Review

Design, mechanism, delivery and therapeutics of canonical and Dicer-substrate siRNA

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A B S T R A C T
Upon the discovery of RNA interference (RNAi), canonical small interfering RNA (siRNA) has been recognized to trigger sequence-specific gene silencing. Despite the benefits of siRNAs as potential new drugs, there are obstacles still to be overcome, including off-target effects and immune stimulation. More recently, Dicer substrate siRNA (DsiRNA) has been introduced as an alternative to siRNA. Similarly, it also is proving to be potent and target-specific, while rendering less immune stimulation. DsiRNA is 25–30 nucleotides in length, and is further cleaved and processed by the Dicer enzyme. As with siRNA, it is crucial to design and develop a stable, safe, and efficient system for the delivery of DsiRNA into the cytoplasm of targeted cells. Several polymeric nanoparticle systems have been well established to load DsiRNA for in vitro and in vivo delivery, thereby overcoming a major hurdle in the therapeutic uses of DsiRNA. The present review focuses on a comparison of siRNA and DsiRNA on the basis of their design, mechanism, in vitro and in vivo delivery, and therapeutics.

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1. Introduction

Most novel anticancer drug approvals focus on already known targets, while relatively few compounds are certified against novel molecules [1]. Thus, only a small number of molecules are targeted due to the cost, time, and difficulties associated with the discovery and validation of novel proteins which might prove to be vital to the pathogenesis of disease [2]. Numerous important proteins remain undiscovered, hinder-
degradation in eukaryotes, resulting in selective protein silencing. This makes RNAi a useful laboratory research aid, both in vitro and in vivo. The development of RNAi library protocols, which use specific reagents to methodically target all genes present in the genome, has facilitated high output screening designed to examine phenotypes linked to the loss of function of several genes concurrently. By overpowering the expression of a gene and hence, the function of its protein, RNAi, at a certain level, models the pharmacological inhibition of the target protein and is thus a potent device for proof-of-concept trials to classify and authenticate the targets of cancer drugs [3].

siRNA is a double-stranded RNA (dsRNA), consists of 21–25 nucleotides in length [4] and is able to silence gene expression in the somatic tissues of mammals. Since the discovery of its ability to silence gene expression, siRNA has offered a novel method to treat genetic-based diseases [5], siRNA has also been used extensively as an investigational tool for the authentication of useful gene targets. siRNAs are loaded into the RNAi-induced silencing complex (RISC) by exploiting the normal RNAi pathway, thus silencing the expression of targeted gene. The inhibition of disease-related genes by siRNA thus renders RNAi a potential mechanism for advanced therapy. Despite the putative benefits of siRNA in new drug therapies, challenges such as off-target effects and immune responses activation have limited its therapeutic application. More recently, Dicer substrate siRNA (DsiRNA) has been introduced as a newer variant of RNAi-based therapeutics which also is proving to be potent, target-specific and generating less immune stimulation.

In the case of DsiRNA, Dicer endonuclease attaches to longer dsRNAs, resulting in the accurate cleavage of dsRNAs into shorter siRNAs [6]. The resultant siRNAs bind to the RISC, targeting homologous mRNA and inducing its degradation. It has also been proposed that apart from the cleavage of longer dsRNAs, Dicer endonuclease plays important roles in the loading of cleaved dsRNA into the RISC [7–9]. This theory has driven the creation of a newer class of siRNA called DsiRNA, an extremely powerful mediator of genespecific silencing. Therefore, the focus of this review will be on the design and delivery of DsiRNA in comparison to siRNA.

2. Discovery of RNAi

RNAi, a phenomenon in which molecules of RNA control the expression of genes, was discovered in plants [10]. It was originally considered a strange phenomenon, initially termed Post-Transcriptional Gene Silencing (PTGS). This phenomenon was previously believed to be limited to certain species [11]. It was revealed accidentally by Napoli et al. [10] at The University of Arizona in the late 1980s during an investigation on transgenic petunia flowers that were anticipated to be darker in their purpl color. The flowers became white or lost their color by the insertion of a gene encoding for a pigment-producing enzyme called chalcone synthase [10]. The study of this phenomenon has since been expanded to bacterial and differentiated, cultured mammalian cells, and the term RNAi is now used to define this phenomenon in animals. RNAi was initially defined in the nematode worm Caenorhabditis elegans (C. elegans). It is a process that induces gene silencing, caused by the cellular insertion of molecules of dsRNA with sequences complementary to those of mRNA. This process can be initiated either experimentally or naturally, via endogenous sources such as replicating viruses, or the deployment of similar genetic elements (transposons, etc.) [12,13].

3. Mechanism of RNAi action

The activation of RNAi by long dsRNAs (longer than 30 bases) has also been observed in Drosophila, though early experiments in vertebrates proved to be partly ineffective owing to the ability of dsRNA to trigger interferon (IFN) response [14]. Long dsRNAs trigger an immune response via protein kinase R (PKR) and IFN pathway [15]. The IFN response results in the general degradation of mRNA and inhibition of post transcriptional gene expression [15,16]. Non-vertebrates lack this mechanism, which allowed for the preliminary activation of RNAi in insects and worms. The non-specific immune stimulation dilemma was mitigated by numerous breakthroughs involving RNAi. An enzyme resembling RNase III called Dicer was discovered, which produces a 19–21 nucleotide duplex (siRNA) with two nucleotide projections at its 3’ end via the cleavage of long dsRNAs [14,17,18]. While the IFN response is triggered by longer dsRNAs, siRNAs generally, have a lower risk of triggering it [19]. Therefore, the analysis of genes in mammalian cells can be conducted using siRNAs. As the Dicer enzyme cleaves dsRNA, the produced siRNA attaches to numerous proteins and forms a nuclelease complex recognized as the RISC [20]. Argonaute proteins are essential to the assembly of the RISC [18]. Despite the scarcity of information regarding Argonaute proteins, they are recognized as nucleases based on their crystal structure. Functional data analysis reveals that Argonaute2 regulates mRNA cleavage activity [21]. siRNA binds to these proteins and facilitates the cleavage of complementary mRNA. Dicer produces double-stranded fragments, but only the antisense strand attaches to Argonaute because mostly this side contains the 2 base 3-overhang and Dicer only processes this side regardless of whether it is labelled at the 5-end of the sense or antisense strand [22]. Thus, during activation of the RISC, the sense strand is degraded. From the 3’ end of the complementary siRNA, the mRNA is cleaved into 12 nucleotides by the RISC. The deactivated mRNA releases the RISC and is then itself degraded by cellular exonucleases [23]. The translation of mRNA is inhibited due to its inexact match to the target sequence [24] (Fig. 1).

Upon discovery that the introduction of long dsRNAs triggered the formation of siRNAs, efforts were made to stimulate the endogenous synthesis of siRNAs. The focus of experiments in which short RNAs were purified from numerous animal species was on the discovery of host genome-encoded short RNAs, and not on the production of proteins. More than 200 genomically-encoded, 19–25 nucleotide-long, single-stranded RNAs were discovered in mice, C. elegans, and Drosophila; these were referred to as micro RNAs (miRNAs) [25–27]. miRNAs comprise 0.5–1% of all predicted gene messaging products that have been identified in the eukaryotes.
that have been studied [28]. Viral mRNA is inactivated in plants using miRNAs [29]. In post-embryonic development, miRNAs control larval-stage transitions in nematodes [30]. miRNAs were also found to be involved in fat metabolism and cell death suppression in Drosophila [31]. miRNA is a 70-nucleotide long, short hairpin RNA (shRNA) molecule which folds onto itself owing to its self-complementary nature. Dicer, which slices siRNA, also cleaves 70-nucleotide long miRNA into 21–22 nucleotide long molecules subsequent to its incorporation into an RNA/protein complex known as the microRNA ribonucleoprotein complex (miRNP). The complex is then sent to the 3’ terminus of target mRNA [27,32]. This 3’-untranslated region includes a number of binding sites to which numerous miRNAs can bind to a particular mRNA [24]. The extent to which transcription is inhibited is associated with the number of miRNA molecules bound to the 3’ terminus of mRNA [33]. It was initially believed that siRNA and miRNA work inversely, with siRNA causing target RNA cleavage, while miRNA does not. Subsequent research has discovered that this hypothesis is incorrect; it has been found in humans that miRNA also induces target-RNA cleavage leading to gene silencing [34].

4. siRNA

siRNA is a 21–25 nucleotide long dsRNA [23]. Its potential to silence expression of target gene in the somatic tissues of mammals has offered many researchers a new approach to treat genetic-based diseases [5]. siRNA has also been widely deployed as an investigational tool in the validation of useful gene targets. In short, administered siRNAs are incorporated into the RISC via a normal RNAi pathway, thereby silencing gene expression.

4.1. Existing strategies in the design of siRNA

siRNA-based RNAi has rapidly become a promising technique in functional genomics research [35]. Delivery of chemically synthesized siRNA leads to extremely robust and sequence-specific silencing of gene expression [35]. Initially, Dicer cuts long dsRNA into 21-nucleotide long dsRNA containing 2-nucleotide 3’-overhangs. siRNA with this conformation has hence been commonly used [36]. These RNAs do not undergo additional Dicer processing; they are directly incorporated into the RISC, facilitating antisense strand selection [37] and target recognition, hence causing cleavage. This characteristic was initially thought to be helpful in that it bypasses a step, allowing for a more rapid approach to the critical stage, from a therapeutic standpoint. The mainstream design of conventional 21-mers has enabled the development of rational design algorithms based on siRNA sequence. To increase stability, several site-specific alterations to this fundamental design have been made through chemical changes [38–45]. These changes are vital for in vivo efficacy but are dependent on chemical modifications in multiple siRNA designs. The use of lengthier siRNA, longer than 30 base pairs (bp), was originally not recommended due to unwanted effects in mammalian cells. Newer reports, however, propose that this preliminary thinking may not be completely true; RNAi effectors (longer than 30 bp) are able to undergo Dicer processing exhibit better activity [8,46,47].

4.2. Therapeutics of siRNA

Researchers have found that chemically synthesized siRNA which is exogenously inserted into cells could mediate the degradation of RNA with high specificity and efficacy. Genes involved in various diseases can be controlled using siRNA,
Table 1 - Strategies of siRNA-mediated therapies for cancer.

| No.  | Strategies                                      | Target gene(s) | References |
|------|------------------------------------------------|----------------|------------|
| 1.   | Inhibition of angiogenesis                      | VEGF           | [149–156]  |
| 2.   | Inhibition of tumor survival and inducing apoptosis | c-REL, Survivin, Kras | [157,158–159,160] |
| 3.   | Enhancing radio- or chemosensitivity by inhibiting multi-drug resistance gene | EZH2, MDR1 | [161,162] |

RNAi can be used to target human immunodeficiency virus (HIV), owing to a better understanding of its gene expression profile and life cycle. Both early and late HIV-encoded RNAs have been targeted using synthetic and expressed siRNAs [55]. An example is the trans-activation response (TAR), an RNA element known to be important in the transactivation of the viral promoter and in replication [56,57]. The regulatory protein trans-activating transcriptional (TAT) considerably enhances the efficiency of viral transcription. RNAi has been found to successfully down-regulate cellular cofactors such as nuclear factor-kappa B [58] and HIV receptor CD4 [59], leading to the inhibition of HIV replication. Furthermore, HIV replication inhibition has been accomplished in primary cells containing T lymphocytes and hematopoietic stem-cell derived macrophages, as well as many human cell lines [60–63]. Moreover, viruses could develop resistance to antiviral drugs. This could also occur in RNAi-based methods, as a single siRNA in the targeted region can inadverently allow for the escape of viruses from the RNAi pathway. This has been observed in poliovirus [64,65], HIV-1 [65–67], and HCV [68]. Certain techniques could be applied to avoid this problem, either by using pools of siRNA or by targeting the untranslated regions of RNA viruses which are vital to viral replication and sensitive to mutation. A point mutation in this untranslated region may cause cell function loss [65]. Besides that, participation of siRNAs in miRNA pathways can mediate off-target effects through the miRNA translational suppression pathway that is directed by 6–7-base matches between a siRNA and non-targeted gene [69]. This could also reduce the silencing effect of siRNAs as antiviral agents.

Unlike in viruses, the use of siRNA is not effective to inhibit bacterial infection, as bacteria largely reproduce outside the host cells without involving the cellular machinery of the host [70,71]. Yet, it has been described that siRNA could be used as a prospective agent to lessen the adverse effects induced by host immune responses and host genes involved in bacterial invasion, such as by decreasing the expression of proinflammatory cytokines [71]. Recently, siRNA was applied to combat Pseudomonas aeruginosa (P. aeruginosa) infection by silencing MexB gene from the MexA-MexB-OprM efflux pump in the pathogen [72]. Over-expression of the pump increases antibiotic efflux capacities, conferring multidrug resistance. In this study, siRNAs targeting against pathogenic MexB gene inhibited the mRNA expression in vitro. Furthermore, the designed siRNA effectively reduced the bacterial load in the in vivo model of chronic lung infection [72]. Therefore, siRNA also promises to be an innovative approach for combating bacterial infections, particularly resistant strains.

4.2.3. siRNA in clinical trials

siRNAs are evolving as next-generation biodrugs because of their selective and potent RNAi triggering potential. The therapeutic potential of siRNA has been reported in numerous studies. To date, over 30 clinical trials involving the application of miRNA and siRNA have been reported [73–75]. Earlier clinical trials have reported their use in treating viral infections, cancers, respiratory diseases, and macular degeneration. For example, different phases of the HIV life cycle were reported to be inhibited by the use of viral mRNA-targeted siRNA [59]. Liver failure implicated in chronic autoimmune
hepatitis was also prevented by Fas-specific siRNA hydrodynamic injection in an animal model [52]. Furthermore, the first clinical trials involving the age-related macular degeneration (AMD) treatment by targeting the VEGF signaling pathway have been conducted. AGN211745, a chemically altered siRNA targeting against VEGF receptor 1 gene, was found to elevate clinically improvement in vision, with minimal side effects, in an appropriate subgroup of patients [76]. In the past decade, more than 14 programs involving RNAi-based therapeutics have entered clinical trials; most of these are for therapies offering localized and topical applications. Further details regarding clinical trials of siRNA therapeutics is reviewed by Chakraborty et al. [73].

4.3. Challenges of siRNA delivery

Given its anionic and hydrophilic nature, siRNA is incapable of entering cells by passive diffusion mechanisms. Due to ineffective uptake by tissues, limited penetration across the capillary endothelium, renal elimination, and rapid enzymatic digestion in plasma, in vitro/in vivo delivery of unprotected siRNA to target sites remains a substantial barrier [77]. To tackle these issues, the development of effective in vitro and in vivo delivery systems is indispensable.

4.3.1. In vitro delivery of siRNA

The success of RNAi-based therapies depends on the ability of the delivery systems to deliver loaded compounds to the cytoplasm, which is the site of action [78]. Delivery of unchanged and unassisted siRNA in cell cultures usually leads to ineffective knockdown of the target gene, as mammalian cells are lacking the operative dsRNA-uptake mechanism which is present in many other species, including C. elegans [79]. siRNA cannot readily cross the lipid bilayers of plasma membranes, as it is a hydrophilic polyanion [80] with a relatively large surface area [81]. In comparison to plasmid DNA (pDNA), siRNA enters the cells by endocytic pathway and is then transported into endosomes and/or lysosomes where it is vulnerable to nuclease degradation [82,83] due to acidic interiors of both vesicles (pH 5–6.5) [84]. Additionally, lysosomes contain hydrolase enzymes, including ribonuclease, deoxyribonuclease, acid phosphatase, phosphodiesterase, and pyrophosphatase [85], which together can degrade siRNA, rendering it incapable of inducing RNAi [79].

Moreover, localization of synthetic siRNA transfected by liposomes was restricted to the perinuclear regions where it could not enter the nucleus even after a prolonged incubation [86–88]. In contrast, directly introduced siRNA into the cytosol (by physical methods e.g. direct injection) moved quickly into the nucleus, another site for siRNA activity apart from cytoplasm [89]. Based on this finding, synthetic or intracellularly-expressed siRNA can be used to administer siRNA into the mammalian cells [83]. For intracellularly-expressed siRNA, siRNA is produced after introducing encoded genetic information into the cells by pDNA or viral siRNA. Delivery of DNA encoders is more difficult to achieve as compared to RNA encoders because it requires delivery to the nucleus, the site for encoded DNA construct is transcribed. Effective in-vivo siRNA delivery is more challenging to achieve when compared to in vitro siRNA delivery, owing to problems with target selectivity and homeostasis [90]. Generally, endogenous substances play important roles in maintaining homeostasis of the body. In case of exogenous drugs, they are not being provided by the body system with an appropriate biodistribution profile. Therefore, their pharmacokinetics is not necessarily optimized to exhibit its pharmacological effects [91]. Furthermore, problems associated with poor circulation stability and unfavorable pharmacokinetics and biodistribution profiles of siRNA are partly contributed to the difficulties [92]. Even so, the cellular membrane is still the main obstacle for efficient siRNA transport into the target site, even for in vitro delivery.

Several strategies have been developed for intracellular siRNA delivery. These include the incorporation of siRNA into cationic polymers or liposomes; manipulation of viral vectors; and distortion of cell membrane integrity by physical methods, including compelling siRNA into the cells (gene gun, magnetofection) or weakening the cell membrane barrier (electroporation, ultrasound) [90,93]. Nevertheless, the application of physical methods like electroporation is known to possibly decrease the cell viability to less than 60%, even if high uptake in the cells is achievable [94]. Furthermore, a number of cellular factors have been demonstrated to affect siRNA transfection into the mammalian cells, including cell type, confluence, and passage number. In the case of cationic carriers, their compatibility with the growth medium has also been reported to impact siRNA transfection efficiency besides other known factors such as toxic effects to the cells and the physical characteristics of their cationic particles [90]. Besides sufficiently small in particle size, the cationic particles should be designed to facilitate cellular uptake via endocytosis and endolysosomal escape to the cytosol.

Recently, smart drug delivery systems have been developed as efficient siRNA delivery vehicles capable of escaping endolysosomal vesicles. Smart polymers that can respond to certain stimuli, such as changes in surrounding pH are currently being used for improving gene silencing efficacy. Swelling/deswelling and degradation of the polymeric systems are the various responses that have been reported [95]. In a previous study by Han and Yin [96], poly(allylamine hydrochloride)-citraconic anhydride (PAH-Cit) was developed to induce disassembly of multi-layered nanocomplexes (MLNs) and facilitate their escape to the cytosol through the charge reversal of PAH–Cit, triggered by the acidity (pH 5.0) of endolysosomal interiors.

Furthermore, modified gold nanoparticles (AuNPs) were also developed as a strategy to overcome problems with poor siRNA stability and low cellular uptake of siRNA [80]. In this study, AuNPs were modified using branched polyethylenimine (bPEI). The results suggested that the modified AuNPs enhanced cellular uptake of siRNA via the “proton sponge” effect of bPEI without significant cytotoxicity. The use of PAH-cit as a charge reversal polymer for AuNPs was reported firstly by Guo et al. [97]. Charge reversal functionalized AuNPs were prepared by layer-by-layer technique from polymers, including PAH-cit, PEI and 11-mercaptoundecanoic acid (MUA). PEI was later deposited onto the functionalized AuNPs to produce PEI/PAH-Cit/PEI/MUA-AuNPs; for improving intracellular delivery of siRNA. Later, efficient cellular uptake of siRNA vectorized by similar charge reversal AuNPs was reported [98]. In this study, AuNPs were reduced and stabi-
lized by chitosan. The positive surface charge of the resultant AuNPs allowed PEI/PAH-cit to be deposited onto their surface via electrostatic interaction, forming a nanosystem (PEI/PAH-Cit/AuNP-Chitosan) that released siRNA efficiently at pH 5.5.

4.3.2. In vivo delivery of siRNA

In several gene therapy experiments, the type of agents to deliver siRNAs seems to be the key hurdle for siRNA technology to progress to clinical trials. Given this challenge, effective RNAi approaches might not require viral vectors because RNAi-based molecules could be directly introduced into tumors or subjects. Liposomes and lipid nanoparticles are commonly employed for in vivo delivery of siRNA [99]. The technology has evolved that many variants have been developed so far; ranging from cationic liposomes to form lipoplexes to stable nucleic acid lipid particles (SNALPs) and the newer generations of SNALPs, including lipid particle using dimethylaminopropane (DLinDMA) with improved properties for siRNA delivery and highly potent lipid nanoparticles for specific tissue targeting such as DLin-KC2-DMA. DLin-KC2-DMA showed favorable accumulation in antigen-presenting cells (APCs) [100], mainly in liver. However, a major limitation of SNALPs is that the systems are accumulated in liver via passive targeting; siRNAs are also distributed to non-target cells in the liver and results in toxicity effects [101]. As an example, uptake and activation of Kupffer cells, the immune cells of liver are likely to cause hepatic toxicity and carcinogenesis [102].

Overcoming the problem regarding hepatocyte targeting could be accomplished by designing a carrier that can reversibly mask the activity of membrane-active polymer until it reaches the acidic environment of endosomes and has the ability to target this modified polymer and its siRNA cargo specifically to hepatocytes in vivo. The carrier is known as Dynamic PolyConjugates (DFC) [101]; consists of siRNA, endosomolytic polymer, shielding agent polyethylene glycol (PEG), targeting ligand and masking chemistry (to release PEG and targeting ligand in endosomes). They demonstrated effective knockdown of two endogenous genes in mouse liver; apolipoprotein B (apoB) and peroxisome proliferator-activated receptor alpha (ppara). Knockdown of apoB resulted in clear phenotypic changes, including a significant reduction in serum cholesterol and increased fat accumulation in the liver, consistent with the known functions of apoB. Knockdown of ppara also resulted in a phenotype consistent with its known function, although with less penetrance than observed in apoB knockdown mice [101]. Schneider et al. [103] formulated the Digoxigenin-siRNA into nanoparticles consisting of DPCs and the resulting complexes enabled siRNA-specific mRNA knockdown with IC50 siRNA values in the low nanomolar range for a variety of siRNAs, and target cells [103].

Besides, the conjugation of lipid nanoparticles with a targeting ligand, N-acetylgalactosamine (GalNAC) mediates cellular uptake by the binding with asialoglycoprotein receptor (ASGPR1) [99]. Tris-GalNAC binds to the ASGPR1 that is highly expressed on hepatocytes, resulting in rapid endocytosis. Enough amounts of siRNAs enter the cytoplasm to induce robust and target selective RNAi responses in vivo. Multiple GalNAC-siRNA conjugates are currently underway for clinical trials, including two phase III trials in treating various diseases [104].

In comparison to methods that use antisense molecules, in vivo RNAi approaches are more advantageous because, unlike single-stranded antisense molecules, duplex siRNAs are more stable so that in vivo RNAi results in better inhibition of gene expression than previous antisense techniques. The effective expression of a firefly reporter gene was achieved initially by using high pressure siRNA as a method of delivery [105]. Intracaudal administration of siRNA-containing pDNA in mice suppressed an HCV gene in the liver by up to 90% [50]. Correspondingly, by targeting siRNA against the FAS gene, reduced concentration of Fas mRNA and protein were observed in the kidney, pancreas, spleen, and lungs [52]. HBV-genome-targeting siRNA was found to successfully inhibit protein production and viral replication in mice [49]. Furthermore, adenoviral-mediated delivery of siRNA targeting the polyglutamine aggregation in polyglutamine diseases, Xia and colleagues [106] revealed that therapeutic RNAi treatment benefited patients with neurodegenerative disorders. Similarly, VEGF-targeting cationic lipid-complexed siRNA was investigated for the inhibition of ocular neovascularization using a mouse eye model [107]. Later, VEGF-targeting free siRNA was injected intravenously, intraperitoneally and subcutaneously, in fibrosarcoma-bearing mice, which caused a 70% reduction in VEGF level and a 66% decrease in the tumor volume within 16 days [108].

For years, a combination therapy of siRNA and chemotherapy drugs has attracted interest as an effective anti-cancer therapy. For example, doxorubicin was co-loaded into siRNA-phospholipids together with cationic lipids and PEG-fused poly(DL-lactic-co-glycolic acid) (PLGA) conjugates that were reported to halt tumor growth in vivo models [109]. Later, MLNs were shown to serve as effective and safe delivery systems to exploit the synergistic effects of chemotherapy drugs (doxorubicin) and therapeutic genes (siRNA) [96]. Briefly, these experiments illustrate that the in vivo delivery of siRNA exhibits great clinical potential.

Over the years, increased numbers of siRNA-based bio-drugs are entering clinical trials, mostly by new biopharmaceutical companies [73]. Tekmira Pharmaceuticals developed SNALPs to deliver siRNAs as potential strategies to treat diseases, including hypercholesterolemia, solid tumors, Ebola and amyloidosis [99]. Tekmira Pharmaceuticals initiated a phase 1 clinical trial to evaluate the safety of apoB-specific siRNA delivered by SNALPs for potential treatment of hypercholesterolemia. In some cases, significant activation of innate immune response was observed although extensive preclinical studies showed little evidence of immunostimulatory potential [99].

The development of linear cyclodextrin-containing polymers (CDPs) for nucleic acid delivery traces back to the mid-1990s by Dr. Mark Davis. Utilizing a siRNA targeting the EWS/Fli1 fusion oncogene and the human transferrin protein as a targeting ligand, the first in vivo proof-of-concept experiments, were performed shortly thereafter in a disseminated murine model of Ewing’s sarcoma [110]. The significant antitumor effect demonstrated in this work motivated the creation of a company, Calando Pharmaceuticals, to further advance this delivery platform (RONDEL) towards therapeutic
candidates suitable for clinical evaluation in human cancer patients. The first such candidate, termed CALAA-01, contained a siRNA targeting the M2 subunit of ribonucleotide reductase (RRM2), a protein involved in DNA replication which function is required to complete cell division. Upon identification of the optimal anti-RRM2 siRNA sequence [111] and evaluation of the in vivo nanoparticle performance [112], an Investigational New Drug (IND) application was submitted to the Food and Drug Administration (FDA) and Calando received approval to initiate a phase I trial of CALAA-01 in patients with solid tumors in 2008.

The first use of synthetic polyplex to deliver siRNA (CALAA-01) intravenously in human for cancer therapy was also initiated by Calando Pharmaceuticals [113]. CALAA-01 was complexed with cyclodextrin and functionalized with PEG and transferrin for shielding and specific targeting, respectively. The formulation was evaluated in patients with solid tumors that were refractory to standard therapy [113,114]. With encouraging interim clinical data in hand, avenues for continued development and improvement of nanoparticles identified, and the emergence of alternative siRNA-containing nanoparticles in the clinic from which all in this field will learn, the future for siRNA-containing nanoparticles based on cyclodextrin-containing polycations appears bright.

5. DsiRNA

In the RNAi cascade, long dsRNA binds with Dicer endonuclease (a member of the RNase III family), which results in accurate cleavage of longer dsRNAs into short and functional siRNAs [115]. These functional siRNAs bind to the RISC, targeting any homologous mRNA and leading to its degradation. It has been proposed that Dicer endonuclease not only cleaves long dsRNAs but also plays a role in loading the dsRNA into the RISC [8,9,116]. This theory has driven the development of a novel class of siRNA called DsiRNA, an extremely powerful mediator of gene-specific silencing.

Numerous experiments have revealed that 25–30 nucleotide dsRNAs are more potent effectors of silencing in particular genes when compared to 21-mers. In a comparison study designed to target the same sequence, 25- to 30-mers were found to be approximately 100-fold more effective than siRNAs of 21-mers [46]. This higher potency seems to rely on Dicer, which processes the longer dsRNAs and cuts them to yield 21-mers. A similar decrease in potency of siRNA was observed when siRNAs of 27-mers were labelled with 6-carboxyfluorescein (6-FAM) to interfere with the cleavage by Dicer [117].

The precise cleavage of 27-mers by Dicer into 21-mers is not the only factor that enhances the effectiveness of the 27-mers. Various 21-mer siRNAs with 2-nucleotide 3’-overhangs were designed and produced to match all profitable yields of Dicer that could possibly obtain from a longer dsRNA. These 21-mers did not yield similar levels of gene silencing compared to those obtained with the 27-mer siRNAs at low concentrations [46]. To be precise, the enhanced cleavage afforded by Dicer does not alone explain the increased gene silencing efficiency. It has been proposed that providing a substrate along with Dicer during the cleavage of 27-mers augments the efficacy of the siRNAs’ entry into the RISC and is also accountable for improving gene silencing [8].

5.1. DsiRNA design

Increased efficiency in longer-than-standard RNAi effectors (25–30 nt size range) has been reported [46]. The IFN induction by in vitro transcribed siRNAs 25- to 27-nucleotides long by bacteriophage T7 polymerase (T7 siRNA) exhibited better efficacy in inhibiting Herpes simplex virus (HSV) at the cellular level than that of synthetic 21-nt siRNAs [118]. Further investigation revealed that the T7 siRNA was a more potent RNAi and IFN inducer than the synthetic 21-nt siRNA. In this circumstance, immune stimulation by siRNA while maintaining its gene silencing efficacy is beneficial in combating viral infections [99]. Similar findings were observed for small RNA hairpins, which can also act as a small synthetic RNA hairpin with a 2-bp 3’-overhang and a 29-bp stem. These molecules were more powerful RNAi inducers than their smaller hairpins [47]. Studies also illustrated that in vitro Dicer undergoes directional processing, starting mainly from the open end of the stem and producing a cleavage products mixture (21- and 22-nt). In the above-mentioned scenario, improved efficacy could be accredited to the effect of Dicer processing, which is believed to enhance effective assimilation into the RISC by the physical bond of Dicer with the Argonaute proteins, known effectors of RNAi. This explanation has been reinforced by biochemical validation in Drosophila melanogaster, signifying the role of Dicer at the initial stages of the RISC assembly [119]. In human cells, the processing of mature RNAi precursors mediated by Dicer increases silencing of targeted genes when combined to a specific RISC assembly of miRNA [120,121]. Dicer products of 27-nt unchanged duplexes are mostly erratic, often leading to the generation of siRNAs with poor activity, i.e., lower than that of an ideal 21-mer. Therefore, there is no assurance that an asymmetrically designed 27-mer will be more effective than the 21-mers. The problem of attaining predictable DsiRNA processing would appear to have been solved by generating coherent designs based on the availability of strategic algorithms. This novel optimization strategy offers directional reactions and exclusivity of processing in the Dicer cleavage step by imitating important characteristics of pre-miRNAs. These pre-miRNAs are natural substrates for Dicer with expanded stem-loop structures with 2-nucleotide 3’-extensions. According to more recent reports, the projections on the exposed side of the stem were bound by Dicer and controlled the processing direction [47]. In a naturally occurring substrate of Dicer, the duplex is closed by a loop at the other end to inhibit Dicer from binding to the end. This characteristic can be mimicked in DsiRNA by reducing the length of the resultant duplex end and presenting two DNA nucleotides in the blunt and sense strand of the duplex. The introduction of a 3’-overhang at one end begins a partiality to commence from that end, whereas in the opposite blunt end the DNA nucleotides impose this irregularity and inhibit processing events concerning two phosphodiester linkages at the terminal. This leads to the expected formation of a single or main 21-nt processing moiety beginning from the overhang terminal. Sometimes this moiety is supplemented with a minor 22-nt product, which results from
processing at the same end [35,47]. Such descriptions of Dicer processing may suggest a certain level of sequence priority near the presumed cleavage site. The above DsiRNA configuration, having a single 2-base, 3′-overhang on the antisense strand and is blunt on the other end provides Dicer with a single favorable binding sites and selective loading of antisense strand into RISC which enhance the potency [99]. Moreover, it was suggested that optimizing the design features of 3′-overhangs by merely altering the 3′-end of the overhang while maintaining the same duplex sequence would allow rational designing of DsiRNA with high potency [122].

5.2. DsiRNA mechanism of action

The silencing of target gene through the activation of RNAi pathway depends on dsRNA as an activator. In this cascade, longer dsRNAs bind to Dicer endonucleases, which cleave the dsRNA into shorter siRNAs and facilitate loading into the RISC. Asymmetric 27-mer dsRNA are loaded into the RNAi processing apparatus (Fig. 2), one step before the conventional siRNA. This irregular structure, with a two-base 3′-overhang on the antisense strand and DNA bases on the opposite blunt end, provides a substrate for Dicer to slice in an expected way. The cleaved small dsRNA is then loaded into the RISC and one of the two strands is selected as a guide or antisense strand, depending on the 3′-overhang [122] and chemical modification of dsRNA [123]. In the RISC assembly, the sense strand of siRNA, also known as the passenger strand, is cut and liberated, while the guide strand is combined into the Argonaute2 protein, which is part of the RISC. The guide strand directs the RISC complex to its respective target mRNA and finally is cleaved by the endonucleolytical role of the Argonaute2 protein.

5.3. DsiRNA delivery

The carrier or transport device is the main constituent in all in vitro and in vivo DsiRNA experiments. The selection of carrier for an active ingredient is important and must also be well-thought-out. Viral vectors are frequently linked with immunogenicity and safety concerns [124]. Researchers are taking more interest in non-viral systems of transfection, which are proving to be more attractive because they provide optimum control over the concentration of the active agent and more predictable patterns of toxicity [125]. Other than these advantages, a transfection agent should protect and deliver DsiRNA to the tissues. Similar to siRNA, nanotechnology offers solutions for the delivery of DsiRNA to the cell cytoplasm.

5.3.1. In vitro delivery of DsiRNA

The actual strength of the administered component is determined by the efficacy of the carrier along with the inherent strength of DsiRNA. Large amount of administered dsRNA increases the possibility of inducing an immune response, particularly when used in conjunction with cationic lipids. The high strength formulations will reduce the number and dose of injections needed to attain functional decrease in the gene expression and protein levels, and thus, reduce possible side effects.

One of the preferred methods of administering DsiRNA therapeutics is via local delivery, which offers direct contact with the macrophages. Nanoparticulate carrier systems such as PLGA nanoparticles have been investigated to efficiently deliver DsiRNA across the plasma membrane of macrophages and into the cytosol. The major drawback of using PLGA nanoparticles as carriers is the immune response stimulation. In a different approach, RNA HIV glycoprotein 120 aptamers were used for delivering and targeting DsiRNA into HIV infected cells [126]. Furthermore, liposomes have been shown to load DsiRNA effectively, for instance, in the targeting of Kupffer cells with DsiRNA-loaded liposomes [127].

Chitosan has also been used effectively to load DsiRNA [128,129]. In a study of an in vitro solid tumor model by Raja et al. [129], DsiRNA loaded into chitosan nanoparticles via an ionic gelation method was shown to significantly knockdown VEGF expression at mRNA and protein levels as a result of uniform penetration and distribution of DsiRNA-chitosan nanoparticles throughout multicellular layers (MCLs) of human colorectal cancer cells (DLD-1). The findings proved that chitosan nanoparticles could effectively deliver DsiRNA to the cytosol and knockdown the targeted gene, thereby pointing to a potential treatment for human colorectal carcinoma [129]. Pluronic F-127 (PF-127) has also been used as a temperature sensitive gel to carry chitosan nanoparticles loaded with DsiRNA to enable their permeation through the skin in the treatment of skin cancer [109]. TAT peptides have also been used to load and deliver DsiRNA into cells [130]. DsiRNA was complexed to TAT via simple complexation to produce small complexes below 200 nm. TAT was also reported to bind siRNA.
strongly, but the resulting complexes were larger than those formed with DsiRNA [131].

Recently, linoleic and caprylic acids-substituted PEI has been developed as a delivery system for DsiRNA to target breast cancer cells [132]. Significant knockdown of hypoxanthine-guanine phosphoribosyltransferase (HGPRT), interleukin-8 (IL8), and cystic fibrosis transmembrane conductance regulator (CFTR) expression at the mRNA and protein levels was also achieved by in vitro delivery of DsiRNA using lipid transfection reagents [133]. In a different study, integrin-targeted nanoparticles were developed to deliver DsiRNA to tumor cells [78]. The αvβ3 integrins are upregulated in tumor cells as compared to healthy cells and represent attractive targets for the development of specific DsiRNA delivery to cancer cells. PEG chitosan was used to enhance the polymer solubility and their respective nanoparticles, while PEI was added to enhance transfection efficiency. The arginine-glycine-aspartate (RGD) peptide, or an RGD peptidomimetic (RGDp) that mimics the RGD motif was conjugated to the distal ends of the PEG chains for targeting integrin receptor specifically. The results obtained suggested that the internalization of these nanoparticles was influenced by the concentration, as a minimum ligand concentration is needed to induce αvβ3 integrin-mediated uptake. In contrast, non-targeted nanoparticles were unable to mediate gene silencing effectively [78].

5.3.2. In vivo delivery of DsiRNA
There are few early reports describing in vivo delivery of DsiRNA [38,134]. The advantage of 27-mer long DsiRNAs in vivo was first revealed in an experiment that used intraperitoneal (IT) injections of cationic lipids formulation to target a peritoneal macrophage-specific TNF-α gene [38]. In a different study, DsiRNAs effectively cured hepatitis C in an in vivo model system in which an intravenous hydrodynamic injection was administered to the liver [134]. The in vivo systemic delivery of siRNAs to tumors and other organs has been performed in a number of methods. However, siRNAs do not permeate the blood-brain barrier (BBB); gene silencing targets in the central nervous system therefore require either the use of a carrier for BBB permeability or direct injection into the cerebrospinal fluid or brain parenchyma.

The efficiency of 27-mer DsiRNAs in decreasing the gene expression of a certain G protein-coupled receptor (GPCR) in the spinal cords of rats was reported previously [135]. In this study, a commercial transfection agent, i-Fect kit containing a low-concentration DsiRNA formulation was administered via IT injection; it continuously reduced the mRNA and protein levels of neurotensin receptor-2 (NTS2) GPCR for three to four days. The decrease in NTS2 resulted in the anticipated behavioral variations in nociception. No apparent toxicity or non-specific adverse effects were noted during the course of the study, and the results generally highlighted the practicality of DsiRNA application in pain management [135].

In the study of viral infections, in vivo delivery of DsiRNA via a chemically synthesized aptamer strongly inhibited HIV-1 replication [22]. The synthesized aptamer was used to complex three different DsiRNAs, achieving effective in vivo delivery; the combination of the three DsiRNAs resulted in inhibition of HIV-1 replication [22]. For non-viral delivery systems, liposomal DsiRNA complexes targeting tumor necrosis factor alpha (TNF-α) were developed to protect against TNF-α-dependent liver toxicity [136]. TNFα is a proinflammatory cytokine that involves in numerous inflammatory diseases including viral encephalitis [136]. DsiRNA has also been used as a prophylactic alternative in a murine model and has been proven effective at partially inhibiting viral replication of human metapneumovirus [137].

For treating cancers using RNAs as a therapy, both in vitro and in vivo small animal studies have shown promise. An important consideration is that any drug delivered must be able to reach the target site with sufficient amount and duration of exposure and does not cause significant toxicity [138]. Khairuddin et al. [138] studied the effectiveness of local versus systemic delivery of immune-stimulating DsiRNAs (IS-DsiRNAs) in a human papillomavirus (HPV)-driven tumor model. Local intratumoral injection of DsiRNA resulted in increased tumor uptake compared to systemic intravenous (IV) delivery and potently activated innate immune responses. Despite increased cellular uptake, intratumoral injection was not as effective as IV in reducing tumor growth.

SNALPs have been shown to be effective delivery systems for siRNAs in vivo [139]. In a study by Dicerna Pharmaceuticals, the cationic lipid and PEG–lipid components of Dicerna’s unique EnCore lipid nanoparticle platform was demonstrated to modulate and improve delivery of DsiRNA to orthotopic and spontaneous liver tumors, as well as xenograft tumors of diverse non-hepatic tissue origin [140].

5.4. Off-target effects
Despite the benefits of siRNAs as potential novel drugs, there are difficulties that must be addressed going forward. One of these challenges is the risk of off-target effects, which lead to the inhibition of genes that should not be targeted. This could occur if a gene shares fractional homology with the siRNA. The silencing of non-target genes may cause complications such as toxicity and difficulty in data interpretation [141]. The selection and design of siRNAs should be performed cautiously to avoid this phenomenon.

Immune stimulation is another challenge with siRNA therapy, a condition wherein the siRNA duplex is recognized by the host’s innate immunity [77]. Introduction of excessive amounts of siRNA is reported to cause non-specific interactions due to the triggering of innate immunity. Given that the dsRNA sensor PKR activates the immune system, it is necessary to design siRNA which can be used at the lowest possible concentration to eliminate off-target effects. Furthermore, some reports propose that the RNAi mechanism can become saturated and subsequently inhibit the appropriate processing of miRNA precursors [142], possibly resulting in toxicity. Non-specific toxicity and other deleterious properties can be mitigated by keeping siRNA at the lowest concentration as possible. For instance, complete activation of the IFN pathway can be avoided by using low concentrations of siRNAs of less than 30 nucleotides in length [35]. It has been reported that higher concentrations of siRNA can trigger proinflammatory responses [143] while several cytoplasmic localized receptors such as PKR recognize longer siRNAs (> 30 nt) that subsequently trigger innate immunity [89]. The longer the length of dsRNA, the stronger is the effect on the host cells [144].
Despite shorter siRNAs (<30 nt) was considered non-immunogenic initially, induction of partial cytokine and type-1 IFN response via toll-like receptors (TLRs) was reported as well, including TLR7 and TLR8 on endosomes [15,145]. Moreover, the off-target effects can be a result of nucleic acids mediated immunostimulation, mainly through TLR3 that binds dsRNA [145]. Delivery strategies involving internalization via endocytosis and localization in the endosomal compartment are more prone to cause immune stimulation through TLR7/8 for example, siRNA complexed with cationic lipids or polymers. Contrarily, shRNAs endogenously synthesized by plasmid and viral vectors are less likely to trigger immune responses [15].

Different strategies are available to minimize the risk of side effects caused by non-specific immune response activated by synthetic siRNA. Chemical modification such as 2'-O-methyl (2'OMe) RNA has been employed because it is naturally occurring RNA variant in mammalian cells; avoiding recognition by endosomal TLRs [99] and showing improved stability against nucleases without loss of potency if the 5’-end of the guide strand is modified with 2'OMe [146]. Other 2’-modifications include 2’Fluoro (2F) and locked nucleic acid (LNA), developed to escape immune detection. The use of asymmetric and shorter sequence siRNAs such as DsiRNA will also minimize the risk in addition to chemical modification [99]. Detailed discussion on strategies to overcome siRNA-mediated innate immune responses could be obtained in an article reviewed by Judge and MacLachlan [147].

In order to further decrease the risk of activating immune responses, DsiRNAs can be developed to avoid initiation of the PKR pathway and proinflammatory cytokines (IFN-α and IFN-β) [148]. In an attempt to validate this, cells were introduced with 27-mer DsiRNA, 21-mer siRNA, or tripolyphosphate-containing single-stranded RNA (ssRNA). The ssRNA acted as a positive control because of its tendency to initiate IFN-α and IFN-β when introduced into cells. As compared to the cells introduced with ssRNA, assays of cell lysates from cells transfected with 27-mer dsRNA or 21-mer siRNA exhibited no measurable levels of IFN-α and IFN-β initiation, and no indication of PKR activation.

Although the risk of other off-target effects still persists, it can be alleviated by applying substances that allow for the use of low nanomolar concentrations of siRNA [143]. Another advantage of DsiRNA is permanence of silencing which can reduce the risk of off-target effects by using low amount of administered DsiRNA or at a lesser frequency besides reducing the potential off-target effects from the passenger strand through selective loading of guide strand in RISC [8]. When mouse embryonic fibroblast (NIH 3T3) cells, which steadily express higher levels of Green Fluorescent Protein (eGFP), were transfected with 27-mers or 21-mers to target the eGFP gene, the resulting suppression of eGFP by the 21-mer persisted for four days, and for up to ten days by the 27-mer [134]. These results are similar with the findings of another study [143].

6. Closing remarks

RNAi-based therapeutics that can permit long-lasting and steadily powerful silencing at low concentrations are desirable in improving efficacy of silencing with low risk of off-target effects. Though there are fewer direct comparisons amongst RNAi-based therapeutics to date, DsiRNAs, designed for processing by Dicer to maximize RNAi efficiency will open wider opportunities for safer and better treatments clinically. Furthermore, the exploitation of an effective delivery system permits these DsiRNAs to be applied at the lowest concentration possible, thus reducing the risk of adverse effects. Solid dispersion is one of the most efficient techniques to improve the dissolution rate of poorly water-soluble drugs, leading to an improvement in the relative bioavailability of their formulations. At present, the solvent method and the melting method are widely used in the preparation of solid dispersions. In general, subsequent grinding, sieving, mixing and granulation are necessary to produce the different desired formulations.

Conflicts of interest

The authors declare that there is no conflicts of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ajps.2018.12.005.

REFERENCES

[1] Collins I, Workma P. New approaches to molecular cancer therapeutics. Nat Chem Biol 2006;2(12):689–700.
[2] Kamb A, Wee S, Lengauer C. Why is cancer drug discovery so difficult? Nat Rev Drug Discov 2006;6(2):115–20.
[3] Iorns E, Lord CJ, Turner N, Ashworth A. Utilizing RNA interference to enhance cancer drug discovery. Nat Rev Drug Discov 2007;6(7):556–68.
[4] Huvägner G, Zamore P. A microRNA in a multiple-turnover RNAi enzyme complex. Science 2002;297(5589):2056–60.
[5] Sloud M. Therapeutic siRNAs. Trends Pharmacol Sci 2004;25(1):22–8.
[6] MacRae JJ, Zhou K, Li F, et al. Structural basis for double-stranded RNA processing by Dicer. Science 2006;311(5758):195–8.
[7] Lee YS, Nakahara K, Pham JW, et al. Distinct roles for Drosophila Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. Cell 2004;117(1):69–81.
[8] Rose SD, Kim DH, Amazighoui M, et al. Functional polarity is introduced by Dicer processing of short substrate RNAs. Nucleic Acids Res 2005;33(13):4140–56.
[9] Sontheimer EJ. Assembly and function of RNA silencing complexes. Nat Rev Mol Cell Biol 2005;6(2):127–38.
[10] Napoli C, Lemieux C, Jorgensen R. Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. Plant Cell 1990;2(4):279–89.
interference in cultured mammalian cells. Nature 2001;411(6836):494–8.

Khvorova A, Reynolds A, Jayasena SD. Functional siRNAs and miRNAs exhibit strand bias. Cell 2003;115(2):209–16.

Schwarz DS, Hütvagner G, Du T, Xu Z, Aronin N, Zamore PD. Symmetry in the assembly of the RNAi enzyme complex. Cell 2003;115(2):199–208.

Amazguioui M, Holen T, Babaei E, Pryzde H. Tolerance for mutations and chemical modifications in a siRNA. Nucleic Acids Res 2003;31(2):589–95.

Czauderna F, Fechtner M, Dames S, et al. Structural variations and stabilising modifications of synthetic siRNAs in mammalian cells. Nucleic Acids Res 2003;31(11):2705–7.

Braasch DA, Jensen S, Liu Y, et al. RNA interference in mammalian cells by chemically-modified RNA. Biochemistry 2003;42(26):7967–75.

Chiu YL, Rana TM. siRNA function in RNAi: a chemical modification analysis. RNA 2003;9(9):1034–48.

Morrissey DV, Lockridge JA, Shaw L, et al. Potent and persistent in vivo anti-HBV activity of chemically modified siRNAs. Nat Biotechnol 2005;23(8):1002–7.

Prakash TP, Allerson CR, Dande P, et al. Positional effect of chemical modifications on short interference RNA activity in mammalian cells. J Med Chem 2005;48(13):4247–53.

Elmén J, Thonberg H, Ljungberg K, et al. Locked nucleic acid (LNA) mediated improvements in siRNA stability and functionality. Nucleic Acids Res 2005;33(1):439–47.

Dande P, Prakash TP, Sioufi N, et al. Improving RNA interference in mammalian cells by 4′-thio-modified small interfering RNA (siRNA): effect on siRNA activity and nuclelease stability when used in combination with 2′-O-alkyl modifications. J Med Chem 2006;49(5):1624–34.

Kim DH, Behlke MA, Rose SD, Chang MS, Choi S, Rossi JJ. Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy. Nat Biotechnol 2004;23(2):222–6.

Siolas D, Lerner C, Burchard J, et al. Synthetic shRNAs as potent RNAi triggers. Nat Biotechnol 2004;23(2):227–31.

Tan FY, Yin JQ, RNAi, a new therapeutic strategy against viral infection. Cell Res 2004;14:460–6.

McCaffrey AP, Nakai H, Pandey K, et al. Inhibition of hepatitis B virus infection by RNA interference. Nat Biotechnol 2003;21(6):639–44.

McCaffrey AP, Meuse L, Pham T-TT, Conklin DS, Hannon GJ, Kay MA. Gene expression: RNA interference in adult mice. Nature 2002;418(6893):38–9.

Randall G, Grakoui A, Rice CM. Clearance of replicating hepatitis C virus replicon RNAs in cell culture by small interfering RNAs. Proc Natl Acad Sci 2003;100(1):235–240.

Song E, Lee SK, Wang J, et al. RNA interference targeting Fas protects mice from fulminant hepatitis. Nat Med 2003;9(3):347–51.

Wilson JA, Jayasena S, Khvorova A, et al. RNA interference blocks gene expression and RNA synthesis from hepatitis C replicons propagated in human liver cells. Proc Natl Acad Sci 2003;100(5):2783–8.

Kapadia SB, Brideau-Andersen C, Chisari FV. Interference of hepatitis C virus RNA replication by short interfering RNAs. Proc Natl Acad Sci 2003;100(4):2014–18.

Jacque JM, Triques K, Stevenson M. Modulation of HIV-1 replication by RNA interference. Nature 2002;418(6896):435–8.

Lee NS, Dohjima T, Bauer G, et al. Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. Nat Biotechnol 2002;20(5):500–5.

Coburn GA, Cullen BR. Potent and specific inhibition of human immunodeficiency virus type 1 replication by RNA interference. J Virol 2002;76(18):9225–31.
Surabhi RM, Gaynor RB. RNA interference directed against viral and cellular targets inhibits human immunodeficiency virus type 1 replication. J Virol 2002;76(24):12963–73.

Novina CD, Murray MF, Dykhxhoorn DM, et al. siRNA-directed inhibition of HIV-1 infection. Nat Med 2002;8(7):681–6.

Martínez MA, Gutiérrez A, Armand-Ugón M, et al. Suppression of chemokine receptor expression by RNA interference allows for inhibition of HIV-1 replication. AIDS 2002;16(18):2385–90.

Capodici J, Kankó K, Weissman D. Inhibition of HIV-1 infection by small interfering RNA-mediated RNA interference. J Immunol 2002;169(9):5196–201.

Banerjea A, Li MJ, Bauer G, Remling, Lee NS, Rossi J, Akkina R. Inhibition of HIV-1 by lentiviral vector-transduced siRNAs in T lymphocytes differentiated in SCID-hu mice and CD34+ progenitor cell-derived macrophages. Mol Ther 2003;8(1):62–71.

Li MJ, Bauer G, Michienzi A, et al. Inhibition of HIV-1 infection by lentiviral vectors expressing Pol III-promoted anti-HIV RNAs. Mol Ther 2003;8(2):196–206.

Gitlin L, Stone JK, Andino R. Poliovirus escape from RNA interference: short interfering RNA-target recognition and implications for therapeutic approaches. J Virol 2005;79(2):1027–35.

van Rij RP, Andino R. The silent treatment: RNAi as a defense against viral infection in mammals. Trends Biotechnol 2006;24(4):186–93.

Boden D, Pusch O, Lee F, Tucker L, Ramratnam B. Human immunodeficiency virus type 1 escape from RNA interference. J Virol 2003;77(21):11531–5.

Das AT, Brummerkamp TR, Westerhout EM, et al. Human immunodeficiency virus type 1 escapes from RNA interference-mediated inhibition. J Virol 2004;78(5):2601–5.

Wilson JA, Richardson CD. Hepatitis C virus replicons escape RNA interference induced by a short interfering RNA directed against the NS5b coding region. J Virol 2005;79(11):7050–8.

Birmingham A, Anderson EM, Reynolds A, et al. 3'UTR seed matches, but not overall identity, are associated with RNAi off-targets. Nat Methods 2006;3(9):199–204.

Lieberman J, Song E, Lee SK, Shankar P. Interfering with disease: opportunities and roadblocks to harnessing RNA interference. Trends Mol Med 2003;9(9):397–403.

Leung RK, Whittaker PA. RNA interference: from gene silencing to gene-specific therapeutics. Pharmacol Ther 2005;107(2):222–39.

Gong FY, Zhang DY, Zhang JG, et al. siRNA-mediated gene silencing of MexB from the MexA-MexB-Oprm efflux pump in Pseudomonas aeruginosa. BMB Rep 2014;47(4):203–8.

Chakraborty C, Sharma AR, Sharma G, Doss CGF, Lee SS. Therapeutic miRNA and siRNA: moving from bench to clinic as next generation medicine. Mol Ther - Nucleic Acids 2017;8:132–43.

Rupaimoole R, Slack FJ. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. Nat Rev Drug Discov 2017;16(3):203–22.

Crooke ST, Witztum JL, Bennett CF, Baker BF. RNA-targeted therapeutics. Cell Metab 2018;27(4):714–39.

DeVincenzo J, Cehelsky JE, Alvarez R, et al. Evaluation of the safety, tolerability and pharmacokinetics of ALN-AMG01, a novel RNAi antiviral therapeutic directed against respiratory syncytial virus (RSV). Antiviral Res 2008;77(3):225–31.

Bumcrot D, Manoharan M, Koteliansky V, Sah DW. RNAi therapeutics: a potential new class of pharmaceutical drugs. Nat Chem Biol 2006;2(12):711–9.

Ragelle H, Colombo S, Pourcelle V, et al. Intracellular siRNA delivery dynamics of integrin-targeted, PE/PEGylated chitosan-poly(ethylene imine) hybrid nanoparticles: a mechanistic insight. J Control Release 2015;190(1):1–9.

Rozema DB, Lewis DL. siRNA delivery technologies for mammalian systems. Targets 2003;2(6):253–60.

Muratovska A, Eccles MR. Conjugate for efficient delivery of short interfering RNA (siRNA) into mammalian cells. FEBS Lett 2004;558(1):63–8.

Shaata H, Mostafa M, Moustafa M, et al. Modified gold nanoparticles for intracellular delivery of anti-liver cancer siRNA. Int J Pharm-Pharmaceutical Nanotechnology 2016;504(1–2):125–39.

Beale G, Hollins AJ, Benboubetta M, Sohali R, Fox SP, Benter I, Akhtar S. Gene silencing nucleic acids designed by scanning arrays: anti-EGFR activity of siRNA, ribozyme and DNA enzymes targeting a single hybridization-accessible region using the same delivery system. J Drug Targeting 2003;11(7):449–56.

Gilmore IR, Fox SP, Hollins AJ, Sohali M, Akhtar S. The design and exogenous delivery of siRNA for post-transcriptional gene silencing. J Drug Targeting 2004;12(6):315–40.

Murthy N, Campbell J, Fausto N, Hoffman AS, Stayton PS. Design and synthesis of pH-responsive polymeric carriers that target uptake and enhance the intracellular delivery of oligonucleotides. J Control Rel 2003;89(3):365–74.

Hudson AJ, Lee W, Porter J, Akhtar J, Duncan R, Akhtar S. Stability of antisense oligonucleotides during incubation with a mixture of isolated lysosomal enzymes. Int J Pharm 1996;133(1):257–63.

Spagnoli S, Miller AD, Keller M. Lipidic carriers of siRNA: differences in the formulation, cellular uptake, and delivery with plasmid DNA. Biochemistry 2004;43(42):13348–56.

Chiu YL, Ali A, Chu CY, Cao H, Rana TM. Visualizing a correlation between siRNA localization, cellular uptake, and RNAi in living cells. Chem Biol 2004;11(8):1165–75.

Keller M. Lipidic carriers of RNA/DNA oligonucleotides and polynucleotides: what a difference a formulation makes!. J Control Release 2005;105(3):227–40.

Schiffelers RM, Wood MC, Scaris P. Pharmaceutical prospects for RNA interference. Pharm Res 2004;21(1):1–7.

Hughes MD, Husain M, Nawaz Q, Sayyed P, Akhtar S. The cellular delivery of antisense oligonucleotides and ribozymes. Drug Discov Today 2001;6(6):303–15.

Yamashita F, Hashida M. Pharmacokinetic considerations for targeted drug delivery. Adv Drug Deliv Rev 2013;65(1):139–47.

Park J, Park J, Pei Y, Xu J, Yeo Y. Pharmacokinetics and biodistribution of recently-developed siRNA nanomedicines. Adv Drug Deliv Rev 2016;104:93–109.

Niidome T, Huang L. Gene therapy progress and prospects: nonviral vectors. Gene Ther 2002;9(24):1647–52.

McManus MT, Haines BB, Dillon CP, et al. Small interfering RNA-mediated gene silencing in T lymphocytes. J Immunol 2002;169(10):5754–60.

Kim TH, Jiang HL, Jere D, et al. Chemical modification of chitosan as a gene carrier in vitro and in vivo. Prog Polym Sci 2007;32(7):726–53.

Han L, Yin CTC. Dual-targeting and pH/redox-responsive multi-layered nanocomplexes for smart co-delivery of doxorubicin and siRNA. Biomaterials 2015;60:42–52.

Guo S, Huang Y, Jiang Q, et al. Enhanced gene delivery and siRNA silencing by gold nanoparticles coated with charge-reversal polyelectrolyte. ACS Nano 2010;4(9):5505–11.

Han L, Zhao J, Zhang X, et al. Enhanced siRNA delivery and silencing gold-chitosan nanosystem with surface
charge-reversal polymer assembly and good biocompatibility. ACS Nano 2012;6(8):7340–51.

[99] Rettig GR, Behlke MA. Progress toward in vivo use of siRNAs-II. Mol Ther 2012;20(3):483–512.

[100] Semple SC, Akinc A, Chen J, et al. Rational design of cationic lipids for siRNA delivery. Nat Biotechnol 2010;28(2):172–6.

[101] Rozema DB, Lewis DL, Wakefield DH, et al. Dynamic polyconjugates for targeted in vivo delivery of siRNA to hepatocytes. Proc Natl Acad Sci USA 2007;104(32):12982–7.

[102] Roberts RA, Caney FE, Ju C, Kamendulis LM, Rusan I, Klauing JG. Role of the Kupffer cell in mediating hepatic toxicity and carcinogenesis. Toxicol Sci 2007;96:2–15.

[103] Schneider B, Grote M, John M, et al. Targeted siRNA delivery and mRNA knockdown mediated by bispecific digoxigenin-binding antibodies. Mol Ther Nucleic Acids 2012;1(9):e46.

[104] Springer AD, Dowdy SF. GalNac-siRNA conjugates: leading the way for delivery of RNAi therapeutics. Nucleic Acid Ther 2018;28(3):109–18.

[105] Lewis DL, Hagstrom JE, Loomis AG, Wolff JA, Herweijer H. Efficient delivery of siRNA for inhibition of gene expression in postnatal mice. Nat Genet 2002;32(1):107–8.

[106] Xia H, Mao Q, Paulson HL, Davidson BL. siRNA-mediated gene silencing in vitro and in vivo. Nat Biotechnol 2002;20(10):1006–10.

[107] Reich S, Fujimoto J, Kuroki A, et al. Small interfering RNA (siRNA) targeting VEGF effectively inhibits ocular neovascularization in a mouse model. Mol Vis 2003;9(5):210–6.

[108] Filleur S, Courtin A, Ait-Si-Ali S, et al. siRNA-mediated inhibition of vascular endothelial growth factor severely limits tumor resistance to antiangiogenic thrombospondin-1 and slows tumor vascularization and growth. Cancer Res 2003;63(14):3919–22.

[109] Liu H, Li Y, Mozhai A, et al. siRNA-phospholipid conjugates for gene and drug delivery in cancer treatment. Biomaterials 2014;35(24):6519–33.

[110] Hu-Lieskovan S, Heidel JD, Bartlett DW, Davis ME, Triche TJ. Sequence-specific knockdown of EWS-FLI1 by targeted, nonviral delivery of small interfering RNA inhibits tumor growth in a murine model of metastatic Ewing’s sarcoma. Cancer Res 2005;65(19):8984–92.

[111] Heidel JD, Liu JY, Yen Y, et al. Potent siRNA inhibitors of ribonucleotide reductase subunit RM2 reduce cell proliferation in vitro and in vivo. Clin Cancer Res 2007;13(7):2207–15.

[112] Heidel JD, Yu Z, Liu JYC, et al. Administration in non-human primates of escalating intravenous doses of targeted nanoparticles containing ribonucleotide reductase subunit M2 siRNA. Proc Natl Acad Sci USA 2007;104(14):5715–21.

[113] Davis ME, Zuckerman JE, Choi CHJ, et al. Evidence of RNAi in mice from systematically administered siRNA via targeted nanoparticles. Nature 2010;464:1067–70.

[114] Ribas A, Kalinoski L, Heidel JD, et al. Systemic delivery of siRNA via targeted nanoparticles in patients with cancer: results from a first-in-class phase I clinical trial. J Clin Oncol 2010;28(suppl15):3022.

[115] MacRae IJ, Zhou K, Li F, et al. Structural basis for double-stranded RNA processing by Dicer. Science 2006;311(5758):195–8.

[116] Lee YS, Nakahara K, Pham JW, et al. Distinct roles for Drospihila Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. Cell 2004;117(1):69–81.

[117] Kim SW, Kim NY, Choi YB, Park SH, Yang JM, Shin S. RNA interference in vitro and in vivo using an arginine peptide/siRNA complex system. J Control Release 2010;143(3):335–43.

[118] Kim DH, Longo M, Han Y, Lundberg P, Cantin E, Rossi JJ. Interferon induction by siRNAs and ssRNAs synthesized by phage polymerase. Nat Biotechnol 2004;22(3):321–5.

[119] Pham JW, Pellino JL, Lee YS, Cartewh RW, Sontheimer EJ. A Dicer-2-dependent 80S complex cleaves targeted mRNAs during RNAi in drosophila. Cell 2004;117(1):83–94.

[120] Gregory RI, Chandrimada TP, Cooch N, Shiekhattar R. Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. Cell 2005;123(4):631–40.

[121] Maniati A, Morelatos Z, human A. ATP-independent, RISC assembly machine fueled by pre-miRNA. Genes Dev 2005;19(24):2979–90.

[122] Zhou J, Song MS, Jacobi AM, Behlke MA, Rossi JJ. Deep Sequencing analyses of DsiRNAs reveal the influence of 3’ terminal overhangs on Dicing polarity, strand selectivity, and RNA editing of siRNAs. Mol Ther Nucleic Acids 2012;1(4):1–17.

[123] Snead NM, Wu X, Li A, Cui Q, Sakurai K, Burnett JC, Rossi JJ. Molecular basis for improved gene silencing by Dicer substrate interfering RNA compared with other siRNA variants. Nucleic Acids Res 2013;41(12):6209–21.

[124] Cotter MJ, Muruve DA. The induction of inflammation by adenovirus vectors used for gene therapy. Front Biosci 2005;10:1098–105.

[125] Anderson RG. The caveolae membrane system. Annu Rev Biochem 1998;67(1):199–225.

[126] Zhou J, Swiderski P, Li H, et al. Selection, characterization and application of new RNA HIV gp 120 aptamers for facile delivery of Dicer substrate siRNAs into HIV infected cells. Nucleic Acids Res 2009;37(9):3094–109.

[127] Pichu S, Krishnamoorthy S, Zhang B, Jing Y, Shishkov A, Ponnappa BC. Dicer-substrate siRNA inhibits tumor necrosis factor alpha secretion in Kupffer cells in vitro: in vivo targeting of Kupffer cells by siRNA-liposomes. Pharmacol Res 2012;65(1):48–55.

[128] Katas H, Raja MAG, Hamid ZA, Razali NA. Physico-chemical properties and in vitro cytotoxicity studies of chitosan as a potential carrier for Dicer-substrate siRNA (DsiRNA). J Nanomater 2013;2013:108.

[129] Raja MAG, Katas H, Hamid ZA. Penetration and silencing activity of VEGF dicer substrate siRNA vectorized by chitosan nanoparticles in monolayer culture and a solid tumor model in vitro for potential application in tumor therapy. J Nanomater 2016;2016:7201204.

[130] Katas H, Siang TT, Raja MAG. Topical temperature-sensitive gel containing DsiRNA-chitosan nanoparticles for potential treatment of skin cancer. Trends Med Res 2017;12(1):1–13.

[131] Katas H, Raja MAG, Choy LE. Comparative characterization and cytotoxicity study of TAT-peptide as potential vectors for siRNA and Dicer-substrate siRNA. Drug Dev Ind Pharm 2014;40(11):1443–50.

[132] Farmar MB, Alibadi HM, Mahdipoor P, et al. Targeting cell cycle proteins in breast cancer cells with siRNA by using lipid-substituted polyethyleneimines. Front Bioeng Biotechnol 2015;3:1–14.

[133] Ramachandran S, Krishnamurthy S, Jacobi AM, et al. Efficient delivery of RNA interference oligonucleotides to polarized airway epithelia in vitro. Am J Physiol Lung Cell Mol Physiol 2013;305(1):L23–32.

[134] Kim M, Shin D, Kim SI, Park M. Inhibition of hepatitis C virus gene expression by small interfering RNAs using a tri-cistronic full-length viral replicon and a transient mouse model. Virus Res 2006;122(1):1–10.

[135] Doré-Savid L, Roussy G, Dansereau MA, et al. Central delivery of Dicer-substrate siRNA: a direct application for pain research. Mol Ther 2008;16(7):1331–9.

[136] Lundberg P, Yang H-J, Jung S-J, Behlke MA, Rose SD, Cantin EM. Protection against TNF-α-dependent liver
toxicity by intraperitoneal liposome delivered DsiRNA targeting TNFs: in vivo. J Control Release 2012;160(2):194–9.

Darniot M, Schildgen V, Schildgen O, et al. RNA interference in vitro and in vivo using DsiRNA targeting the nucleocepсид N mRNA of human metapneumovirus. Antiviral Res 2012;93(3):364–73.

[138] Khairuddin N, Blake SJ, Firdaus F, et al. In vivo comparison of local versus systemic delivery of immunostimulating siRNA in HPV-driven tumours. Immunol Cell Biol 2014;92:156–63.

[139] Kanasty R, Dorkin JR, Vegas A, Anderson D. Delivery materials for siRNA therapeutics. Nat Mater 2013;12:967–77.

[140] Abrams M, Ganesh S, Ying B, et al. EnCore-LNP mediated tumor delivery of MYC and CTNNB1 dicer substrate RNAs (DsiRNAs). Mol Cancer Res 2015;13(10 Suppl):S20.

[141] Janas MM, Schlegel MK, Harbison CE, et al. Selection of GalNac-conjugated siRNAs with limited off-target-driven rat hepatotoxicity. Nat Commun 2018;9(1):723.

[142] Bitko V, Musiyenko A, Shulayeva O, Barik S. Inhibition of respiratory viruses by nasally administered siRNA. Nat Med 2004;11(1):50–5.

[143] Persengiev SP, Zhu X, Green MR. Nonspecific, concentration-dependent stimulation and repression of mammalian gene expression by small interfering RNAs (siRNAs). RNA 2004;10(12):12–18 12–8.

[144] Guo F, Coban O, Sneed NM, Trebley J, Shu Y. Engineering RNA for targeted siRNA delivery and medical application. Adv Drug Deliv Rev 2010;62(6):650–66.

[145] Karikó K, Bhuyan P, Capodici J, Weissman D. Small interfering RNAs mediate sequence-independent gene suppression and induce immune activation by signaling through toll-like receptor 3. J Immunol 2004;172(11):6545–9.

[146] Choug S, Kim YJ, Kim S, Park HO, Choi YC. Chemical modification of siRNAs to improve serum stability without loss of efficacy. Biochem Biophys Res Commun 2006;342(3):919–27.

[147] Judge A, MacLachlan I. Overcoming the innate immune response to small interfering RNA. Hum Gene Ther 2008;19(2):111–24.

[148] Kim DH, Behlke MA, Rose SD, Chang M-S, Choi S, Rossi JJ. Synthetic dsiRNA Dicer substrates enhance RNAi potency and efficacy. Nat Biotechnol 2004;23(2):222–6.

[149] Florinas S, Kim J, Nam K, Janát-Amsbury MM, Kim SW. Ultrasound-assisted siRNA delivery via arginine-grafted bioreducible polymer and microbubbles targeting VEGF for ovarian cancer treatment. J Control Release 2014;183: 1–8.

[150] Pittella F, Miyata K, Maeda Y, Suma Y, Kataoka K. Pancreatic cancer therapy by systemic administration of VEGF siRNA contained in calcium phosphate/charge-convensional polymer hybrid nanoparticles. J Control Release 2012;161(3):868–74.

[151] Hadi-Slimane R, Lepelletier Y, Lopez N, Garbay C, Raynaud F. Short interfering RNA (siRNA), a novel therapeutic tool acting on angiogenesis. Biochimie 2007;89(10):1234–44.

[152] Xie FY, Woodle MC, Lu FY. Harnessing in vivo siRNA delivery for drug discovery and therapeutic development. Drug Discov Today 2006;11(11):67–73.

[153] Das B, Yeger H, Tsuchida R, et al. A hypoxia-driven vascular endothelial growth factor/FIT1 autocrine loop interacts with hypoxia-inducible factor-1α through mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2 pathway in neuroblastoma. Cancer Res 2005;65(16):7267–75.

[154] List AF, Glinsmann-Gibson B, Stadheim C, Meuillet EJ, Bellamy W, Powis G. Vascular endothelial growth factor receptor-1 and receptor-2 initiate a phosphatidylinositol 3-kinase–dependent clonogenic response in acute myeloid leukemia cells. Exp Hematol 2004;32(6):526–35.

[155] Kou R, SenBanerjee S, Jain MK, Michel T. Differential regulation of vascular endothelial growth factor receptors (VEGFR) revealed by RNA interference: interactions of VEGFR-1 and VEGFR-2 in endothelial cell signaling. Biochemistry 2005;44(45):15064–73.

[156] Takei Y, Kadomatsu K, Yuzawa Y, Matsuo S, Muramatsu T. A small interfering RNA targeting vascular endothelial growth factor as cancer therapeutics. Cancer Res 2004;64(10):3365–70.

[157] Mohammad SM, Mohammadnejad D, Feizi AAH, Movassaghpour AA, Nozad H. Charoude Inhibition of c-REL using siRNA increased apoptosis and decreased proliferation in pre-B ALL blasts: therapeutic implications. Leuk Res 2017;61:53–61.

[158] Chen X, Zhang Y, Tang C, Tian C, Zhang C. Co-delivery of paclitaxel and anti-survivin siRNA via redox-sensitive oligopeptide liposomes for the synergistic treatment of breast cancer and metastasis. Int J Pharm 2017;529(1–2):102–15.

[159] Salzgno G, Rielhe R, Navarro G, Perche F, Torchlin VP. Polymeric micelles containing reversibly phospholipid-modified anti-survivin siRNA: a promising strategy to overcome drug resistance in cancer. Cancer Lett 2014;343(2):224–31.

[160] Wen ZM, Ji J, Zhang Y, Liu H, Peng LP. A self-assembled polyulginan nanoparticle loaded with doxorubicin and anti-Kras siRNA for attenuating multidrug resistance in human lung cancer. Biochem Biophys Res Commun 2017;493(4):1430–7.

[161] Lee SY, Yang CY, Peng CL, Wei MF, Shieh MJ. A theranostic micelleplex co-delivering SN-38 and VEGF siRNA for colorectal cancer therapy. Biomaterials 2016;86:92–105.

[162] Xia Y, Wang X, Cheng H, Fang M, Song H. A polycation coated liposome as efficient siRNA carrier to overcome multidrug resistance. Colloids Surf B Biointerfaces 2017;159(1):427–36.

[163] Tebes SJ, Krusk FA. The genesis of RNA interference, its potential clinical applications, and implications in gynecologic cancer. Gynecol Oncol 2005;99(3):736–41.