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Review

Development of new RNAi therapeutics

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Summary. RNAi-mediated gene inactivation has become a cornerstone of the present day gene function studies that are the foundation of mechanism and target based drug discovery and development, which could potentially shorten the otherwise long process of drug development. In particular, the coming of age of "RNAi drug" could provide new promising therapeutics bypassing traditional approaches. However, there are technological hurdles need to overcome and the biological limitations need to consider for achieving effective therapeutics. Major hurdles include the intrinsic poor pharmacokinetic property of siRNA and major biological restrictions include off-target effects, interferon response and the interference with endogenous miRNA. Recent innovations in nucleic acid chemistry, formulations and delivery methods have gradually rendered it possible to develop effective RNAi-based therapeutics. Careful design based on the newest RNAi/miRNA biology can also help to minimize the potential tissue toxicity. If successful with systemic application, RNAi drug will no doubt revolutionize the whole drug development process. This review attempts to describe the progress in this area, including applications in preclinical models and recent favorable experience in a number of human trials of local diseases, along with the discussion on the potential limitations of RNAi therapeutics.

Key words: RNAi therapeutics, Delivery, RNA modification, AMD

Introduction

Since confirmed the gene silencing effect of RNAi in mammalian cells just a few years ago, RNA interference (RNAi) has become the tool of choice for gene function study. A variety of technologies based on RNAi have demonstrated essential utilities in today's drug target identification and validation. Due to the very fact that RNAi mimicking antagonistic effect of drugs, siRNA itself can also be directly exploited as therapeutics for human diseases bypassing the usually costly, complex and long process of drug discovery, after the target is identified and validated. Therefore, RNAi based therapeutics provides a promising new perspective for treating human diseases.

RNA interference or RNAi gene silencing is mediated by siRNA (short interference RNA of 19 base pair with two 3' nucleotide overhangs at both ends) through the degradation of their homologous mRNAs (Filipowicz, 2005; Zhou et al., 2006), an effect evolutionarily conserved throughout eukaryotes. With the rapid advancement in RNAi biology, now we understand that the exogenously introduced RNAi in mammalian cells is achieved by taking advantage of an evolutionarily conserved biochemical pathway of microRNA (miRNA). In mammalian cells, the endogenous miRNA gene is transcribed to yield pri-miRNA which is then processed into pre-miRNA by Drosha in nucleus. Pre-mRNA is then exported to cytoplasm via Exportin 5, where pre-miRNA matures into miRNA, a 19 base pair double stranded RNA with two 3' nucleotide overhangs at both ends, by the cytoplasmic process mediated by Dicer (Fig. 1). miRNA will incorporate into the RISC complex (RNA induced silencing complex) and thus regulate gene expression via translation inhibition or degradation of the complementary RNA. miRNA plays important physiological roles and has been linked to early development and cancer. A variety of current RNAi strategies are in fact the exogenously introduced miRNA intermediates that are processed into siRNA using the machineries of miRNA pathway such as Exportin 5, Dicer and RISC (Fig. 1).

Effective gene silencing of siRNA depends on its sequences and delivering methods. The homology to the target, the internal stability at 5' end of the antisense-strand (lower stability is favored for higher potency) (Khvorova et al., 2003), as well as the position-specific sequences may contribute to the optimal activity (Ui-Tei et al., 2004). Public domains as well as commercial entities (e.g. Dharmacon, Ambion, Qiagene and Invitrogen) provide designing tools using algorithms based on the above factors. siRNA can be delivered into

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cells in two forms: chemically synthesized siRNA and RNAi expression cassettes (or RNAi gene, usually expressing short hairpin RNA, or shRNA) via vectors (Fig. 1). Synthesized siRNA/shRNA provides simple but transient (<1 week) gene inactivation. RNAi genes can potentially provide stable gene silencing. Due to its unparalleled robustness and simplicity in gene silencing, RNAi has become the most popular tool for drug target discovery and validation in vitro and increasingly also *in vivo* (Liu et al., 2006).

Pharmacological potentials of RNAi

Built on the rapid accumulated knowledge of RNAi, scientists in both academia and industry are actively pursuing pharmacological applications of RNAi for human diseases. As compared to traditional small molecules and antibodies, RNAi therapeutics have the following advantages: 1) time from target to drug is significantly shortened since the drugs can readily be rationally designed according to target sequences; 2) synthetic routes are basically the same for any RNAi drug; 3) RNAi therapy can target any genes including the "non-druggable genes", as well as non-protein targets; 4) high specificity for "disease gene target" can be achieved as RNAi-mediated allele-specific gene inactivation has been demonstrated; 5) off-target effects can be avoided by careful site selection; 6) any RNAi drug with the same chemistry/formulation should have similar pharmacokinetics. Indeed, because of all these advantages, a number of biotech companies are actively working in this area to make RNAi-based drugs a reality. Some of the current siRNA drugs in clinical trials are summarized in Table 1.

Issues regarding RNAi therapeutics

Despite all the enthusiasm and some promising progress, there are still major obstacles that need to be overcome for a wide application of pharmacologically active RNAi drugs. RNAi drugs, like all the other nucleic acid based drugs, exhibit the same poor pharmacokinetic properties. Additionally, RNAi genes as drugs will also have the same delivery issues that plague gene therapy.

Delivery and cellular uptake of siRNA

The plasma stability of siRNA molecules, although significantly higher than that of single stranded RNA molecules, remains too low as a practical systemic drug. This is mainly due to the nuclease mediated degradation in plasma where nucleases are abundant. Cellular uptake is another obstacle needs to be addressed since nucleic acids do not pass through cell membrane and enter cells easily without transfection agents, many of which usually are toxic for *in vivo* use. However, progress in nucleic acid chemistry and formulation are recently being made to improve the pharmacokinetics of siRNA drugs by enhancing stability and cellular uptake:
1) Chemical modifications of siRNA molecules increase siRNA resistance to nucleases (de Fougerolles et al., 2005). Several different backbone modifications of siRNA, e.g. boranophosphate (Hall et al., 2004) and 2'-

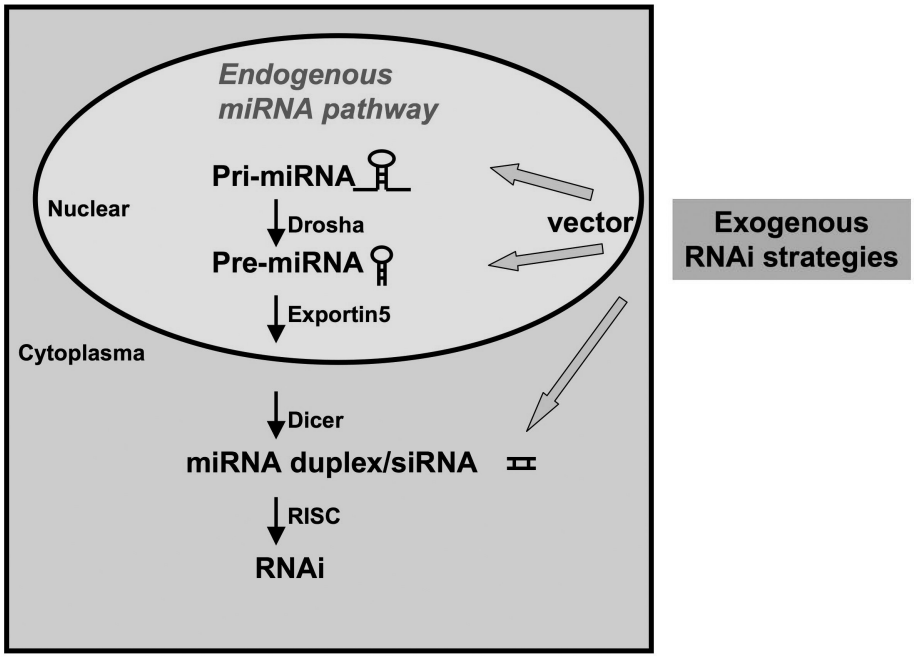


Fig. 1. Gene silencing mediated by exogenous RNAi and endogenous miRNA pathway. miRNA biogenesis requires multiple cellular factors, which are shared by the exogenous RNAi strategies, either RNAi gene (mimicking pri-miRNA or pre-miRNA) or siRNA. High levels of exogenous RNAi can potentially compete with endogenous miRNA for the limited miRNA pathway, thus interfere with miRNA-mediated normal cellular and tissue physiology *in vivo* and cause cell or tissue toxicity.

fluoro modification (Layzer et al., 2004), have been described, which showed increased stability of siRNA molecules, and even potency in some cases.

2) New formulations are also being developed to increase plasma stability. For example, noncovalent complexation of synthetic siRNAs with low molecular weight polyethylenimine (PEI) efficiently stabilizes siRNAs. *In vivo* systemic delivery of the PEI-complexed siRNAs targeting HER2 reduced tumor growth (Urban-Klein et al., 2005).

3) New formulations are also being developed to facilitate cellular uptake of siRNA molecules. Conjugation with cholesterol derivatives to generate lipophilic siRNA greatly enhanced the cellular uptake in liver cells *in vitro* (Lorenz et al., 2004). There are also reports showing that peptide conjugation improved siRNA cellular uptake (Juliano, 2005).

4) Recently, an antibody-conjugated siRNA technique was described that demonstrated cell type specific systemic delivery (Song et al., 2005). First, Song et al. constructed a conjugate composed of an antibody (Fab) against a pre-selected cell surface receptor (e.g., HIV envelop protein, or tumor specific receptor) and a basic cellular protein protamine that readily interacts with net negatively charged nucleic acids. Second, this conjugate was non-covalently linked to siRNA by simple mixing. After incubating with cells, the conjugate was found only up-taken by the cells that express the specific receptor. This system was further tested *in vivo*. HIV envelope glycoprotein expressing melanoma cells were injected into mice to form tumor. Then siRNAs targeting c-Myc, MDM2 and VEGF were mixed with the protamine-Fab (antibody against human immunodeficiency virus (HIV) envelop protein) conjugate, and the siRNA/protamine-Fab was injected intravenously or intratumorally into the mice. Response to siRNA drug was observed for the HIV-env expressing tumors as compared to the control tumor without HIV-env expression. Therefore, this study provides a simple method to achieve cell type specific delivery of siRNA *in vivo*.

5) Routs of administration should be carefully considered to reach desired bio-distribution and satisfactory potency of siRNA drugs. Essentially the routes can be broadly divided into two catogories: local and systemic administrations. Successful local delivery has been described in several animal models, including intraocular injection for the wet form of age related macular degeneration (wet AMD), intratumoral injection for cancer, intranasal delivery for treatment of asthma, intracerebellar injection for treatment of neuropathic pain (Dorn et al., 2004), and local electroporation for treatment of collagen-induced arthritis (Schiffelers et al., 2005). As compared to local delivery, systemic delivery of siRNA encountered obstacles of poor blood stability and biodistribution. Intravenous delivery *via* hydrodynamic injection of siRNA has been successfully shown to inhibit hepatitis B virus (HBV) infection in liver in animals (Giladi et al., 2003). However, the risk of this delivery method cannot be accepted for human therapy at present and therefore still needs further investigation (Toumi et al., 2006). In addition, stable delivery of RNAi via viral vector into liver also demonstrated severe toxicity due to interference of miRNA pathway and liver tissue regeneration (see below). Caution should therefore be advised for systemic application of siRNA drugs. Nevertheless, there have been several reports describing effective gene-silencing via intravenous injection of siRNA/shRNA into mice (Soutschek et al., 2004; Morrissey et al., 2005; Song et al., 2005).

A more recent report described a successful intravenous injection of lipid-encapsulated siRNA targeting apoB into cynomolgus monkeys, which caused decreased serum cholesterol levels with a single injection (Zimmermann et al., 2006). Zimmermann et al. used a liposomal formulation to stabilize siRNA that targets apoB, a protein involved in lipid metabolism. They delivered the siRNA by intravenous administration into cynomolgus monkeys at doses of 1 or 2.5 mg/kg. The treated animals were evaluated for pharmacokinetics, efficacy and safety of this drug. The result showed that the siRNA specifically silenced liver ApoB expression in a fast on-set rate (48 hours after treatment, apoB mRNA was reduced by ~68% and 90% for doses of 1 mg/kg and 2.5 mg/kg respectively).

Table 1. RNAi based therapeutics.

Indication	Company	RNAi platform (target)	Clinical stage
Wet AMD	Acuity	Modified siRNA (VEGFR)	Phase II
	Sirna	Modified siRNA (VEGF)	Phase I/II
	Alnylam	siRNA	Preclinical/Phase I
Infectious disease	Alnylam	siRNA for RSV (viral gene)	Phase I
	Benitec	multiple RNAi for HIV (viral gene)	Preclinical
	Phytovation	siRNA for HIV/HCV	Preclinical
	Combimatrix	siRNA for HCV/HIV	Preclinical
	Nucleonicsinc	siRNA for HCV/HBV	Preclinical
Inflammation	Sirna	siRNA for asthma (IL-4R)	Preclinical
Cancer	Intradigm	Nanoparticle siRNA for solid tumor (VEGF)	Preclinical

Moreover, this potent silencing effect had been maintained for 11 days after a single injection. Consistent with the mRNA silencing effect, the siRNA drug also decreased plasma LDL level dramatically (up to 82% reduction) and rapidly (as early as 24 hour post injection), which had also lasted for 11 days. This study, for the first time, demonstrated that single-dosage systemic delivery of siRNA to non-human primates can achieve a rapid and lasting silencing effect, which provides insight into clinical RNAi drugs *via* systemic delivery.

Safety issues of siRNA drugs

The high specificity of siRNA renders the acceptance of siRNA as a potential safe and specific therapeutic agent. However, there are still certain safety concerns. First, double stranded RNAs have been known to cause interferon response causing non-specific inhibition of transcription and translation, and thus cell apoptosis. There have been reports that the lipid vehicles, as well as certain specific siRNA sequences, enhance interferon response in animals. Optimal designs of siRNA have been demonstrated to minimize the induction of interferon response (Judge et al., 2005). Second, for viral vector based siRNA gene delivery, there are potential virus-associated immunogenicity problems. Although retrovirus mediated RNAi for liver delivery has been reported, the safety of such vector in long-term treatment is always a concern because it integrates into the host cell chromosome (Judge et al., 2005; Ma et al., 2005). Third, siRNA causing off-target effect is now a widely accepted phenomenon, and this may also cause certain safety concern (Birmingham et al., 2006). Cautions are advised during the testing of candidate RNAi strategies. Fourth, since siRNA-mediated RNAi shares the components used for the endogenous miRNA-induced gene-inactivation, whether the introduction of the exogenous siRNA at potentially high levels would interfere with normal cellular physiology *in vivo* has also become a concern (Fig. 1). Both design and testing, particularly *in vivo*, may be particularly important for developing potential treatment by RNAi gene-mediated stable silencing without inducing cell/tissue toxicity. A recent study tested the long-term effects of high-level shRNA expression in livers of adult mice (Grimm et al., 2006). Grimm et al. evaluated 49 distinct shRNA in adeno-associated virus (AAV) vector, targeting 6 different genes. After intravenous infusion, 36 mice exhibited dose-dependent liver injury, with 23 ultimately dead. Moreover, they also associated the morbidity with the down-regulation of liver miRNAs, likely causing the failure of liver tissue regeneration. The study also suggested that the likely bottleneck where high level exogenous shRNA competes with endogenous miRNA effect is exportin 5. These observations suggest that the high cellular shRNA expression may compete with the endogenous miRNA for the limited cellular factors, and therefore interferes with the physiological function of miRNA and thus causes severe toxicity. Measures must be taken to minimize the associated risks for RNAi therapeutic strategy, including careful selection of the adequate dose, the design of certain si/shRNA sequence, intended targets, etc.

RNAi therapeutics in different disease indications

RNAi-based therapy is being tested in various disease indications (Table 1). Great progress in RNAi therapeutic research has been made recently in several disease areas. Because of the above mentioned issues, RNAi therapeutics have so far mainly focused on the localized diseases in human, such as the ones in the targetable organs and tissues, *via* local delivery. These organs include eye, brain, upper-respiratory tract, liver, etc (Fig. 2).

Exudative form of age-related macular degeneration (wet AMD)

Age-related macular degeneration (dry AMD) affects 13 million of the aging population leading to gradual loss of their central vision. Nearly 10% of them further develop into the exudative form of age-related macular disease (the wet form of AMD), characterized by excessive blood vessel growth in the choroids beneath macular resulting in breaks through Bruch's membrane (choroidal neovascularization, CNV). Leakage of the vessels results in accumulation of fluid and retinal tears causing central vision disruption, and the eventual scarring formation causing permanent damage in retina. Wet AMD is the leading cause of blindness in the industrial countries. The VEGF (Vascular endothelial growth factor) pathway has been shown to play a central role of the angiogenesis as well as the vessel permeability. It was also found to be directly involved in wet AMD in eyes of aging population, where increased levels have closely correlated to the lesions. Recently, anti-VEGF antibodies (e.g. Avastin (Bevacizumab), Lucentis (ranibizumab)) have been demonstrated to effectively inhibit CNV and improve patient vision *via* intravitreal injection. VEGF and its receptors VEGF-R1 are well validated target molecules for wet AMD.

Local delivery of siRNA obviates many pharmacokinetic challenges associated with systemic delivery. Thus, it is not surprising that the most successful application of RNAi is the treatment of wet AMD via intraocular injection thus far. Sirna-027 is a modified siRNA developed by Sirna Therapeutics specifically targeting VEGF-R1 of human, mouse, rat, monkey and pig (sequence see Fig. 3). The chemically-modified siRNA was found to enhance stability, improve intraocular PK ($T_{1/2}$~16 hours) as well as specificity. Subretinal delivery of Sirna-027 has shown inhibition of CNV (66%) in several rodent models, which correlated with the VEGF-R1 decrease at both mRNA (~57%) and

protein levels. No toxicity was observed locally and negligible systemic exposure after intraocular injection. Sirna-027 is currently in phase I clinical trial and is the first human experience of a chemically-modified siRNA. The preliminary data suggest that it is safe and well tolerated, and it lead to visual acuity stabilization and/or improvement (Guerchiolini, 2006).

In separate studies, siRNAs targeting VEGF also significantly inhibited laser-induced CNV in a number of animal models, e.g. mouse and non-human primates (Reich et al., 2003). Several intravitreal injections of siRNA against VEGF for the treatment of wet AMD are also currently in clinical trials (Acuity Pharmaceuticals (modified siRNA), Alnylam Pharmaceuticals (unmodified siRNA)). Recently, Acuity Pharmaceuticals reported positive initial phase II results, similar to that of the antibody therapeutics against VEGF. and expected to enter phase III trial next year.

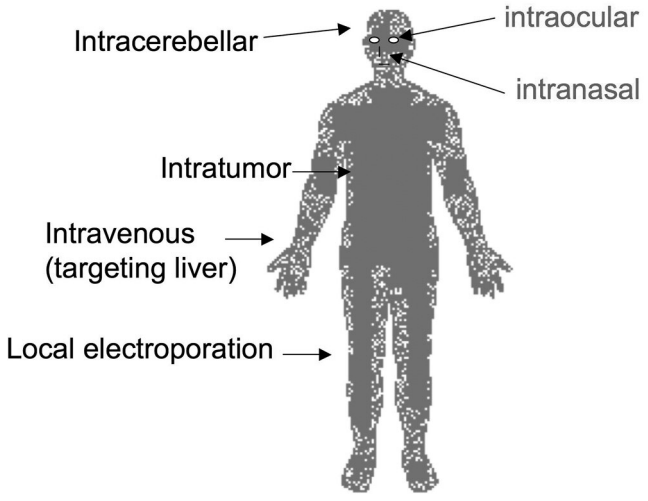
Infectious diseases

The application of RNAi technology has been intensively studied in the infectious diseases areas since RNAi-regulated virus RNA transcription was found to decrease virus replication. In this area, the most successful application of siRNA deals with respiratory syncytial virus (RSV) infection. RSV infects nearly every child at least once by the age of two years and accounts for more than 100,000 hospitalizations per year in the U.S. RSV infects both the upper and lower respiratory tracts, and leads to cold like symptoms first, which may develop into croup, pneumonia and bronchiolitis. Currently, no effective vaccine or antiviral drugs are available to treat RSV (Maggon and Barik, 2004), therefore novel therapeutics are in significant need.

Two recent studies using siRNA against RSV viral genes (Bitko et al., 2005; Zhang et al., 2005) significantly inhibited RSV infection when the siRNA was intranasally delivered into mice. Similarly, Alnylam Pharmaceuticals Inc. developed a siRNA (ALN-RSV01) that selectively and potently silences a RSV gene that is essential for RSV replication. Preclinical data showed that intranasally delivered ALN-RSV01 specifically inhibits RSV replication in animals and is active in the prevention and treatment of RSV infection. Alnylam has initiated a Phase I study in U.S. and Europe to evaluate the human safety of the siRNA.

Previous studies demonstrated that siRNA against HBV inhibited viral gene expression in co-expressing cells. However, this did not prove that RNAi would be effective in an estabolished HBV animal model. Liver is considered to be readily targeted by systemic delivery by either vectors or siRNA molecules, as compared to other organs or tissues. A recent study reported that intravenous injection of adenoviral vector encoding siRNA against HBV into mice with estabolished chronic HBV infection inhibited viral gene expression and replication to almost undetectable levels for at least 26 days. Although liver is a relatively easy organ to target, these results still provide proof-of-principle for silencing HBV by RNAi strategy (Uprichard et al., 2005).

Progress has also been made on inhibition of HIV infection using RNAi. Multiple studies have demonstrated decreased viral replication using siRNAs that target viral genes (Boden et al., 2004). However, there are several additional challenges, one being that HIV can mutate to escape RNAi, another being the finding that HIV-1 evades elicited RNAi through the newly recognized function of its Tat protein (the HIV-1-encoded activator of the viral LTR). Tat has been shown to interfere with Dicer activity (Bennasser et al., 2005). To address the first concern, one can use DNA-directed RNAi (ddRNAi) that can encode multiple shRNAs to target multiple gene sequences of the virus. The broad targeting of viral genes may maximally avoid virus resistance, and a cocktail of siRNAs can be made in a single formulation. Currently, several companies are testing this approach in preclinical studies (Benitec, Nucleonics, and Sirna Therapeutics). In order to overcome the second hurdle, one could use siRNA of the exact length of 21mer, instead of shRNA, since both the longer siRNA and hairpin RNA will require Dicer activity. One can also target host genes that facilitate virus entry (Qin et al., 2003; Anderson and Akkina, 2005). The *in vivo* feasibility and efficacy of these strategies remain to be tested.



Fig, 2. Delivery routes of RNAi drugs. Delivery routs that are currently in clinical trials are in red color. Delivery routs that are still in preclinical trials (animal studies) are in black color.

5′–BCUGAGUUUAAAAGGCACCCTTB–3′
3′–T꜀TGACUCAAAUUUUCCGUGGG–5′

Fig. 3. The sequence of Sirna-027.

Oncology

Another active area for RNAi based therapy is Oncology. One advantage of RNAi for cancer therapy is that it can potentially silence a disease allele specifically without affecting the wild-type allele, therefore achieving tumor-specific therapy. For example, many human cancers have point mutations in the p53 tumor suppressor and/or Kras oncogene. siRNAs have been shown to distinguish between the mutant and wild-type p53 and Kras in cells expressing both forms, and were tested for suppressing the mutant forms but not the wild-type forms (Martinez et al., 2002; Ke et al., 2004). Multiple targets that are involved in the disease development have been tested for anti-tumor activity by siRNA in animal models. Genes regulating cell proliferation and apoptosis, such as raf-1 (Pal et al., 2005) and bcl-2 (Yano et al., 2004), were silenced by synthetic siRNA, which led to reduced tumor growth. RNAi-mediated silencing of VEGF (Takei et al., 2004) or CXCR4 (Liang et al., 2005) were shown to prevent tumor growth or metastasis. Also, siRNA targeting multi drug transporter (MDR1) has been used *in vitr*o to decrease the drug resistance of cells. So far siRNA application for cancer treatment has been limited to the preclinical stage.

Conclusions

RNAi technology has become an essential tool in today's drug discovery and development. It makes drug development significantly simplified, accelerated and optimized. RNAi library based combinatorial gene inactivation identifies the effective gene targets of specific human diseases, which can be validated by RNAi-based animal models. While the verified gene or gene product can be targeted by antagonists e.g. small molecules, the potent siRNA/shRNA validated during these process can also potentially be explored as therapeutics for these diseases. This coupling of target identification and drug discovery bypasses lengthy and costly process involved in traditional drug discovery and development. RNAi-based drugs, rendering targetable the traditionally considered non-druggable genes, have started to demonstrate therapeutic effectiveness in a number of localized human diseases. Although still in its infancy, once the pharmacokinetic properties are significantly improved, RNAi technology will no doubt reshape the future of medicine.

References

Anderson J. and Akkina R. (2005). HIV-1 resistance conferred by siRNA cosuppression of CXCR4 and CCR5 coreceptors by a bispecific lentiviral vector. AIDS Res. Ther. 2, 1.

Bennasser Y., Le S.Y., Benkirane M. and Jeang K.T. (2005). Evidence that HIV-1 encodes an siRNA and a suppressor of RNA silencing. Immunity 22, 607-619.

Birmingham A., Anderson E.M., Reynolds A., Ilsley-Tyree D., Leake D., Fedorov Y., Baskerville S., Maksimova E., Robinson K., Karpilow J., Marshall W.S. and Khvorova A. (2006). 3' UTR seed matches, but not overall identity, are associated with RNAi off-targets. Nat. Methods 3, 199-204.

Bitko V., Musiyenko A., Shulyayeva O. and Barik S. (2005). Inhibition of respiratory viruses by nasally administered siRNA. Nat. Med. 11, 50-55.

Boden D., Pusch O. and Ramratnam B. (2004). HIV-1-specific RNA interference. Curr. Opin. Mol. Ther. 6, 373-380.

de Fougerolles A., Manoharan M., Meyers R. and Vornlocher H.P. (2005). RNA interference in vivo: toward synthetic small inhibitory RNA-based therapeutics. Methods Enzymol. 392, 278-296.

Dorn G., Patel S., Wotherspoon G., Hemmings-Mieszczak M., Barclay J., Natt F.J., Martin P., Bevan S., Fox A., Ganju P., Wishart W. and Hall J. (2004). siRNA relieves chronic neuropathic pain. Nucleic Acids Res. 32, e49.

Filipowicz W. (2005). RNAi: the nuts and bolts of the RISC machine. Cell 122, 17-20.

Giladi H., Ketzinel-Gilad M., Rivkin L., Felig Y., Nussbaum O. and Galun E. (2003). Small interfering RNA inhibits hepatitis B virus replication in mice. Mol. Ther. 8, 769-776.

Grimm D., Streetz K.L., Jopling C.L., Storm T.A., Pandey K., Davis C.R., Marion P., Salazar F. and Kay M.A. (2006). Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. Nature 441, 537-541.

Guerchiolini (2006). Small Interfering RNA against VEGFR-1 as treatment for age-related macular degeneration. IBC 3rd International Conference on Ocular Anti-angiogenesis.

Hall A.H., Wan J., Shaughnessy E.E., Ramsay Shaw B. and Alexander K.A. (2004). RNA interference using boranophosphate siRNAs: structure-activity relationships. Nucleic Acids Res. 32, 5991-6000.

Judge A.D., Sood V., Shaw J.R., Fang D., McClintock K. and MacLachlan I. (2005). Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. Nat. Biotechnol. 23, 457-462.

Juliano R.L. (2005). Peptide-oligonucleotide conjugates for the delivery of antisense and siRNA. Curr. Opin. Mol. Ther. 7, 132-136.

Ke N., Albers A., Claassen G., Yu D.H., Chatterton J.E., Hu X., Meyhack B., Wong-Staal F. and Li Q.X. (2004). One-week 96-well soft agar growth assay for cancer target validation. Biotechniques 36, 826-828, 830, 832-823.

Khvorova A., Reynolds A. and Jayasena S.D. (2003). Functional siRNAs and miRNAs exhibit strand bias. Cell 115, 209-216.

Layzer J.M., McCaffrey A.P., Tanner A.K., Huang Z., Kay M.A. and Sullenger B.A. (2004). In vivo activity of nuclease-resistant siRNAs. Rna 10, 766-771.

Liang Z., Yoon Y., Votaw J., Goodman M.M., Williams L. and Shim H. (2005). Silencing of CXCR4 blocks breast cancer metastasis. Cancer Res. 65, 967-971.

Liu G., Wong-Staal F., Li Q.-X. (2006). Recent development of RNAi in drug target discovery and validation. Drug Discov. Today (In Press).

Lorenz C., Hadwiger P., John M., Vornlocher H.P. and Unverzagt C. (2004). Steroid and lipid conjugates of siRNAs to enhance cellular uptake and gene silencing in liver cells. Bioorg. Med. Chem. Lett. 14, 4975-4977.

Ma Z., Li J., He F., Wilson A., Pitt B. and Li S. (2005). Cationic lipids enhance siRNA-mediated interferon response in mice. Biochem. Biophys. Res. Commun. 330, 755-759.

Maggon K. and Barik S. (2004). New drugs and treatment for respiratory

syncytial virus. Rev. Med. Virol. 14, 149-168.

Martinez L.A., Naguibneva I., Lehrmann H., Vervisch A., Tchenio T., Lozano G. and Harel-Bellan A. (2002). Synthetic small inhibiting RNAs: efficient tools to inactivate oncogenic mutations and restore p53 pathways. Proc. Natl. Acad. Sci. USA 99, 14849-14854.

Morrissey D.V., Lockridge J. A., Shaw L., Blanchard K., Jensen K., Breen W., Hartsough K., Machemer L., Radka S., Jadhav V., Vaish N., Zinnen S., Vargeese C., Bowman K., Shaffer C.S., Jeffs L.B., Judge A., MacLachlan I. and Polisky B. (2005). Potent and persistent in vivo anti-HBV activity of chemically modified siRNAs. Nat. Biotechnol. 23, 1002-1007.

Pal A., Ahmad A., Khan S., Sakabe I., Zhang C., Kasid U.N. and Ahmad I. (2005). Systemic delivery of RafsiRNA using cationic cardiolipin liposomes silences Raf-1 expression and inhibits tumor growth in xenograft model of human prostate cancer. Int. J. Oncol. 26, 1087-1091.

Qin X.F., An D.S., Chen I.S. and Baltimore D. (2003). Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. Proc. Natl. Acad. Sci. USA 100, 183-188.

Reich S. J., Fosnot J., Kuroki A., Tang W., Yang X., Maguire A.M., Bennett J. and Tolentino M.J. (2003). Small interfering RNA (siRNA) targeting VEGF effectively inhibits ocular neovascularization in a mouse model. Mol. Vis. 9, 210-216.

Schiffelers R.M., Xu J., Storm G., Woodle M.C. and Scaria P.V. (2005). Effects of treatment with small interfering RNA on joint inflammation in mice with collagen-induced arthritis. Arthritis Rheum. 52, 1314-1318.

Song E., Zhu P., Lee S.K., Chowdhury D., Kussman S., Dykxhoorn D.M., Feng Y., Palliser D., Weiner D.B., Shankar P., Marasco W.A. and Lieberman J. (2005). Antibody mediated in vivo delivery of small interfering RNAs via cell-surface receptors. Nat. Biotechnol. 23, 709-717.

Soutschek J., Akinc A., Bramlage B., Charisse K., Constien R., Donoghue M., Elbashir S., Geick A., Hadwiger P., Harborth J., John M., Kesavan V., Lavine G., Pandey R.K., Racie T., Rajeev K.G., Rohl I., Toudjarska I., Wang G., Wuschko S., Bumcrot D., Koteliansky V., Limmer S., Manoharan M. and Vornlocher H.P. (2004). Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. Nature 432, 173-178.

Takei Y., Kadomatsu K., Yuzawa Y., Matsuo S. and Muramatsu T. (2004). A small interfering RNA targeting vascular endothelial growth factor as cancer therapeutics. Cancer Res. 64, 3365-3370.

Toumi H., Hegge J., Subbotin V., Noble M., Herweijer H., Best T.M. and Hagstrom J.E. (2006). Rapid intravascular injection into limb skeletal muscle: a damage assessment study. Mol. Ther. 13, 229-236.

Ui-Tei K., Naito Y., Takahashi F., Haraguchi T., Ohki-Hamazaki H., Juni A., Ueda R. and Saigo K. (2004). Guidelines for the selection of highly effective siRNA sequences for mammalian and chick RNA interference. Nucleic Acids Res. 32, 936-948.

Uprichard S.L., Boyd B., Althage A. and Chisari F.V. (2005). Clearance of hepatitis B virus from the liver of transgenic mice by short hairpin RNAs. Proc. Natl. Acad. Sci. USA 102, 773-778.

Urban-Klein B., Werth S., Abuharbeid S., Czubayko F. and Aigner A. (2005). RNAi-mediated gene-targeting through systemic application of polyethylenimine (PEI)-complexed siRNA in vivo. Gene Ther. 12, 461-466.

Yano J., Hirabayashi K., Nakagawa S., Yamaguchi T., Nogawa M., Kashimori I., Naito H., Kitagawa H., Ishiyama K., Ohgi T. and Irimura T. (2004). Antitumor activity of small interfering RNA/cationic liposome complex in mouse models of cancer. Clin. Cancer Res. 10, 7721-7726.

Zhang W., Yang H., Kong X., Mohapatra S., San Juan-Vergara H., Hellermann G., Behera S., Singam R., Lockey R.F. and Mohapatra S.S. (2005). Inhibition of respiratory syncytial virus infection with intranasal siRNA nanoparticles targeting the viral NS1 gene. Nat. Med. 11, 56-62.

Zhou D., He Q.S., Wang C., Zhang J. and Wong-Staal F. (2006). RNA interference and potential applications. Curr. Top. Med. Chem. 6, 901-911.

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., Judge A.D., Lam K., McClintock K., Nechev L.V., Palmer L.R., Racie T., Rohl I., Seiffert S., Shanmugam S., Sood V., Soutschek J., Toudjarska I., Wheat A.J., Yaworski E., Zedalis W., Koteliansky V., Manoharan M., Vornlocher H.P. and MacLachlan I. (2006). RNAi-mediated gene silencing in non-human primates. Nature 441, 111-114.

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