antisense stand with a 2-nt overhang at the 3′-end (25D/27-nt), could be directly cleaved by Dicer in a single 21-nt siRNA product, and exhibited a potent gene-silencing effect (Amarzguioui et al. 2006). Although these results, structural modifications of siRNAs are not enough to solve problems of poor cell uptake and low resistance against nuclease degradation. Protection against nuclease degradation can be provided externally through use of a delivery vehicle such as liposomes, peptides or nanoparticles, or intrinsically through chemical modifications of the nucleic acid itself.

3'-dTdT overhangs

The single-stranded 3'-overhangs present in traditional 21-mer siRNA designs are particularly susceptible to nuclease degradation that probably initiates its attack at the 3'-overhangs and proceeds in a 3 → 5′direction (Zou et al. 2008). Deoxythymidine (dT) or another deoxyribonucleotide overhang protect against exonuclease degradation (Elbashir et al. 2001). However, this outlook has no unanimity. Strapps and colleagues (Strapps et al. 2010), for example, have demonstrated that the thymidine overhangs are consistently detrimental to obtaining maximum duration of silencing from the siRNAs both in vitro and in vivo and should therefore be avoided for any siRNA that is intended to be developed for therapeutic purposes or for extended action in any context. The authors exactly argue that the presence of thymidine residues may expose the siRNA to DNAses. It is also possible that dTdT overhangs bind less stably to the Argonaute (Lee et al. 2007), and thus reducing the stability of RISC complexes.

Chemical modifications

Several chemical modifications, extensively used in antisense oligonucleotides, can be potentially employed in siRNAs to improve nuclease stability. Modifying directly the internucleotide phosphate linkage by replacing an oxygen with sulfur (PS), boron (boranophosphate), nitrogen (phosphoramidate), or metyl (methylphosphonate groups can provide nuclease resistance to siRNAs (Behlke 2008).

Modification of the 2'-position of the ribose can also improve nuclease resistance of the internucleotide phosphate bond and increase duplex stability. This modification can be acquired by 2'-O-methyl (2'OMe), 2'-fluoro (2'-F), 2'-O-(2-methoxyethyl) (2'-MOE) or 2'-fluoro-ß-D-arabinonucleotide (FANA) incorporation at specific positions of sense or antisense strands (Allerson et al. 2005; Choung et al. 2006; Dowler et al. 2006). These modifications can be combined for improving stability in serum and in vivo performance (Behlke 2008).

Locked nucleic acids (LNAs) which contain a methylene bridge linking the 2'-O with the 4'C of the ribose and "locking" the sugar in the 3'-end conformation provide both duplex stability and nuclease resistance to siRNAs (Grunweller et al. 2003; Mook et al. 2007).

Table 1. RNAi antiviral therapeutics currently in clinical trials*.

Clinical setting	Drug	Disease	Target(s)	Delivery/Vehicle	Company	Status
Antiviral	ALN-RSV01	RSV in volunteers	RSV nucleocapsid	Naked siRNA in intranasal adminstration	Alnylam Pharm	Completed, Phase II
	ALN-RSV01	RSV in lung transplant patients	RSV nucleocapsid	Naked siRNA by nebulization	Alnylam Pharm	Completed, Phase I
	ALN-RSV01	RSV in lung transplant patients	RSV nucleocapsid	Naked siRNA by nebulization	Alnylam Pharm	Active, Phase II
	ALN-RSV01	RSV in lung transplant patients	RSV nucleocapsid	Naked siRNA by nebulization	Alnylam Pharm	Active, Phase II
	pHIV7-shl-TAR-CCR5RZ	HIV-1 in infected patients	HIV-1 tat/rev genes, CCR5RZ	lentivirus-transduced T-cell per infusion	City of Hope Medical Center	Completed, Phase 0
	AVI-7100	Influenza A (H1N1) in heal thy volunteers	Influenza A virus M1 and M2 genes	siRNA incorporated into PMO*plus*™ per infusion	AVI Biopharma Inc.	Active, Phase I
	TKM-Ebola	Ebolavirus	Ebolavirus L polymerase, VP24 and VP35 genes	siRNA incorporated into lipid nanoparticle intravenous injection	Tekmira Pharm. Corp.	Active, Phase I

RSV, respiratory syncytial virus; PMO*plus*™ phos phor odiami date mor pholino oligomer containing three modifi ed linkages ; CR5RZ, host T cell CCR5 cytokine receptor.
* From www.clinicaltrials.gov

The 2'OMe modification is a naturally occurring RNA variant and its use in synthetic siRNAs is not expected to present significant toxicity (Behlke 2008). Contrarily, 2'-F, 2'-MOE or FANA modifications are not naturally occurring and their potential toxicity, therefore, needs to be considered. To date, no evident toxicity has been observed after using of 2'-F nucleic acids in vivo. In contrast, some dose-dependent hepatic toxicity has been reported when LNA-oligonucleotides were administrated to mice (Fluiter et al. 2003; Swayze et al. 2007).

The presence of specific chemical modifications, including those present in mammalian tRNAs and rRNAs as pseudouridine, N6-methyl-A, 2'OMe and 2'-F modified ribose, can allow synthetic siRNAs to evade immune response (Behlke 2008). Inclusion of only two or three 2'OMe modified residues in an RNA duplex can be sufficient to prevent immune activation, and modification of rU or rG residues is most effective (Judge et al. 2006).

A single 2'OMe residue at position 2 of the guide strand may also reduce or abolish off-target effect due to the participation of siRNAs in miRNA pathways (Jackson et al. 2006b). Replacement of entire seed region by DNA residues may similarly maintain wanted silencing of target genes while reduce off-target knockdown related with the homology of the seed region of siRNA (Ui-Tei et al. 2008a).

Future directions and conclusions

Optimism over the potential for RNAi therapeutics remains undiminished despite the numerous obstacles encountered since its discovery. In theory, siRNAs have extraordinary abilities as to be developed to silence any gene; to be developed incredibly fast; to have picomolar efficacy; to have an effect that may last for weeks. Nevertheless, vari¬ous complex barriers to achieving efficient therapy based on RNAi have become evident. These hurdles include: specificity for the target gene; stability of the siRNAs; and delivery to the correct cell or tissues. With the emergence of newer technologies such as chemical modification of siRNAs, non-specific effects, degradation and difficulties with delivery of siRNAs may become manageable. Although the clinical utility of RNAi has not yet been concretized, encouraging results have been provided by a number of patient trials, notably for treating skin, cancers, ocular and kidney disorders, and viral infections. To date, there have been more than 32 clinical trials involving 16 diseases and 23 different siRNA or shRNAs, seven of them against viral diseases (Table 1). Moreover, since 2008, the development pipeline has not only grown in size (18 active clinical candidates today), but more importantly it has improved in quality concomitant with a shift from local to systemic delivery: 7 of the 14 new clinical candidates since 2008 were delivered systemically, compared to only 1 of the 8 before (Haussecker 2012). This is largely the result of the clinical entry of the most advanced systemic delivery platforms, stable nucleic acid lipid particles (SNALP) and AtuPLEX. SNALP alone accounts for six clinical candidates (ALN-VSP02, TKMApoB, ALN-TTR01, TKM-PLK1, ALN-PCS02, TKM-EBOLA) and one more is expected to enter the clinic in the near future (ALN-TTR02). Up to now, more advanced results have been obtained targeting accessible tissues such as liver, skin or ocular mucosa because delivery still remains the most significant barrier to the widespread use of RNAi

therapeutics.

In summary, although the clinical applications of RNAi technology have not yet been fully conquered it has made extraordinary progress in less than fifteen years since the first demonstration of gene silencing in mammalian cells. Many hurdles remain for using RNAi-based therapeutics, one of the most significant one being probably delivery. However, very encouraging results are showing how far RNAi technology will go.

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