NUCLEIC ACID THERAPEUTICS
Volume 24, Number 4, 2014

DOI: 10.1089/nat.2014.0480

# Development of RNA Interference–Based Therapeutics and Application of Multi-Target Small Interfering RNAs

Tiejun Li,[1–3] Meihua Wu,[1–3] York Yuanyuan Zhu,[2,3] Jianxin Chen,[2,3] and Li Chen[1]

RNA interference (RNAi) has been proven in recent years to be a newly advanced and powerful tool for development of therapeutic agents toward various unmet medical needs such as cancer, in particular, a great attention has been paid to the development of antineoplastic agents. Recent success in clinical trials related to RNAi-based therapeutics on cancer and ocular disease has validated that small interfering RNAs (siRNAs) constitute a new promising class of therapeutics. Currently, a great wealth of multi-target based siRNA structural modifications is available for promoting siRNA-mediated gene silencing with low side effects. Here, the latest developments in RNAi-based therapeutics and novel structural modifications described for siRNAs—in particular multi-target siRNAs—are reviewed.

## Introduction

RNA interference (RNAi) is the process of post-transcriptional highly specific gene silencing triggered by 19–23 base pairs (bp) double-stranded RNA (dsRNA) present in different organisms (Fire et al., 1998; Hammond et al., 2000; Elbashir et al., 2001a). The process is initiated upon cleavage of long dsRNA into mall interfering RNA (siRNA) duplexes with two overhanged nucleotides at 3′ ends by specific ribonuclease Dicer (Zhang et al., 2002; MacRae et al., 2008; Noland et al., 2011). Then siRNA duplexes incorporate into RNA-induced silencing complex (RISC) (Tomari et al., 2004a; Sakurai et al., 2011). In RISC, one strand (''sense'' or ''passenger'') undergoes cleavage and dissociates from the complex upon RISC activation, the other strand (''antisense'' or ''guide'') which remains in the complex recognizes and cleaves RNA targets with the help of Argonaute-2 (*AGO-2*) (Song et al., 2004; Hutvagner et al., 2008) and other related RISC proteins: R2D2 (Liu et al., 2003), protein kinase RNA activator (Kok et al., 2007), and TAR RNA binding protein (Tomari et al., 2004b; Chendrimada et al., 2005; Liu et al., 2006; Kini and Walton, 2009; Gredell et al., 2010). The discovery of RNAi has broadened scientists' perspectives in the development of therapeutic drugs. An approach to address multigenic targets simultaneously by using combined siRNAs in one payload is considered to be a better way of controlling complex disease systems (Zimmermann et al., 2007), and thus such a strategy as applying multi-target siRNAs has become popular in RNAi-based therapeutic research (Sharp, 2001). In this review, the focus is on recent development of multi-target siRNAs for applications in RNAi-based therapeutics, novel siRNA structural designs and modifications.

## RNAi-Based Therapeutics

RNAi technology used to suppress expression of pathologically and physiologically important genes is applicable for the treatment of human diseases caused by specific genes that are difficult to treat through traditional approaches. Since the first description of RNAi phenomenon in mammalians, development of RNAi-based therapies has rapidly been advanced with many powerful new drug candidates in clinical trials against various human diseases, such as age-related macular degeneration (AMD) (McFarland et al., 2004; Mccullough et al., 2005; Gu et al., 2010), respiratory syncytial virus (RSV) infection (Bitko et al., 2005), neurodegenerative disorders (Raoul et al., 2006) and various cancers (Takeshita and Ochiya, 2006; Aigner, 2007; Davidson and McCray, 2011).

### *Candidates of RNAi-based drugs*

Increasing popularity in the development of RNAi-based therapeutics over recent years has evidenced many siRNA preclinical and clinical trials, currently with about 30 siRNA therapeutic candidates reaching various clinical trials stages for treatment of at least 16 diseases (DeVincenzo et al., 2008; Davis et al., 2010; Leachman et al., 2010; Burnett et al., 2011; Davidson and McCray, 2011; Burnett and Rossi, 2012). Some major global pharmaceutical companies such as Novartis

[1]Department of Pathological Anatomy, and [2]Small RNA Technology and Application Institute, Nantong University, Nantong, China.
[3]Department of Life Science Center, Biomics Biotechnologies Co., Ltd., Nantong, China.

Table 1. Current Status of siRNA Therapeutics in Clinical Trials

| *Drug* | *Disease* | *Delivery* | *Route* | *Target* | *Phase* | *Status* | *Company* |
|---|---|---|---|---|---|---|---|
| siRNA-EphA2-DOPC | Advanced cancer<br>Advanced solid tumors | Neutral liposome | IV | *EPHA2* | 1 | Not yet recruiting | UT MDA/OCRF |
| iPsiRNA | Metastatic melanoma | Electroporation | *Ex vivo*, Intradermal | *LMP2, LMP7, MECL1* | 1 | Active | Duke University |
| SV40 vectors carrying siRNA | CML | SV40 vectors | IV | *BCR-ABL* | 1 | Completed | HMO/DOD |
| CALAA-01 | Solid cancer | RONDEL | IV | *RRM2* | 1 | Active | Calando |
| Atu027 | Advanced solid cancer | AtuPLEX | IV | *PKN3* | 1 | Completed | Silence |
| QPI-1007 | Optic atrophy | Naked siRNA | IVT | *CASPASE-2* | 1 | Completed | Quark |
| PRO-040201 | Hypercholesterolemia | SNALP | IV | *APO B* | 1 | Terminated | Tekmira |
| TKM-080301 | Cancer | SNALP | IV | *PLK1* | 1 | Recruiting | Tekmira |
| ND-L02-s0201 injection | Liver cirrhosis | VA-lip | Interventional | *HSP47* | 1 | Recruiting | NDT |
| TD101 | Pachyonychia congenital | Naked siRNA | Interventional | *Keratin 6A (N171K)* | 1 | Completed | TransDerm/ IPCC |
| ALN-TTR01 | ATTR | SNALP | IV | *TTR* | 1 | Completed | Alnylam |
| EZN-2968 | Carcinoma; lymphoma | Naked LNA | IV | *HIF-1A* | 1 | Completed | Enzon |
| EZN-2968 | Neoplasms; liver metastases | Naked LNA | IV | *HIF-1A* | 1 | Completed | NCI |
| ALN-PCS02 | Hypercholesterolemia | SNALP | IV | *PCSK9* | 1 | Completed | Alnylam |
| ALN-VSP02 | Liver cancer/solid tumors | SNALP | IV | *KSP* and *VEGF* | 1/1 | Completed | Alnylam |
| I5NP | AKI/DGF | Naked siRNA | IV | *P53* | 1/1 | Completed/terminated | Quark |
| SYL1001 | Ocular pain<br>Dry eye syndrome | Naked siRNA | Ophthalmic drops | *TRPV1* | 1/2 | Recruiting | Sylentis |
| I5NP | DGF/other Complication of kidney transplant | Naked siRNA | IV | *P53* | 1/2 | Active, not recruiting | Quark |
| AGN211745/Sirna-027 | AMD | Naked siRNA | IVT | *VEGF-R1* | 2 | Terminated | Allergan/Sirna |
| Bevasiranib/Cand5 | DME | Naked siRNA | IVT | *VEGF* | 2 | Completed | Opko Health |
| SYL040012 | Ocular hypertension, Open angle glaucoma | Naked siRNA | Ophthalmic drops | *ADRB2* | 2 | Completed | Sylentis |
| siG12D LODER | Pancreatic ductal Adenocarcinoma<br>Pancreatic cancer | LODER | EUS biopsy needle | *KRASG12D* | 2 | Not yet recruiting | Silenseed |
| PF-04523655 | DME | Naked siRNA | IVT | *RTP801* | 2 | Active, not recruiting | Quark/Pfizer |
| ALN-RSV01 | RSV infection after lung transplantion | Naked siRNA | Nebulization | *RSV Nucleocapsid* | 2 | Completed | Alnylam |
| PF-04523655 | Wet AMD | Naked siRNA | IVT | *RTP801* | 2 | Completed | Quark/Pfizer |
| FANG vaccine | Ovarian cancer<br>Colon cancer | Electroporation | Interventional | *FURIN* and *GM-CSF* | 2/2 | Recruiting, Recruiting | Gradalis |
| Bevasiranib | Wet AMD | Naked siRNA | IVT | *VEGF* | 3 | Terminated | Opko Health |
| ALN-TTR02 | ATTR | SNALP | IV | *TTR* | 3 | Recruiting | Alnylam |

ADRB2, adrenergic receptor beta-2; AKI, acute kidney injury; AMD, age-related macular degeneration; *APOB*, apolipoprotein B; ATTR, amyloidosis; *BCR-ABL*, breakpoint cluster region-abelson murine leukemia; CML, chronic myeloid leukemia; DGF, delayed graft function; DME, diabetic macular edema; DOD, Department of Defense; DOPCC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; *EPHA2,* ephrin type-A receptor 2; EUS, endoscopic ultrasound; *GM-CSF,* granulocyte-macrophage colony-stimulating factor; *HIF-1A*, hypoxia-inducible factor 1-alpha; HMO, Hadassah Medical Organization; *HSP47*, heat shock protein 47; IPCC, International Pachyonychia Congenita Consortium; iPsiRNA, inducible Proteasomal subunit-specific siRNA; IV, intravenous; IVT, intravitreal; *KRASG12D*, K-Ras mutation G12D; *KSP*, kinesin spindle protein; *LMP2*, large multifunctional protease 2; *LMP7*, large multifunctional protease 7; LNA, locked nucleic acid; LODER, local drug eluter; *MECL1*, multicatalytic endopeptidase complex-like 1; NCI, National Cancer Institute; NDT, Nitto Denko Corporation; OCRF, Ovarian Cancer Research Fund; *PCSK9*, proprotein convertase subtilisin/kexin type 9; *PKN3*, protein kinase N3; *PLK1*, polo-like kinase 1; RONDEL, RNA/oligonucleotide nanoparticle delivery; *RRM2*, ribonucleoside-diphosphate reductase subunit M2; RSV, respiratory syncytial virus; siRNA, small interfering RNA; SNALP, stable nucleic acid lipid particle; *TRPV1*, transient receptor potential vanilloid 1; TTR, transthyretin; UT MDA, M.D. Anderson Cancer Center; VA-lip, vitamin A–coupled liposome; *VEGF*, vascular endothelial growth factor; *VEGF-R1*, vascular endothelial growth factor receptor 1.

and Merck have also been involved in clinical applications of RNAi-based therapeutics. RNAi is considered to be an exceptional technology for knock-down or silencing of disease related target genes, and scientists anticipate that it will significantly shorten the drug development timeline. Furthermore, clinical pipelines of RNAi-based therapies using siRNAs have been gradually growing since approximately 2011 as the technology matures. As shown in Table 1, there are many candidates of RNAi-based drugs under clinical development in 2013.

### *RNA delivery systems*

RNAi-based drugs rely on safe and efficient delivery systems without unwanted side effects. Up to now, there are a number of efficient delivery systems which can be categorized as *ex vivo*, topical/local and systemic methods (Peer D and Lieberman, 2011; Vicentini et al., 2013). Local delivery of siRNA is practicable for tissues that are external and/or locally restricted including ocular, epidermal, pulmonary, and bladder. Additionally, local delivery may be suitable for noninvasive therapies that require administration by patients, such as eye drops and nasal sprays. The treatment of AMD and diabetic macular edema using intravitreal injections were the first clinical applications of siRNAs as these drugs could be delivered directly to ocular tissue (Barakat and Kaiser, 2009; Kaiser et al., 2010). Other therapeutic applications using siRNAs have been developed via topical delivery and are currently in clinical trials (QPI-1007, TD101, SYL040012, SYL1001, ALN-RSV01). In the past 5 years, the development pipeline has shifted from local to systemic delivery as more advanced delivery vehicles become available for systemic applications, including stable nucleic acid lipid particles (SNALP) (Zimmermann et al., 2006) and RNA/oligonucleotide nanoparticle delivery (RONDEL) (Heidel et al., 2007; Davis et al., 2010). These technologies have demonstrated to be effective *in vivo* (Zimmermann et al., 2006; Judge et al., 2009; Davis et al., 2010), and significant progress is being achieved in some clinical trials (PRO-040201, TKM-080301, ALN-TTR01, ALN-PCS02, ALN-VSP02, CALAA-01) (Table 1).

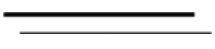
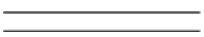
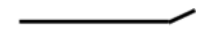
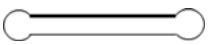
## Structural Modifications of siRNA

To improve the silencing efficiency of siRNAs, researchers tried different ways to modify structures of siRNAs. A variety of structural siRNA modifications has been reported for gene silencing (Table 2).

### *Shorter siRNAs mimicking Dicer cleavage products*

The structure of most canonical siRNAs mimics features of the products from Dicer natural cleavage, which comprises two annealed strands, one is a 21-nucleotide (nt) guiding strand antisense to a target RNA and the other is a complementary passenger strand. These two strands form an siRNA duplex upon annealing with a 19-bp dsRNA stem and 2-nt 3′-overhangs at both ends (Elbashir et al., 2001b). Depending on sequence features, some studies reported that siRNAs shorter than canonical siRNAs could efficiently trigger RNAi in mammalian cells and sometimes led to better silencing effect even than that of canonical siRNAs. Reduction of nonspecific responses and immune stimulation as well as enhanced internalization with shorter siRNAs has also been reported. Some variations in the shorter modification on siRNAs include blunt 19-bp siRNA (Czauderna et al., 2003; Prakash

Table 2. Structural Modifications of siRNA with Different Features

| *Structural siRNAs* | *Structures*[a] | *Reference* |
|---|---|---|
| Canonical siRNA |  | Elbashir et al., 2001a,b |
| Blunt-ended siRNA |  | Czauderna et al., 2003; Prakash et al., 2005; Hogrefe et al, 2006; Ghosh et al., 2009 |
| shRNA |  | Siolas et al., 2005; Ge et al., 2010 |
| 16 nt siRNA |  | Chu and Rana, 2008 |
| asiRNA |  | Sun et al., 2008 |
| aiRNA |  | Sun et al., 2008 |
| ss-siRNA |  | Martinez et al., 2002; Holen et al., 2003; Hall et al., 2006 |
| dgRNA |  | Hossbach et al., 2006 |
| sisiRNA |  | Bramsen et al., 2007 |
| Fork siRNA |  | Hohjoh, 2004; Petrova Kruglova et al., 2010 |
| Dumbbell siRNA |  | Abe et al., 2007 |
| Bulge siRNA |  | Dua et al., 2011 |
| 25- to 27-mer siRNA |  | Doré-Savard et al., 2008 |

[a]*Bold line*: sense strand, *thin line*: antisense strand.

aiRNA, asymmetrical siRNA; dgRNA, double-guide siRNAs; shRNA, short hairpin RNA; sisiRNA, small internally segmented interfering RNA; ss-siRNA, single-stranded siRNA.

et al., 2005; Hogrefe et al., 2006; Ghosh et al., 2009), short hairpin siRNA (Siolas et al., 2005; Ge et al., 2010), 16-mer siRNA (Chu and Rana, 2008), asymmetrical shorter-duplex siRNA (Sun et al., 2008), and asymmetrical siRNA (Sun et al., 2008). Other modified siRNAs used in research and drug development are single-stranded siRNAs (Martinez et al., 2002; Holen et al., 2003; Hall et al., 2006), double-guide siRNAs (Hossbach et al., 2006), small internally segmented interfering RNAs (sisiRNA) (Bramsen et al., 2007), fork siRNAs (Hohjoh, 2004; Petrova Kruglova et al., 2010), dumbbell-shaped circular siRNA (or dumbbell siRNA) (Abe et al., 2007), and bulge-siRNA (Dua et al., 2011).

### *Long dsRNA designed as Dicer-substrate for gene silencing*

RNAi effectors longer than standard siRNA were reported in 2005 by Kim et al. and Hannon et al. These longer siRNAs that structurally mimic various Dicer substrates to enhance incorporation into RNAi pathways, are termed ''Dicer-substrate siRNA'' (Kim et al., 2005; Rose et al., 2005; Siolas et al., 2005; Amarzguioui et al., 2006; Collingwood et al., 2008; Hefner et al., 2008; Tanudji et al., 2009). An investigation of cellular interferon induction caused by *in vitro* transcribed siRNAs showed that limited concentrations of some 25- to 27-mer siRNAs had greater potency than all of the possible synthetic 19-bp duplex and 2-base 3′-overhangs siRNAs that could be generated from the larger duplex (Kim et al., 2005). Hannon et al. (2005) also found that synthetic dsRNA with 25- to 30-mer lengths could be up to 100-fold more potent than corresponding conventional 19-bp duplex and 2-base 3′-overhangs siRNAs and would not activate protein kinase R or induce interferon (*IFN*). The enhanced potency of the longer duplexes is attributed to the fact that they are substrates of Dicer, linking the production of siRNAs to incorporation in the RISC. Foster et al. (2012) evaluated the *in vitro* and *in vivo* activities of siRNAs and dual-target siRNAs (dsiRNAs) targeting phosphatase and tensin homolog and factor 7 with over 250 compounds representing both siRNA and dsiRNA structures. They identified highly active compounds from 25-/27-mer dsiRNAs both *in vitro* and *in vivo* (Foster et al., 2012). This study further demonstrated that *in vitro* processing by Dicer is direct, starting predominantly from the open end of the stem and generating a mixture of 19-bp duplex and 2-base 3′-overhangs cleavage products. Increased potency could be attributed to Dicer processing, which might promote more efficient incorporation into RISC through physical association of Dicer with Ago proteins, the effectors of RNAi. This interpretation is supported by biochemical evidence in *Drosophila melanogaster*, indicating a role for Dicer in the initial stages of RISC assembly (Pham et al., 2004).

Although structurally modified siRNAs have been utilized successfully, more investigations and development may be required to further validate such modified and functionalized siRNAs as therapeutic agents. Some of these siRNAs could be loaded directly into RISC whereas others serve as substrates for Dicer and can be processed into shorter species before loading into the RISC. Doré-Savard et al. (2008) demonstrated that a low dose of 27-mer Dicer-substrate siRNA resulted in effective silence of target genes that are related to central nervous system disorders.

## Multi-targeting siRNAs for Therapeutics

Drugs designed to act on individual molecular targets cannot usually address multigenic or multi-factor diseases such as cancer, or diseases that affect multiple tissues or cell types such as diabetes and immune-inflammatory disorders (Zimmermann et al., 2007). Thus, multi-targeting therapeutics has been considered to be an attractive approach toward complex diseases. Such an approach as addressing multiple targets has been used to regulate multiple nodes of disease network resulting in a synergistic effect (Bolognesi et al., 2009; Efferth and Koch, 2011) and to design medications against atherosclerosis (Lahoute et al., 2011), cancer (Lu et al., 2012), depression (Maes et al., 2012), and psychosis (Kroken et al., 2014) as well as neurodegenerative diseases (Flight, 2013). Another approach focused on ''systemic'' drugs suggested that development of novel computational and mathematical concepts be certainly required for suitable modeling of complex data (Schrattenholz et al., 2010).

### *Combination of multi-target siRNAs for disease therapeutics*

Various design strategies with multiple-target siRNAs have been used in many gene-mediated disorders or viral infectious research (Sharp, 2001; Holen et al., 2002). Use of multiple siRNA duplexes in targeting CXC chemokine receptor type 4 (*CXCR4*) and fatty acid synthase ligand (*FASL*) was first reported to prevent human immunodeficiency virus-1 (HIV-1) infection. An enhanced gene silencing with the multiple siRNAs was demonstrated not only at protein or mRNA expression level, but also at functional level by evoking greater inhibition of HIV infection as well as more reduction of *FASL*-mediated apoptosis than that using single siRNA ( Ji et al., 2003). Menendez et al. (2004) identified a molecular link between fatty acid synthase (*FAS*) and human epidermal growth factor receptor 2 (*HER2*) oncogene by using concurrent RNA-mediated silencing of *FAS* and *HER2* genes, they found that simultaneous inhibition of *FAS* and *HER2* genes by corresponding siRNAs led to apoptosis synergistically in *HER2* overexpressing cancer cells. In an anti-prostate cancer study, multi-target siRNAs based on the homologous region of the DNA methyl transferases 3A and 3B (*DNMT3A/B*) family were designed and their effects on proliferation, migration, and invasion of TSU-PR1 prostate cancer cells were investigated *in vitro* (Du et al., 2012). The results showed that *DNMT3B* alone apparently played a key role in maintaining unfavorable behavior of prostate cancer cells, thereby implying the potential significance of *DNMT3B* as a promising therapeutic target, while *DNMT3A* was simply a helper. To evaluate the effect of dual gene silencing from heat shock protein 27 (*HSP27*) and cellular Fas-associated death domain protein (*FADD*)-like interleukin-1β-converting enzyme inhibitory protein (*C-FLIP*) on doxazosin-induced apoptosis of prostate cancer cell, Kim et al. (2013) used a mixture of siRNAs corresponding to *HSP27* and *C-FLIP*. They showed that dual silencing of *C-FLIP* and *HSP27* enhanced apoptosis even under 1 μM—a rather low concentration—of doxazosin in prostate cancer PC-3 cells. In another study (Tai et al., 2010), dual silencing of *HER2* and vascular endothelial growth factor (*VEGF*) genes by multi-target siRNAs exhibited significant changes in cell morphology and substantial suppression on migration, spreading, cell adhesion, and proliferation *in vitro*

Table 3. Combination of Multi-target siRNAs for Disease Therapeutics

| *Targets* | *Disease* | *Delivery route* | *References* |
|---|---|---|---|
| *CXCR4, FAS*-ligand (*FASL*) | HIV infection | siRNA, transfection *in vitro* | Ji et al., 2003 |
| *FAS, HER2* | Breast and ovarian cancer | siRNA, transfection *in vitro* | Menendez et al., 2004 |
| *C-MYC, MDM2, VEGF* | Melanoma | Protamine-antibody fusion protein, IT/IP | Song et al., 2005 |
| *XIAP, BCL2, BCL-X(L)* | Bladder cancer | siRNA, transfection *in vitro* | Kunze et al., 2008 |
| *EPHA2, FAK, SRC* | Ovarian cancer | siRNA-DOPC lipsomes, IP | Shahzad et al., 2009 |
| *EZH2, P110α* | Prostate cancer | siRNA-atelocollagen, IV | Takeshita et al., 2005 |
| *N-RAS, EREG* | Hepatocellular carcinoma | siRNA, transfection *in vitro* | Zhao et al., 2009 |
| *HER2, VEGF* | Breast cancer | siRNA, transfection *in vitro* | Tai et al., 2010 |
| *DNMT3A, DNMT3B* | Prostate cancer | siRNA, transfection *in vitro* | Du et al., 2012 |
| *HSP27, C-FLIP* | Prostate cancer | siRNA, transfection *in vitro* | Kim et al., 2013 |
| *VEGF, KSP* | Liver cancer | SNALP, IV | Tabernero et al., 2013 |
| *VEGF, HER2* | Gastric cancer | siRNA, transfection *in vitro* | Liu et al., 2014 |

*BCL-X (L)*, B-cell lymphoma-extra large; *C-FLIP*, cellular Fas-associated death domain protein-like interleukin-1β-converting enzyme inhibitory protein; *CXCR4*, CXC chemokine receptor type 4; *DNMT3A*, DNA methyltransferases 3A; *DNMT3B*, DNA methyltransferases 3B; *EREG*, epiregulin; *EZH2*, enhancer of zeste homolog 2; *FAK*, focal adhesion kinase; *FAS*, fatty acid synthase; *FAS-ligand (FASL)*, FAS ligand; *HER2*, human epidermalgrowth factor receptor-2; HIV, human immunodeficiency virus; *HSP27*, heat shock protein 47; IP, intraperitoneal; IT, intratumoral; *MDM2*, mouse double minute 2 homolog; *P110α*, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha; *XIAP*, X-linked inhibitor of apoptosis protein).

breast cancer model. These results suggested that *HER2* positive breast cancer could be treated more effectively by inhibition of both *HER2* and *VEGF* genes simultaneously. Also in gastric cancer, Liu et al. (2014) demonstrated that co-down-regulation of *VEGF* and *HER2* by a siRNA cocktail resulted in significant inhibition of gastric cancer growth and migration *in vitro*. Thus, inhibition of *VEGF* and *HER2* expression could induce apoptosis of gastric cancer cells. Other examples of applying combinations or cocktails of siRNAs for multiple genes targeting are presented in Table 3. These investigations showed that two or more siRNAs could be synthesized and mixed for therapeutic purposes.

### *Dicer substrate-based designs for multi-target siRNAs*

Alternatively to the above modification methods, conventional double-stranded siRNAs can be structurally modified to bear multiple targeting RNA sequences. In a number of studies, such siRNAs with quite unique structures have been designed and used as Dicer substrates for therapeutic evaluations (Table 4).

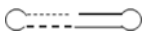
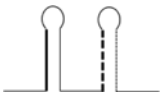
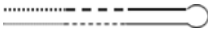
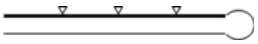
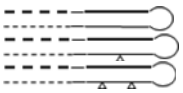
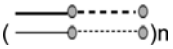
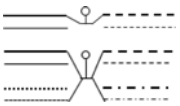
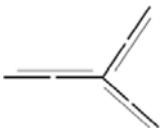
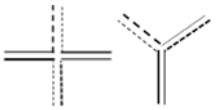
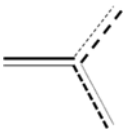
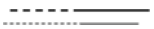
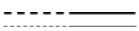
### *Short hairpin siRNAs*

Anderson et al. (2003) reported that bispecific short hairpin siRNAs containing an 8-nucleotide intervening spacer targeted against either *CXCR4* and cluster of differentiation 4 or C-C chemokine receptor type 5 (*CCR5*) and *CXCR4*, which are known HIV-1 cell surface receptors. After being transfected with the bispecific siRNAs, *MAGI-CXCR4* and *CCR5* cells exhibited significant downregulation of their respective co-receptors. In other approach, a double siRNA structure with a single construct called hairpin RNA (hpRNA)—produced by a cytomegalovirus promoter-driven DNA template—was used to block expression of matrix metalloproteases *(MMP)-9* and cathepsin B genes (Lakka et al., 2004). The hpRNAs were found to reduce the invasive behavior of glioblastoma cell line, which was attributed to the inhibition of the *MMP-9* and cathepsin B expression. Direct intratumoral (IT) injections of plasmid DNA expressing hpRNA inhibited growth of established glioma tumor model significantly and reduced invasion of intracranial tumors in *in vivo* models. On the other hand, intraperitoneal (IP) injections of plasmid DNA expressing hpRNA not only repressed preestablished tumors greatly without any indication of tumor cells present but also kept its effect for a period of 4 months. Gondi et al. (2004) showed that, in both *in vitro* and *in vivo* models, glioma cell invasion and angiogenesis could be inhibited when an hpRNA targeting the receptor of urokinase-type plasminogen activator (*UPAR*) and cathepsin B was used, IT injections of plasmid vectors expressing the hpRNA for *UPAR* and cathepsin B resulted in the suppression of preestablished intracranial tumors. Other hpRNAs designed for targeting *UPAR, MMP-9,* and *UPAR* were also tested for treatment of meningioma (Tummalapalli et al., 2007; Kargiotis et al., 2008) and breast cancer (Kunigal et al., 2007). All these studies showed satisfactory inhibition effects of multi-target hpRNAs on cancers *in vivo*.

### *Long hairpin siRNAs*

Long siRNAs with hairpins for multiple gene targets were designed to achieve sustained gene silencing. DNA-directed RNAi (ddRNAi) technology, by which long siRNA with hairpin transcripts were expressed after introduction of ddRNA constructs into cells, had recently been developed (Rice et al., 2005) and successfully applied for anti–hepatitis-C (HCV) therapeutics in a clinical trial (Denise et al., 2014). Others also reported various designs of long siRNAs with hairpin structures (Akashi et al., 2005; Liu et al., 2007; Sano et al., 2008). Novel modified hairpin RNAs (mhRNAs) more than 100 bp in length were designed (Akashi et al., 2005) and featured by multiple specific point mutations in the sense strand. Such mhRNAs induced RNAi and suppressed replication of multiple HCVs effectively without inducing the *IFN* pathway. Liu et al. (2007) introduced the so-called extended short hairpin RNAs (e-shRNAs) that encode two effective siRNAs against HIV-1 variants in a construct. The study indicated that, from activity assays and RNA processing analyses, positioning of the two siRNAs on the hairpin stem was critical to generation of the two corresponding functional siRNAs. Efficient processing of the e-shRNAs into

Table 4. Dicer Substrate-Based Designs for Multi-target siRNAs

| *Multi-target siRNA* | *Structure* | *Targets* | *References* |
|---|---|---|---|
| Bispecific-siRNA |  | *CD4, CXCR4, CCR5* | Anderson et al., 2003 |
| hpRNA |  | *MMP-5*, cathepsin B, *UPAR* | Gondi et al., 2004; Lakka et al., 2004, 2005; Tummalapalli et al., 2007; Kunigal et al., 2007; Gondi et al., 2007; Kargiotis et al., 2008 |
| ddRNAi |  | *β-ACTIN*, hRluc | Rice et al., 2005 |
| mhRNA[a] |  | Hepatitis C virus | Akashi et al., 2005 |
| e-shRNAs[a] |  | HIV-1 *gag, pol, net* gene | Liu et al., 2007 |
| lhRNA |  | HIV-1 *tat, rev* gene | Sano et al., 2008 |
| DGT multi-siRNA[b] |  | *VEGF, SURVIVIN, BCL2* | Lee et al., 2011 |
| Branched RNA[c] |  | *TNF-α* | Aviñó et al., 2011 |
| T-tiRNAs |  | *SURVIVIN, STAT3* | Chang et al., 2012a |
| Branched RNA |  | Firefly luciferase gene | Nakashima et al., 2011 |
| tiRNA |  | *LAMIN A/C, DBP, TIG3* | Chang et al., 2012b |
| tsiRNA |  | EGFP, renilla luciferase | Shin et al., 2009 |
| dsiRNA |  | *TIG3, LAMIN* | Chang et al., 2009 |
| DGT siRNA multi-siRNAs |  | *NET-1, VEGF; SURVIVIN, BCL2* | Peng et al., 2013; Wu et al., 2013 |

[a] △, point mutations in sense (mhRNA) or antisense strand (e-shRNAs).
[b] ●, DTME, a cleavable crosslinker.
[c] ○, dT or RNA, DNA nucleosides.
DBP, D site of albumin promoter (albumin D-box) binding protein; ddRNAi, DNA-directed RNAi; DGT siRNA, dual gene targeting siRNA; DNA, deoxyribonucleic acid; dsiRNA, dual-target siRNA; dT, deoxythymidine; DTME, dithiobismaleimidoethane; EGFP, enhanced green fluorescent protein; e-shRNAs, extended short hairpin RNAs; hpRNA, hairpin RNA; lhRNA, long hairpin siRNA; mhRNA, modified hairpin RNA; multi-siRNA, multimeric siRNA; RNA, ribonucleic acid; tiRNA, tripartite-interfering RNA; tsiRNA, tandem siRNA; T-tiRNAs, tripodal interfering RNAs.

two effective siRNAs could be achieved, which led to better inhibition of virus reproduction. A similar form of long hairpin siRNAs (lhRNAs), termed promoter-expressed long hairpin RNAs, was evaluated (Sano et al., 2008). Long hairpin RNA could be processed into multiple siRNAs that are deemed as effective agents for treatment of rapidly mutating viruses such as HIV. Human U6 promoter-driven lhRNAs of 50, 53, and 80 bp targeting contiguous sequences within the *tat* and *rev* genes of HIV-1 have been generated and the results showed that such lhRNAs could be stably and functionally expressed for a long time (48 days) in HIV-1 susceptible T cells, where they provided potent inhibition of HIV replication against both nonmutant and mutant variants of HIV-1.

With rapid advancement in chemical synthesis and modification methods, chemically synthetized siRNAs other than

vector-based siRNAs have been widely used for investigations of RNAi-based therapeutics. In addition, synthetic multi-target siRNAs could also be conjugated with other moieties by linkages. In one case, Lee et al. (2011) reported that use of conjugates formed from dual gene targeting multimeric siRNA (DGT multi-siRNA) provided more effective inhibition of the two corresponding target genes simultaneously than that from a cocktail of mixed multimerized siRNA conjugates. The DGT multi-siRNA conjugates, which were chemically cross-linked via cleavable disulfide linkages, exhibited significantly higher gene silencing efficiency with negligible stimulation of immune response against *VEGF* and green fluorescent protein (GFP) genes at expression levels of both mRNAs and proteins than a physical mixture of naked siRNAs. DGT multi-siRNAs against *SURVIVIN* and *BCL2* genes were shown to induce enhanced apoptotic effect in a similar fashion.

### *Branched siRNAs*

Branded siRNAs are another class of novel structural designs that could be obtained by using symmetric doubler phosphoramidites (Frieden et al., 2004) to form branches with two or four strands from RNAs. The branched siRNAs had similar inhibitory capacity to those of unmodified siRNA duplexes, as evidenced by tumor necrosis factor-α (*TNF-α*) gene silencing experiments (Aviñó et al., 2011). A variant of branched siRNAs, called tripodal interfering RNAs (T-tiRNAs) (Chang et al., 2012a), was constructed on a trebler-phosphoramidite core structure (Shchepinov et al., 1997) and extended with short DNA linker. Such T-tiRNAs could silence simultaneously up to three different mRNAs or miRNAs by harboring three siRNA or antagomir units. T-tiRNAs could be incorporated into RISC efficiently and trigger efficient RNAi in mammalian cells and achieve greater gene silencing and delivery efficiency than conventional siRNAs. There were also other branched RNAs with three- or four-way junctions (Nakashima et al., 2011) designed by assembling single-stranded RNA for RNA interference. These unique RNAs could be cleaved by Dicer as well into about 20-bp conventional siRNA species that lead to a potent silencing effect. Thus, a branched, nonlinear tripartite-interfering RNA (tiRNA) structure was designed by Chang et al. (2012b), and the study showed that the tiRNA could induce better silencing potency on multiple target genes than the corresponding classical siRNAs. Surprisingly, the gene silencing observed on the use of tiRNA did not require Dicer-mediated processing into smaller RNA units and was thought to be triggered by the 38-nt-long guide strands through the RNAi machinery in mammalian cells.

### *Linear multi-target siRNAs*

In contrast to the above hairpin and branched structures of siRNAs, linear multi-target siRNAs have also been designed and evaluated for silencing of various gene targets. Tandem siRNA (tsiRNA) (Shin et al., 2009), which was composed by a duplex RNA harboring two siRNA units by using an expression system with convergent H1 and U6 polymerase 3 promoters, was used to target HCV (Lavender et al., 2012; Suhy et al., 2012). TsiRNAs were optimized to be processed by the intracellular ribonuclease Dicer into functional siRNAs targeting different sequences in a given gene or different genes. *In vitro*, inhibition of HCV protein could be achieved by tsiRNAs as efficiently as a single 19-bp duplex and 2-base 3′-overhangs siRNA without affecting miRNA maturation or induction of an *IFN* response. Chang et al. (2009) showed that synthetic dsiRNAs with a length as long as 38 bp could induce specific silencing of two target genes simultaneously without causing nonspecific antiviral *IFN* responses. Long double-stranded dual-target or multiplex siRNAs (DGT-siRNAs or multi-siRNAs) (Wu et al., 2013; Peng et al., 2013) were reported for gene silencing through a sequence-specific RNAi process without inducing significant immune responses. A gap feature structurally designed in either of the nucleotide strands of the multi-siRNAs was essential toward silencing target genes and avoiding immune responses.

## Conclusion and Perspectives

Here, we have summarized the current states of RNAi therapeutics and development of siRNA structural modifications, in particular multi-target siRNAs. By using these structurally modified siRNAs, gene silencing efficiency has been improved with low or even no induction of innate immune responses. Some of them can even enhance delivery efficiency compared with canonical siRNA. With rapid growth of RNAi therapy applications, many RNAi-based drug candidates have moved into clinical trials in recent years. Although significant progress has been made toward delivering siRNAs for therapeutic applications as evidenced by the clinical trials, much more effort is still needed to ensure safe and efficient means of delivery of siRNAs *in vivo*. siRNA agents have been successfully delivered into many organs in clinical trial studies, such as eyeball, skin, liver, and lung which may be easier targets than solid tumors in internal organs.

With better understanding of disease mechanisms, novel multi-target drugs have been developed against multiple sites within the pathological process. The RNAi-based therapeutics has also been investigated for multi-target therapies by many researchers. A number of applications using multi-target siRNAs for antiviral and antitumor therapeutics have achieved substantial efficacy and safety outcomes. Some of them have moved into the clinic trials (Table 1). Multi-target-based siRNA therapeutics opens up new therapeutic possibilities and may help to improve treatment effectiveness. Additionally, single- or multi-target siRNA could be used as a pharmaceutical ingredient by combining with other biomedical and/or chemical agents such as monoclonal antibody drugs and/or chemotherapy agents for diseases treatments.

## Author Disclosure Statement

No competing financial interests exist.

## Acknowledgments

This work was supported by Foundation of the Production–Study Research Prospective Joint Research Programs of Jiangsu Province, China (2013042-06), the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), the Science Foundation of Nantong City, Jiangsu Province, China (HS2012070), the Postgraduation Advanced Project Program of Nantong University (2012-29 and 22292679), the National Science and

Technology Major Project from the National Natural Science Foundation of China (2011ZX09401-012), and the National High Technology Research and Development Program of China (863 Program) (2012AA022501).

## References

ABE, N., ABE, H., and ITO, Y. (2007). Dumbbell-shaped nanocircular RNAs for RNA interference. J. Am. Chem. Soc. **129,** 15108–15109.

AIGNER, A. (2007). Applications of RNA interference: current state and prospects for siRNA-based strategies *in vivo*. Appl. Microbiol. Biotechnol. **76,** 9–21.

AKASHI, H., MIYAGISHI, M., YOKOTA, T., WATANABE, T., HINO, T., NISHINA, K., KOHARA, M., and TAIRA, K. (2005). Escape from the interferon response associated with RNA interference using vectors that encode long modified hairpin-RNA. Mol. Biosyst. **1,** 382–390.

AMARZGUIOUI, M., LUNDBERG, P., CANTIN, E., HAGSTROM, J., BEHLKE, M.A., and ROSSI, J.J. (2006). Rational design and *in vitro* and *in vivo* delivery of Dicer substrate siRNA. Nat. Protoc. **1,** 508–517.

ANDERSON, J., BANERJEA, A., and AKKINA, R. (2003). Bispecific short hairpin siRNA constructs targeted to CD4, CXCR4, and CCR5 confer HIV-1 resistance. Oligonucleotides **13,** 303–312.

AVIÑÓ, A., OCAMPO, S.M., PERALES, J.C., and ERITJA, R. (2011). Branched RNA: a new architecture for RNA interference. J. Nucleic Acids **2011,** 586935.

BARAKAT, M.R., and KAISER, P.K. (2009). VEGF inhibitors for the treatment of neovascular age-related macular degeneration. Expert Opin. Investig. Drugs **18,** 637–646.

BITKO, V., MUSIYENKO, A., SHULYAYEVA, O., and BARIK, S. (2005). Inhibition of respiratory viruses by nasally administered siRNA. Nat. Med. **11,** 50–55.

BOLOGNESI, M.L., CAVALLI, A., and MELCHIORRE, C. (2009). Memoquin: a multi-target-directed ligand as an innovative therapeutic opportunity for Alzheimer's disease. Neurotherapeutics **6,** 152–162.

BRAMSEN, J.B., LAURSEN, M.B., DAMGAARD, C.K., LENA, S.W., BABU, B.R., WENGEL, J., and KJEMS, J. (2007). Improved silencing properties using small internally segmented interfering RNAs. Nucleic Acids Res. **35,** 5886–5897.

BURNETT, J.C., and ROSSI, J.J. (2012). RNA-based therapeutics: current progress and future prospects. Chem. Biol. **19,** 60–71.

BURNETT, J.C., ROSSI, J.J., and TIEMANN, K. (2011). Current progress of siRNA/shRNA therapeutics in clinical trials. Biotechnol. J. **6,** 1130–1146.

CHANG, C.I., KANG, H.S., BAN, C., KIM, S., and LEE, D.K. (2009). Dual-target gene silencing by using long, synthetic siRNA duplexes without triggering antiviral responses. Mol. Cells **27,** 689–695.

CHANG, C.I., LEE, T.Y., KIM, S., SUN, X., HONG, S.W., YOO, J.W., DUA, P., KANG, H.S., KIM, S., LI, C.J., and LEE, D.K. (2012a). Enhanced intracellular delivery and multi-target gene silencing triggered by tripodal RNA structures. J. Gene Med. **14,** 138–146.

CHANG, C.I., LEE, T.Y., YOO, J.W., SHIN, D., KIM, M., KIM, S., and LEE, D.K. (2012b). Branched, tripartite-interfering RNAs silence multiple target genes with long guide strands. Nucleic Acid Ther. **22,** 30–39.

CHENDRIMADA, T., GREGORY, R., KUMARASWAMY, E., NORMAN, J., COOCH, N., NISHIKURA, K., and SHIEKHATTAR, R. (2005). TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. Nature **436,** 740–744.

CHU., C.Y., and RANA, T.M. (2008). Potent RNAi by short RNA triggers. RNA **14,** 1714–1719.

COLLINGWOOD, M.A., ROSE, S.D., HUANG, L., HILLIER, C., AMARZGUIOUI, M., WIIGER, M.T., SOIFER, H.S., ROSSI, J.J., and BEHLKE, M.A. (2008). Chemical modification patterns compatible with high potency Dicer-substrate small interfering RNAs. Oligonucleotides **18,** 187–200.

CZAUDERNA, F., FECHTNER, M., DAMES, S., AYGÜN, H., KLIPPEL, A., PRONK, G.J., GIESE, K., and KAUFMANN, J. (2003). Structural variations and stabilising modifications of synthetic siRNAs in mammalian cells. Nucleic Acids Res. **31,** 2705–2716.

DAVIDSON, B.L., and MCCRAY, P.B. (2011). Current prospects for RNA interference-based therapies. Nat. Rev. Genet. **12,** 329–340.

DAVIS, M.E., ZUCKERMAN, J.E., CHOI, C.H., SELIGSON, D., TOLCHER, A., ALABI, C.A., YEN, Y., HEIDEL, J.D., and RIBAS, A. (2010). Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. Nature **464,** 1067–1070.

DENISE, H., MOSCHOS, S.A., SIDDERS, B., BURDEN, F., PERKINS, H., CARTER, N., STROUD, T., KENNEDY, M., FANCY, S.A., LAPTHORN, C., et al. (2014). Deep sequencing insights in therapeutic shRNA processing and siRNA target cleavage precision. Mol. Ther. Nucleic Acids. **3,** e145.

DEVINCENZO, J., CEHELSKY, J.E., ALVAREZ, R., ELBASHIR, S., HARBORTH, J., TOUD JARSKA, I., NECHEV, L., MURUGAIAH, V., VAN VLIET, A., VAISHNAW, A.K., and MEYERS, R. (2008). Evaluation of the safety, tolerability and pharmacokinetics of ALN-RSV01, a novel RNAi antiviral therapeutic directed against respiratory syncytial virus (RSV). Antiviral Res. **77,** 225–231.

DORÉ-SAVARD, L., ROUSSY, G., DANSEREAU, M.A., COLLINGWOOD, M.A., LENNOX, K.A., ROSE, S.D., BEAUDET, N., BEHLKE, M.A., and SARRET, P. (2008). Central delivery of Dicer-substrate siRNA: a direct application for pain research. Mol. Ther. **16,** 1331–1339.

DU, Y.F., LIANG, L., SHI, Y., LONG, Q.Z., ZENG, J., WANG, X.Y., and HE, D.L. (2012). Multi-target siRNA based on DNMT3A/B homologous conserved region influences cell cycle and apoptosis of human prostate cancer cell line TSU-PR1. Genet. Mol. Biol. **35,** 164–171.

DUA, P., YOO, J.W., KIM, S., and LEE, D.K. (2011). Modified siRNA structure with a single nucleotide bulge overcomes conventional siRNA-mediated off-target silencing. Mol. Ther. **19,** 1676–1687.

EFFERTH, T., and KOCH, E. (2011). Complex interactions between phytochemicals. The multi-target therapeutic concept of phytotherapy. Curr. Drug Targets **12,** 122–132.

ELBASHIR, S.M., LENDECKEL, W., and TUSCHL, T. (2001B). RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev. **15,** 188–200.

ELBASHIR, S.M., HARBORTH, J., LENDECKEL, W., YALCIN, A., WEBER, K., and TUSCHL, T. (2001A). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature **411,** 494–498.

FIRE, A., XU, S., MONTGOMERY, M.K., KOSTAS, S.A., DRIVER, S.E., and MELLO, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature **391,** 806–811.

FLIGHT, M.H. (2013). Neurodegenerative diseases: New kinase targets for Alzheimer's disease. Nat. Rev. Drug Discov. **12,** 739.

FOSTER, D.J., BARROS, S., DUNCAN, R., SHAIKH, S., CANTLEY, W., DELL, A., BULGAKOVA, E., O'SHEA, J., TANEJA, N., KUCHIMANCHI, S., et al. (2012). Comprehensive evaluation of canonical versus Dicer-substrate siRNA *in vitro* and *in vivo*. RNA **18,** 557–568.

FRIEDEN, M., AVIÑÓ, A., TARRASÓN, G., ESCORIHUELA, M., PIULATS, J., and ERITJA, R. (2004). Synthesis of oligonucleotide-peptide conjugates carrying the c-myc peptide epitope as recognition system. Chem. Biodivers. **1,** 930–938.

GE. Q., ILVES, H., DALLAS, A., KUMAR, P., SHORENSTEIN, J., KAZAKOV, S.A., and JOHNSTON, B.H. (2010). Minimal-length short hairpin RNAs: the relationship of structure and RNAi activity. RNA **16,** 106–117.

GHOSH, P., DULLEA, R., FISCHER, J.E., TURI, T.G., SARVER, R.W., ZHANG, C., BASU, K., DAS, S.K., and POLAND, B.W. (2009). Comparing 2-nt 3′ overhangs against blunt-ended siRNAs: a system biology based study. BMC Genomics **10,** S17.

GONDI, C.S., LAKKA, S.S., DINH, D.H., OLIVERO, W.C., GUJRATI, M., and RAO, J.S. (2004). RNAi-mediated inhibition of cathepsin B and uPAR leads to decreased cell invasion, angiogenesis and tumor growth in gliomas. Oncogene **23,** 8486–8496.

GONDI, C.S., LAKKA, S.S., DINH, D.H., OLIVERO, W.C., GUJRATI, M., and RAO, J.S. (2007). Intraperitoneal injection of an hpRNA-expressing plasmid targeting uPAR and uPA retards angiogenesis and inhibits intracranial tumor growth in nude mice. Clin. Cancer Res. **13,** 4051–4060.

GREDELL, J.A., DITTMER, M.J., WU, M., CHAN, C., and WALTON, S.P. (2010). Recognition of siRNA asymmetry by TARRNA binding protein. Biochemistry **49,** 3148–3155.

GU, L., CHEN, H., TUO, J., GAO, X., and CHEN, L. (2010). Inhibition of experimental choroidal neovascularization in mice by anti-VEGFA/VEGFR2 or non-specific siRNA. Exp. Eye Res. **91,** 433–439.

HALL, A.H., WAN, J., SPESOCK, A., SERGUEEVA, Z., SHAW, B.R., and ALEXANDER, K.A. (2006). High potency silencing by single-stranded boranophosphate siRNA. Nucleic Acids Res. **34,** 2773–2781.

HAMMOND, S.M., BERNSTEIN, E., BEACH, D., and HANNON, G.J. (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature **404,** 293–296.

HANNON, G.J., and CLEARY, M.A. (2005). Synthetic shRNAs as potent RNAi triggers. Nat. Biotechnol. **23,** 227–231.

HEFNER, E., CLARK, K., WHITMAN, C., BEHLKE, M.A., ROSE, S.D., PEEK, A.S., and RUBIO, T. (2008). Increased potency and longevity of gene silencing using validated Dicer substrates. J. Biomol. Tech. **19,** 231–237.

HEIDEL, J.D., YU, Z., LIU, J.Y., RELE, S.M., LIANG, Y., ZEIDAN, R.K., KORNBRUST, D.J., and DAVIS, M.E. (2007). Administration in non-human primates of escalating intravenous doses of targeted nanoparticles containing ribonucleotide reductase subunit M2 siRNA. Proc. Natl. Acad. Sci. U. S. A. **104,** 5715–5721.

HOGREFE, R.I., LEBEDEV, A.V., ZON, G., PIROLLO, K.F., RAIT, A., ZHOU, Q., YU, W., and CHANG, E.H. (2006). Chemically modified short interfering hybrids (siHYBRIDS): nanoimmunoliposome delivery *in vitro* and *in vivo* for RNAi of HER-2. Nucleic Acids **25,** 889–907.

HOHJOH, H. (2004). Enhancement of RNAi activity by improved siRNA duplexes. FEBS Lett. **557,** 193–198.

HOLEN, T., AMARZGUIOUI, M., BABAIE, E., and PRYDZ, H. (2003). Similar behaviour of single-strand and double-strand siRNAs suggests they act through a common RNAi pathway. Nucleic Acids Res. **31,** 2401–2407.

HOLEN, T., AMARZGUIOUI, M., WIIGER, M.T., BABAIE, E., and PRYDZ, H. (2002). Positional effects of short interfering RNAs targeting the human coagulation trigger Tissue Factor. Nucleic Acids Res. **30,** 1757–1766.

HOSSBACH, M., GRUBER, J., OSBORN, M., WEBER, K., and TUSCHL, T. (2006). Gene silencing with siRNA duplexes composed of target-mRNA-complementary and partially palindromic or partially complementary single-stranded siRNAs. RNA Biol. **3,** 82–89.

HUTVAGNER, G., and SIMARD, M.J. (2008). Argonaute proteins: key players in RNA silencing. Nat. Rev. Mol. Cell Biol. **9,** 22–32.

JI, J., WERNLI, M., KLIMKAIT, T., and ERB, P. (2003). Enhanced gene silencing by the application of multiple specific small interfering RNAs. FEBS Lett. **552,** 247–252.

JUDGE, A.D., ROBBINS, M., TAVAKOLI, I., LEVI, J., HU, L., FRONDA, A., AMBEGIA, E., MCCLINTOCK, K., and MACLACHLAN, I. (2009). Confirming the RNAi-mediated mechanism of action of siRNA-based cancer therapeutics in mice. J. Clin. Invest. **119,** 661–673.

KAISER, P.K., SYMONS, R.C., SHAH, S.M., QUINLAN, E.J., TABANDEH, H., DO, D.V., et al. (2010). RNAi-based treatment for neovascular age-related macular degeneration by Sirna-027. Am. J. Ophthalmol. **150,** 33–39.

KARGIOTIS, O., CHETTY, C., GOGINENI, V., GONDI, C.S., PULUKURI, S.M., KYRITSIS, A.P., GUJRATI, M., KLOPFENSTEIN, J.D., DINH, D.H., and RAO, J.S. (2008). uPA/uPAR downregulation inhibits radiation-induced migration, invasion and angiogenesis in IOMM-Lee meningioma cells and decreases tumor growth *in vivo*. Int. J. Oncol. **33,** 937–947.

KIM, D.H., BEHLKE, M.A., ROSE, S.D., CHANG, M.S., CHOI, S., and ROSSI, J.J. (2005). Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy. Nat. Biotechnol. **23,** 222–226.

KIM, S.S., CHO, H.J., CHO, J.M., KANG, J.Y., YANG, H.W., and YOO, T.K. (2013). Dual silencing of Hsp27 and c-FLIP enhances doxazosin-induced apoptosis in PC-3 prostate cancer cells. Scientific World Journal **2013,** 174392.

KINI, H.K., and WALTON, S.P. (2009). Effect of siRNA terminal mismatches on TRBP and Dicer binding and silencing efficacy. FEBS J. **276,** 6576–6585.

KOK K.H., NG M.H., CHING Y.P., and JIN D.Y. (2007). Human TRBP and PACT directly interact with each other and associate with Dicer to facilitate them production of small interfering RNA. J. Biol. Chem. **282,** 17649–17657.

KROKEN, R.A., LØBERG, E.M., DRØNEN, T., GRÜNER, R., HUGDAHL, K., KOMPUS, K., SKREDE, S., JOHNSEN, E. (2014). A critical review of pro-cognitive drug targets in psychosis: convergence on myelination and inflammation. Front Psychiatry **5,** 11.

KUNIGAL, S., LAKKA, S.S., GONDI, C.S., ESTES, N., and RAO, J.S. (2007). RNAi-mediatged downregulation of urokinase plasminogen activator receptor and matrix metalloprotease-9 in human breast cancer cells results in decreased

tumor invasion, angiogenesis and growth. Int. J. Cancer **121,** 2307–2316.

KUNZE, D., WUTTIG, D., FUESSEL, S., KRAEMER, K., KOTZSCH, M., MEYE, A., et al. (2008). Multi-target siRNA inhibition of antiapoptotic genes (XIAP, BCL2, BCL-X(L)) in bladder cancer cells. Anticancer Res. **28,** 2259–2263.

LAHOUTE, C., HERBIN, O., MALLAT, Z., and TEDGUI, A. (2011). Adaptive immunity in atherosclerosis: mechanisms and future therapeutic targets. Nat. Rev. Cardiol. **8,** 348–358.

LAKKA, S.S., GONDI, C.S., DINH, D.H., OLIVERO, W.C., GUJRATI, M., RAO, V.H., et al. (2005). Specific interference of uPAR and MMP-9 gene expression induced by double-stranded RNA results in decreased invasion, tumor growth and angiogenesis in gliomas. J. Biol. Chem. **280,** 21882–21892.

LAKKA, S.S., GONDI, C.S., YANAMANDRA, N., OLIVERO, W.C., DINH, D.H., GUJRATI, M., and RAO, J.S. (2004). Inhibition of cathepsin B and MMP-9 gene expression in glioblastoma cell line via RNA interference reduces tumor cell invasion, tumor growth and angiogenesis. Oncogene **23,** 4681–4689.

LAVENDER H., BRADY K., BURDEN F., DELPUECH-ADAMS O., DENISE H., PALMER A., PERKINS, H., SAVIC, B., SCOTT, S., SMITH-BURCHNELL, C., et al. (2012). *In vitro* characterization of the activity of PF-05095808 a novel biological agent for hepatitis C virus therapy. Antimicrob. Agents Chemother. **56,** 1364–1375.

LEACHMAN, S.A., HICKERSON, R.P., SCHWARTZ, M.E., BULLOUGH, E.E., HUTCHERSON, S.L., BOUCHER, K.M., HANSEN, C.D., ELIASON, M.J., SRIVATSA, G.S., and KORNBRUST, D.J. (2010). First-in-human mutation-targeted siRNA phase 1b trial of an inherited skin disorder. Mol. Ther. **18,** 442–446.

LEE, S.H., MOK, H., JO, S., HONG, C.A., and PARK, T.G. (2011). Dual gene targeted multimeric siRNA for combinatorial gene silencing. Biomaterials **32,** 2359–2368.

LIU, K., CHEN, H.L., YOU, Q.S., SHI, H., and WANG, Z.W. (2014). The siRNA cocktail targeting VEGF and HER2 inhibition on the proliferation and induced apoptosis of gastric cancer cell. Mol. Cell. Biochem. **386,** 117–24.

LIU, Q.H., RAND, T.A., KALIDAS, S., DU, F.H., KIM, H.E., SMITH, D.P., and WANG, X. (2003). R2d2, a bridge between the initiation and effector steps of the *Drosophila* RNAi pathway. Science **301,** 1921–1925.

LIU, X., JIANG, F., KALIDAS, S., SMITH, D., and LIU, Q. (2006). Dicer-2 and R2D2 coordinately bind siRNA to promote assembly of the siRISC complexes. RNA **12,** 1514–1520.

LIU, Y.P., HAASNOOT, J., and BERKHOUT, B. (2007). Design of extended short hairpin RNAs for HIV-1 inhibition. Nucleic Acids Res. **35,** 5683–5693.

LU, J.J., PAN, W., HU, Y.J., and WANG, Y.T. (2012). Multi-target drugs: the trend of drug research and development. PLoS One **7,** e40262.

MACRAE, I.J., MA, E., ZHOU, M., ROBINSON, C.V., and DOUDNA, J.A. (2008). *In vitro* reconstitution of the human RISC-loading complex. Proc. Natl. Acad. Sci. U. S. A. **105,** 512–517.

MAES, M., FIŠAR, Z., MEDINA, M., SCAPAGNINI, G., NOWAK, G., and BERK, M. (2012). New drug targets in depression: inflammatory, cell-mediated immune, oxidative and nitrosative stress, mitochondrial, antioxidant, and neuroprogressive pathways. And new drug candidates–Nrf2 activators and GSK-3 inhibitors. Inflammopharmacology **20,** 127–150.

MARTINEZ, J., PATKANIOWSKA, A., URLAUB, H., LÜHRMANN, R., and TUSCHL, T. (2002). Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. Cell **110,** 563–574.

MCCULLOUGH, C., PHARMACEUTICALS, A., PHARMACEUTICALS, A., and DAVIDSON, B. (2005). A crucial test. Nat. Med. **11,** 243–244.

MCFARLAND, T.J., ZHANG, Y., APPUKUTTAN, B., and STOUT, J.T. (2004). Gene therapy for proliferative ocular diseases. Expert Opin. Biol. Ther. **4,** 1053–1058.

MENENDEZ, J.A., VELLON, L., MEHMI, I., OZA, B.P., ROPERO, S., COLOMER, R., and LUPU, R. (2004). Inhibition of fatty acid synthase (FAS) suppresses HER2/neu (erbB-2) oncogene overexpression in cancer cells. Proc. Natl. Acad. Sci. U. S. A. **101,** 10715–10720.

NAKASHIMA, Y., ABE, H., ABE, N., AIKAWA, K., and ITO, Y. (2011). Branched RNA nanostructures for RNA interference. Chem. Commun. (Camb.) **47,** 8367–8369.

NOLAND, C.L., MA, E., and DOUDNA, J.A. (2011). siRNA repositioning for guide strand selection by human dicer complexes. Mol. Cell **43,** 110–121.

PEER, D., and LIEBERMAN, J. (2011). Special delivery: targeted therapy with small RNAs. Gene Ther. **18,** 1127–1133.

PENG, W., CHEN, J., QIN, Y., YANG, Z., and ZHU, Y.Y. (2013). Long double-stranded multiplex siRNAs for dual genes silencing. Nucleic Acid Ther. **23,** 281–288.

PETROVA KRUGLOVA, N.S., MESCHANINOVA, M.I., VENYAMINOVA, A.G., ZENKOVA, M.A., VLASSOV, V.V., and CHERNOLOVSKAYA, E.L. (2010). 2′-O-methyl-modified anti-MDR1 fork-siRNA duplexes exhibiting high nuclease resistance and prolonged silencing activity. Oligonucleotides **20,** 297–308.

PHAM, J.W., PELLINO, J.L., LEE, Y.S., CARTHEW, R.W., and SONTHEIMER, E.J. (2004). A Dicer-2-dependent 80s complex cleaves targeted mRNAs during RNAi in Drosophila. Cell **117,** 83–94.

PRAKASH, T.P., ALLERSON, C.R., DANDE, P., VICKERS, T.A., SIOUFI, N., JARRES, R., BAKER, B.F., SWAYZE, E.E., GRIFFEY, R.H., and BHAT, B. (2005). Positional effect of chemical modifications on short interference RNA activity in mammalian cells. J. Med. Chem. **48,** 4247–4253.

RAOUL, C., BARKER, S.D., and AEBISCHER, P. (2006). Viral-based modelling and correction of neurodegenerative diseases by RNA interference. Gene Ther. **13,** 487–495.

RICE, R.R., MUIRHEAD, A.N., HARRISON, B.T., KASSIANOS, A.J., SEDLAK, P.L., MAUGERI, N.J., GOSS, P.J., DAVEY, J.R., JAMES, D.E., and GRAHAM, M.W. (2005). Simple, robust strategies for generating DNA-directed RNA interference constructs. Methods Enzymol. **392,** 405–419.

ROSE, S.D., KIM, D.H., AMARZGUIOUI, M., HEIDEL, J.D., COLLINGWOOD, M.A., DAVIS, M.E., ROSSI, J.J., and BEHLKE, M.A. (2005). Functional polarity is introduced by dicer processing of short substrate RNAs. Nucleic Acids Res. **33,** 4140–4156.

SAKURAI, K., AMARZGUIOUI, M., KIM, D., ALLUIN, J., HEALE, B., SONG, M., GATIGNOL, A., BEHLKE, M.A., and ROSSI, J.J. (2011). A role for human Dicer in pre-RISC loading of siRNAs. Nucleic Acids Res. **39,** 1510–1525.

SANO, M., LI, H., NAKANISHI, M., and ROSSI, J.J. (2008). Expression of long anti-HIV-1 hairpin RNAs for the generation of multiple siRNAs: advantages and limitations. Mol. Ther. **16,** 170–177.

SCHRATTENHOLZ, A., GROEBE, K., and SOSKIC, V. (2010). Systems biology approaches and tools for analysis of interactomes and multi-target drugs. Methods Mol. Biol. **662,** 29–58.

SHAHZAD, M.M., LU, C., LEE, J.W., STONE, R.L., MITRA, R., MANGALA, L.S., LU, Y., BAGGERLY, K.A., DANES, C.G., NICK, A.M., et al. (2009). Daul targeting of EphA2 and FAK in ovarian carcinoma. Cancer Biol. Ther. **8,** 1027–1034.

SHARP, P.A. (2001). RNA interference—2001. Genes Dev. **15,** 485–490.

SHCHEPINOV, M.S., UDALOVA, I.A., BRIDGMAN, A.J., and SOUTHERN, E.M. (1997). Oligonucleotide dendrimers: synthesis and use as polylabelled DNA probes. Nucleic Acids Res. **25,** 4447–4454.

SHIN, D., LEE, H., KIM, S.I., YOON, Y., and KIM, M. (2009). Optimization of linear double-stranded RNA for the production of multiple siRNAs targeting hepatitis C virus. RNA **15,** 898–910.

SIOLAS, D., LERNER, C., BURCHARD, J., GE, W., LINSLEY, P.S., PADDISON, P.J., HANNON, G.J., and CLEARY, M.A. (2005). Synthetic shRNAs as potent RNAi triggers. Nat. Biotechnol. **23,** 227–231.

SONG, E., ZHU, P., LEE, S.K., CHOWDHURY, D., KUSSMAN, S., DYKXHOORN, D.M., FENG, Y., PALLISER, D., WEINER, D.B., SHANKAR, P., et al. (2005). Antibody mediated *in vivo* delivery of small interfering RNA via cell surface receptors. Nature Biotechnol. **23,** 709–717.

SONG, J.J., SMITH, S.K., HANNON, G.J., and JOSHUA-TOR, L. (2004). Crystal structure of Argonaute and its implications for RISC slicer activity. Science **305,** 1434–1437.

SUHY D.A., KAO S.C., MAO T., WHITELEY L., DENISE H., SOUBERBIELLE B., BURDICK, A.D., HAYES, K., WRIGHT, J.F., LAVENDER, H., et al. (2012). Safe, long-term hepatic expression of anti-HCV shRNA in a nonhuman primate model. Mol. Ther. **20,** 1737–1749.

SUN, X., ROGOFF, H.A., and LI, C.J. (2008). Asymmetric RNA duplexes mediate RNA interference in mammalian cells. Nat. Biotechnol. **26,** 1379–1382.

TABERNERO, J., SHAPIRO, G.I., LORUSSO, P.M., CERVANTES, A., SCHWARTZ, G.K., WEISS, G.J., PAZARES, L., CHO, D.C., INFANTE, J.R., ALSINA, M., et al. (2013). First-in-humans trial of an RNA interference therapeutic targeting VEGF and KSP in cancer patients with liver involvement. Cancer Discov. **3,** 406–417.

TAI, W., QIN, B., and CHENG, K. (2010). Inhibition of breast cancer cell growth and invasiveness by dual silencing of HER-2 and VEGF. Mol. Pharm. **7,** 543–556.

TAKESHITA, F., and OCHIYA, T. (2006). Therapeutic potential of RNA interference against cancer. Cancer Sci. **97,** 689–696.

TAKESHITA, F., MINAKUCHI, Y., NAGAHARA, S., HONMA, K., SASAKI, H., HIRAI, K., TERATANI, T., NAMATAME, N., YAMAMOTO, Y., HANAI, K. et al. (2005). Efficient delivery of small interfering RNA to bone-metastatic tumors by using atelocollagen *in vivo*. Proc. Natl. Acad. Sci. U. S. A. **102,** 12177–12182.

TANUDJI, M., MACHALEK, D., ARNDT, G.M., and RIVORY, L. (2009). Competition between siRNA duplexes: impact of RISC loading efficiency and comparison between conventional-21 bp and Dicer-substrate siRNAs. Oligonucleotides **20,** 27–32.

TOMARI, Y., DU, T., HALEY, B., SCHWARZ, D., BENNETT, R., COOK, H., KOPPETSCH, B.S., THEURKAUF, W.E., and ZAMORE, P.D. (2004A). RISC assembly defects in the Drosophila RNAi mutant armitage. Cell **116,** 831–841.

TOMARI, Y., MATRANGA, C., HALEY, B., MARTINEZ, N., and ZAMORE, P.D. (2004B). A protein sensor for siRNA asymmetry. Science **306,** 1377–1380.

TUMMALAPALLI, P., GONDI, C.S., DINH, D.H., GUJRATI, M., and RAO, J.S. (2007). RNA interferencemediated targeting of urokinase plasminogen activator receptor and matrix metalloproteinase-9 gene expression in the IOMM-Lee malignant meningioma cell line inhibits tumor growth, tumor cell invasion and angiogenesis. Int. J. Oncol. **31,** 5–17.

VICENTINI, F.T., BORGHETI-CARDOSO, L.N., DEPIERI, L.V., DE MACEDO MANO, D., ABELHA, T.F., PETRILLI, R., and BENTLEY, M.V. (2013). Delivery systems and local administration routes for therapeutic siRNA. Pharm. Res. **30,** 915–931.

WU, Y.Y., CHEN, L., WANG, G.L., ZHANG, Y.X., ZHOU, J.M., HE, S., QIN, J., and ZHU, Y.Y. (2013). Inhibition of hepatocellular carcinoma growth and angiogenesis by dual silencing of NET-1 and VEGF. J. Mol. Histol. **44,** 433–445.

ZHANG, H., KOLB, F.A., BRONDANI, V., BILLY, E., and FILIPOWICZ, W. (2002). Human Dicer preferentially cleaves dsRNAs at their termini without a requirement for ATP. Embo. J. **21,** 5875–5885.

ZHAO, M., HE, H.W., SUN, H.X., REN, K.H., and SHAO, R.G. (2009). Dual knockdown of N-ras and epiregulin synergistically suppressed the growth of human hepatoma cells. Biochem. Biophys. Res. Commun. **387,** 239–244.

ZIMMERMANN, G.R., LEHÁR, J., and KEITH, C.T. (2007). Multi-target therapeutics: when the whole is greater than the sum of the parts. Drug Discov. Today **12,** 34–42.

ZIMMERMANN, T.S., LEE, A.C., AKINC, A., BRAMLAGE, B., BUMCROT, D., FEDORUK, M.N., HARBORTH, J., HEYES, J.A., JEFFS, L.B., JOHN, M., et al. (2006). RNAi-mediated gene silencing in non-human primates. Nature **441,** 111–114.

Address correspondence to:
*Li Chen*
*Department of Pathological Anatomy*
*Nantong University*
*19 Qixiu Road*
*Nantong, 226001*
*China*

*E-mail:* bl1@ntu.edu.cn

Received for publication January 23, 2014; accepted after revision April 4, 2014.