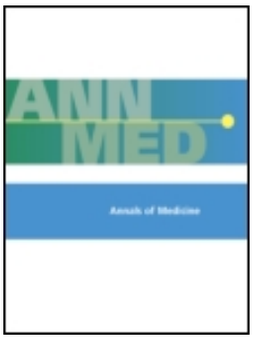

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Development of gene-specific double-stranded RNA drugs

Sailen Barik

A relatively recent entrant into molecular biology – double-stranded RNA (dsRNA) – as a class exhibits a unique set of properties: relative stability, affinity for specific proteins and enzymes, ability to activate the interferon pathway and finally, RNA interference (RNAi). In RNAi, unique double-stranded short interfering RNA molecules (siRNA) destroy the corresponding target RNA with exquisite potency and selectivity, thus causing post-transcriptional gene silencing (PTGS). An understanding of the design of gene-specific dsRNA and development of techniques to deliver dsRNA in the cell and in live animals has heralded a new age of gene therapy without gene knockout. This review first summarizes the biological synthesis, metabolism and effect of the dsRNA with special emphasis on siRNA and RNAi. This is followed by the clinical, pharmacological and pharmaceutical prospects of the development of the dsRNA as a drug. It is clear that the dsRNA holds an enormous promise in the treatment of a large number of metabolic and infectious diseases including but not limited to cancer, macular degeneration, diabetic retinopathy, Alzheimer's and other neural disorders, autoimmune diseases, and all viral infections including AIDS (acquired immune deficiency syndrome), hepatitis and respiratory syncytial virus (RSV).

Keywords: AIDS; antiviral; cancer therapy; double-stranded RNA; gene silencing; hepatitis; interferon; miRNA; RNA interference; siRNA

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Introduction: *dsRNA, siRNA, miRNA and RNAI*

The prospects of dsRNA as a drug cannot be appreciated without an understanding of the basic aspects of its biology. In principle, dsRNA can form whenever overlapping transcription occurs from the complementary strands of a given segment of DNA. A flurry of recent research has indeed led to the estimate that in a normal cell nearly 2,500 kinds of antisense RNA may play a variety of regulatory roles including genomic imprinting, translation regulation, alternative splicing, X-chromosome inactivation and RNA editing (1). The actual formation of a dsRNA between the sense and antisense transcripts, however, has not always been demonstrated. It is thus a fair assumption that the dsRNA will receive increasing attention in biology in the years ahead as more and more examples come to light.

Nonetheless, considerably large amounts of specific dsRNA can be generated in mammalian cells from exogenous origins, mainly through transfected of synthetic or recombinant molecules, and this can trigger RNAi (2–4). The mechanism of the RNAi pathway is illustrated in Figure 1, whereby a long dsRNA is chopped by Dicer, a member of the RNase III superfamily of bidentate endonucleases, to produce 21–24 nt long dsRNA fragments with 3′-overhangs, called siRNA. Chemically synthesized siRNA, directly introduced into the cell by transfection or electroporation, bypasses this step. The siRNA duplex contains 5′-phosphate and 3′-OH, of which the 5′-phosphate is crucial for function. In fact, synthetic siRNA with 5′-OH gets phosphorylated within a few minutes after introduction into human cells (5–7). The siRNA is then incorporated into a protein complex called RNA-induced silencing complex (RISC). This is followed by unwinding of the complex by a RISC associated RNA helicase activity to form the activated RISC, denoted as RISC* (with an asterisk). The unwinding is asymmetric, i.e., it preferentially initiates from the terminus with the lower melting temperature, i.e., the one that contain more A-U base pairs as opposed to G-C (8–10). The strand whose 5′-end is at this terminus becomes part of RISC*, and the other strand gets degraded. The

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RISC*-associated strand then acts as a guide RNA and recruits the RISC* to its complementary target RNA for the cleavage and degradation of the latter, resulting in knockdown or silencing of the corresponding gene function. The various steps of this pathway require energy, making RNAi a significant consumer of adenosine triphosphate (ATP).

More recently, a related class of single-stranded cellular RNA, named microRNA (miRNA), has been discovered that is also 21–24 nucleotide long and produced from unique endogenous genes (11–15). The miRNAs are produced through sequential processing by two enzymes of the RNaseIII superfamily (14, 16, 17) (Fig 1). A nuclear enzyme, named Drosha in human, cleaves the long primary transcript (pri-miRNA) into an approximately 70-nucleotide long pre-miRNA that is essentially a short hairpin RNA (shRNA). The pre-miRNA is further processed in the cytoplasm by Dicer to generate the final miRNA.

Accumulating evidence continues to blur the distinction between siRNA and miRNA (3, 9, 14). Thus, biogenesis of both requires some form of dsRNA and RNase-mediated processing reactions that are fundamentally similar. Depending on the degree to which they are complementary to their mRNA targets, both can direct cleavage or translational repression of the target mRNAs. In general, siRNA and miRNA that are a perfect or near-perfect match with their target degrade the target through RISC, whereas those with mismatches repress translation instead. Major examples of miRNA are *let-7* and *lin-4* of the nematode (*C. elegans*), mutations in which cause developmental defects (3, 14). Although the exact number of the small non-coding RNAs of the siRNA/miRNA family in a cell remains unknown, the current estimate for humans runs in the hundreds (3, 14, 15). It is quite likely that they regulate a variety of cellular pathways, including differentiation and development. Because of their extraordinary efficiency and target specificity (see later), the siRNA and miRNA have gained considerable attention as tools for gene knockdown, and hence, therapeutic use.

Key messages

- **The prospect of dsRNA as a gene therapy tool and drug owes its origin to the recently recognized phenomenon of RNA interference. In this mechanism, 21–25 nucleotide long dsRNA molecules associate with multi-subunit protein complexes and eventually degrade complementary target RNA, resulting in post-transcriptional gene silencing (PTGS).**
- **PTGS, mediated by dsRNA, is highly sequence-specific and thus, holds enormous promise in therapeutics. In general, it is useful wherever knockdown of specific cellular or foreign genes may lead to a cure, for example, in cancer and viral infections.**
- **The development of dsRNA as drug will benefit from further research concerning its specificity, delivery, stability, and potential to induce the interferon pathway.**

dsRNA, siRNA and the IFN pathway

In mammalian cells, dsRNAs that are roughly 35 base pairs or longer also tend to trigger at least two cellular stress response pathways, both of which lead to a general and non-specific abrogation of protein synthesis (6, 12). In emulating the IFN pathway, long dsRNA binds to and activates the dsRNA-activated protein kinase (PKR) that phosphorylates the translation initiation factor, eIF2α, leading to global translational shut-off and often, apoptosis. This mechanism, therefore, also serves to eliminate virus-infected cells. Activated PKR also regulates transcription factors such as nuclear factor-kappa B (NF-κB), interferon regulatory factor-1, NF-90, p53 and STAT (signal transducers and activators of transcription), and thus may regulate a plethora of cellular genes some of which are indeed pro-apoptotic. In the second pathway, dsRNA activates 2′- 5′ oligoadenylate synthetase which catalyzes the formation of 2′- 5′ oligoadenylate that bond to and activate RNase L, resulting in non-specific degradation of intracellular RNA. To sum up, long dsRNAs may adopt either or both pathways to cause a general repression of all protein synthesis, viral as well as cellular.

As the transcription of many viral genomes produces dsRNA, viruses have evolved a variety of mechanisms to defeat both of these pathways so that viral translation can continue unabated in the infected cell (18, 19). These include: masking of the dsRNA by viral dsRNA-binding proteins, direct blocking of the PKR active site (pseudosubstrate mechanism), degradation of PKR, disruption of PKR dimerization, and specific inhibition of eIF-2α phosphorylation.

In contrast to dsRNA, the siRNA and miRNA, because of their shorter length, do not activate the IFN or RNase L pathways, which is key to their target-specific abrogation of translation (4). A direct analysis of eIF-2α in fact revealed no elevated phosphorylation at 18 h after transfection of synthetic siRNA into cultured cells (20). In apparent contrast, some groups reported activation of PKR and other IFN-stimulated genes (ISGs) by siRNA or shRNA, although the effect subsided by about 90 min (21–23). Regardless, the silencing effect of the siRNA in these

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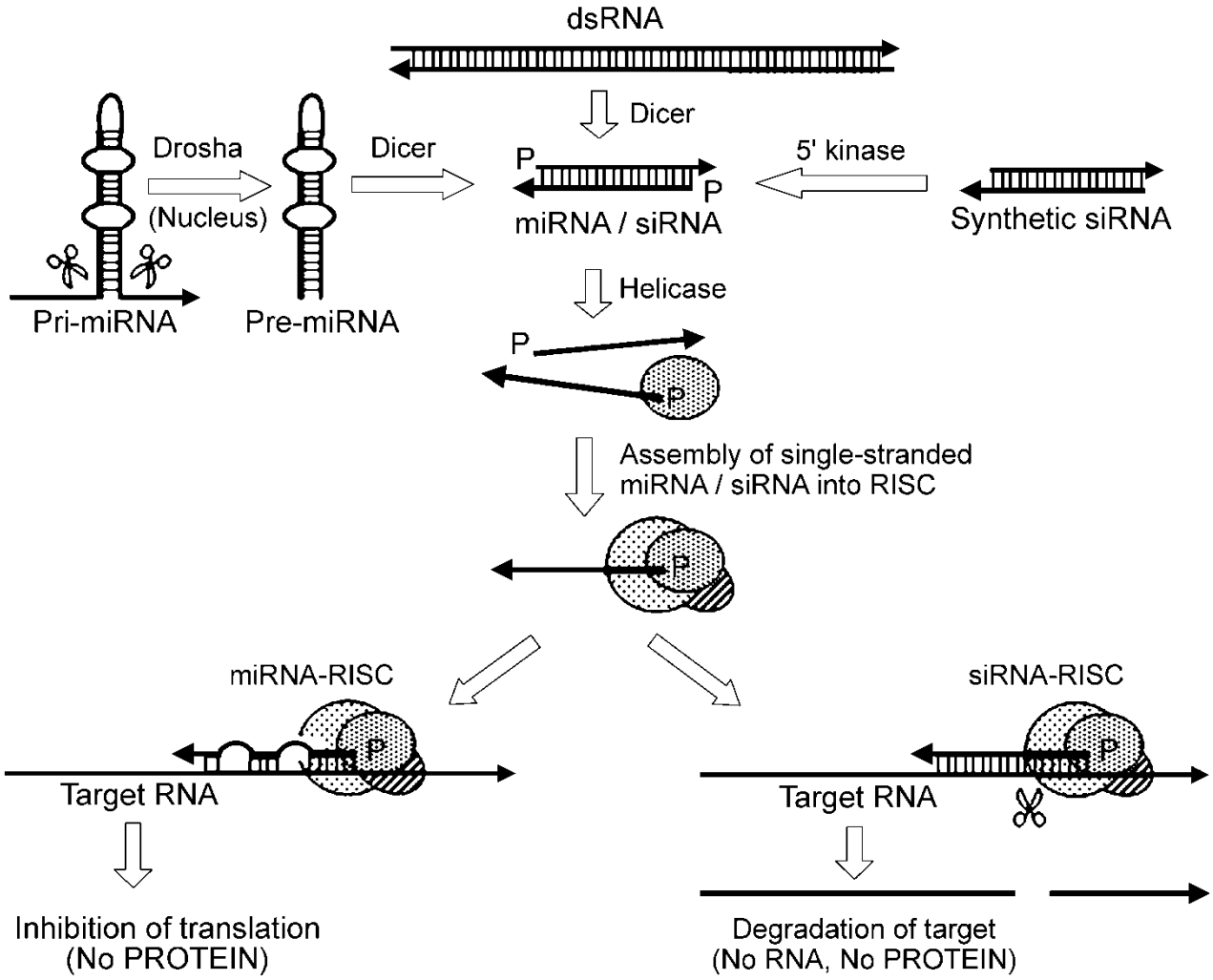

Figure 1. The pathways of gene silencing by a double-stranded RNA in human cell (see text for details) (2, 3, 5). For this review, any double-stranded RNA is considered under the term 'dsRNA' although the emphasis as drug is on siRNA/miRNA. All the steps described here occur in the cytoplasm, the only exception being the cleavage of pri-miRNA by Drosha, which occurs in the nucleus. 'P' represents 5′-phosphate, essential for the assembly of the siRNA into RISC. The arrowheads signify the 3′-ends of the RNA strands. During assembly, one strand (thin line) is always destroyed; the other strand (thick line) eventually engages in the RISC. The miRNA shares the same pathway as siRNA but generally differs in its structure and effect (see text); the mismatched regions of the miRNA are depicted as bulges. The scissors illustrate sites of endonucleolytic cleavage. The circles represent the various protein subunits of the RISC, and are not meant to accurately represent the number, size, or shape of the subunits.

studies was still target-specific and irrelevant mRNA remained unaffected. Part of the controversy was resolved when it was found that transcription from viral vectors generated RNA strands with 5′-triphosphates and that siRNA containing 5′-triphosphates were indeed robust activators of the IFN pathway (24). Removal of the triphosphates with phosphatase abolished the activation of IFN and restored target-specific silencing. In the few studies where siRNA did activate IFN the degree of activation was proportional to the amount of siRNA (22). The corollary is that for a given siRNA one should use the minimum amount needed for optimal RNAi effect and avoid excess. In any case, these studies draw attention to the potential of siRNA to trigger the IFN response, which should be taken into account especially when evaluating siRNA as specific drugs against viruses that are naturally sensitive to IFN.

The exact parameters that dictate the relative extent to which a siRNA will promote specific versus non-specific gene silencing remain to be understood. Nevertheless, one can speculate that once a long dsRNA enters a human cell, two cellular dsRNA-binding proteins vie for its attention: Dicer and PKR (18, 21). If Dicer wins, the dsRNA is led to the RNAi pathway eventually causing degradation of specific mRNAs; on the other hand, if PKR wins, it leads to the activation of the IFN pathway, causing a general repression of all mRNA translation. A shorter dsRNA or a siRNA, in contrast, is less likely to induce the IFN pathway and if properly designed, can act as a specific drug for gene silencing. Clearly, an

Table 1. dsRNA against animal viruses.

Virus	Target gene/ sequence	Fold inhibition
DNA virus		
Hepatitis B	Core, HBsAg, Pol, X	5–20 (25–30)
Herpes	ORF 45, Rta	100 (31)
Papilloma	E7, E6	Apoptosis, effect on cell growth (32)
RNA virus (Retrovirus)		
HIV-1	Gag	4–10 (33–35)
	Pol (RT)	10 (34)
	Rev, Tat	10–10,000 (36–39)
	Env	100 (40)
	Nef, Vif	10 (41)
	HIV receptors: CXCR4, CCR5	2–7 (39, 42–45)
RNA virus (Non-retrovirus)		
Dengue	capsid, E, NS1, NS5, PrM	10–100 (46–48)
Hepatitis C	capsid, NS3, -4B, -5A, -5B, 5′UTR	10–100 (49–53)
Hepatitis delta	Delta antigen	5–10 (54)
Influenza	M, M2, NS, PA, PB1, PB2	10–100 (55–58)
Polio	capsid, 3Dpol	100 (59)
Rhinovirus	Various genes	3–10 (60)
Rotavirus	VP4	10–100 (61)
RSV	viral P, cellular profilin	1000–10,000 (20, 62, 63)
Rous sarcoma	Gag	5–10 (64)
Semliki forest	Nsp-1, Nsp-2, Nsp-4	2 (48)
West Nile	capsid, NS5	3–5 (56)

Only selected viruses are shown to conserve space. Virus abbreviations: HIV = human immunodeficiency virus; RSV = respiratory syncytial virus. A retroviral polymerase (pol) is a reverse transcriptase (RT), while non-retroviral polymerases (e.g., polioviral 3Dpol) are RNA-dependent RNA polymerase (RdRP). The reductions represent the ratio of some parameter of virus growth (titer, viral protein, or number of infected cells) after and before dsRNA treatment. For example, if viral titer dropped from 2×10^5/ml to 10^5/ml, it is a 2-fold reduction. Fold values equal to or greater than 10 were rounded to the nearest ten. For some dsRNA, the range of reductions for different targets and from multiple experiments are compiled together to conserve space. All infections were done in cell or tissue culture, except where the following live animals were infected: mosquito (46), mice (27, 57, 58). Plant viruses were not included.

experimental evidence for the competition between Dicer and PKR will offer new insights into siRNA design.

dsRNA as antiviral drug

The first report on RNAi-mediated inhibition of virus replication in animal cells appeared in 2001 (20). Since then, such effects have been documented in more than a dozen viruses of various genomic structures (25–64). It is now clear that dsRNA/siRNA can inhibit viral replication at several stages of infection, including very early stages when viruses are most susceptible, thus providing a novel form of intracellular immunity. Infection can be blocked by targeting either essential viral genes or host genes that are involved in the viral life cycle. Table 1 provides a short list of DNA and RNA viruses against which dsRNA/siRNA have been used with success. The targets varied from viral transcription factors (e.g., RSV P protein, viral polymerases) to essential proteins of the virion architecture (e.g., capsid proteins) as well as host proteins needed for viral growth (e.g., viral receptors, profilin). The beauty of these studies is that the dsRNA drug, while inhibiting the virus, also validated the knockdown phenotype of the target gene and thus, contributed to the basic knowledge of the function of the gene. The need for new drugs is particularly acute in RNA viruses where high mutation rate and complex immunology have been roadblocks to the development of effective antivirals and reliable vaccines (20, 63). For the sake of conserving space, three model viruses will be discussed here: RSV, HIV, and hepatitis viruses.

RSV, an RNA virus, is one of the biggest serial killers of mankind known and claims a few millions of lives throughout the world annually (65). Currently, there is no reliable drug or vaccine that can prevent RSV infection. The most prescribed treatment of RSV-infected children is a humanized antibody, Synagis® (palivizumab), developed by MedImmune, which is an expensive and difficult treatment regimen (65). The problem is exemplified by the recent closure of a number of anti-RSV biopharma programs as described in a recent comprehensive review (65). Three siRNA drugs have been tested on RSV-infected lung epithelial cells so far: two against the subunits L and P of the viral RNA-dependent RNA polymerase (RdRP), and one against the fusion protein, F (Table 1 and unpublished observation on L) (20, 63). Silencing of L and P

reduced the progeny viral titer by 10^3–10^4-fold, and that of F, by about 10^3-fold also (20). RSV-infected cells in which L and P were knocked down behaved essentially like uninfected cells with no cytopathic effect detectable. It is thus quite likely that animals expressing the corresponding dsRNA would be RSV-resistant, but this needs to be tested.

HIV-1, the causative agent of AIDS, is a retrovirus and has an RNA genome. Multi-drug cocktails including reverse transcriptase inhibitors show limited efficacy in AIDS therapy. However, toxicity to patients and viral resistance remain vexing problems (66), which have led to the testing of dsRNA as potential new therapy. Table 1 lists the various tested targets of HIV-1 that include the viral polymerase (RT or Pol) and structural (Gag, Env), regulatory (Tat, Rev), and accessory proteins (Nef, Vif). Consistent with the notion that viral growth could be inhibited through the loss of essential cell factors, silencing of the cellular receptor (CD4) and coreceptor (CXCR4, CCR5) of HIV also reduced HIV infection of T cells (Table 1).

Both hepatitis B (HBV) and C (HCV) viruses are major public health hazards and substantial risk factors for chronic hepatitis, cirrhosis and hepatocellular carcinoma. The number of HBV carriers worldwide is estimated to be 350 million. Current treatments include interferon or nucleoside analogs such as Lamivudine, Entecavir, and Adefovir dipivoxil (67). The success rate of these treatments, however, is low and re-infections are frequent. HBV is a member of the *Hepadnaviridae* family with a double-stranded circular DNA genome. Expression of shRNA as well as siRNA against specific viral genes inhibited HBV growth in cultured cells and/or mouse liver (25–29). In one study (26), a stable HBV-producing cell line was used which closely approximated chronic hepatitis. HCV, a *Flaviviridae* with a small RNA genome, is a particularly difficult virus to study mainly because of the lack of a definitive cell culture system for its growth. Recently, siRNA was shown to be effective against HCV replicons in the Huh-7 and HepG2 human hepatoma cell lines (49–53). Introduction of synthetic siRNA through electroporation resulted in a virus-resistant state that lasted for about 3 days, but this could be extended to 3 weeks when stable expression of complementary strands of the siRNA was driven from a bicistronic expression vector (51).

In contrast to viral mRNAs, the genomic RNAs of many viruses are resistant to dsRNA; this could be due to the encapsidation of the genome by RNA-binding proteins such as in negative-strand RNA viruses (20, 63), or an extensive secondary structure or exclusive replication of the genome in the nucleus such as in the hepatitis delta virus (54). Early studies had confirmed that the RNAi machinery is exclusively cytoplasmic and fails to knock down unexported nuclear pre-mRNA (68).

For highly mutable viruses, the classic multi-drug approach using multiple dsRNA would be a prudent choice. In this scenario, one can target different parts of the same RNA, or multiple viral RNA, or viral and accessory host RNA. For example, RSV growth was inhibited by silencing of either RSV phosphoprotein, or fusion protein, or cellular profilin (20, 62). Thus, simultaneously targeting two or more of these functions with dsRNA drugs will ensure that the rare RSV mutants that will escape one dsRNA will still be inhibited by the others. In a variation of the theme, dsRNA targeting pro-inflammatory or apoptotic signaling molecules such as Fas, TNF (tumor necrosis factor) or caspases could be paired with anti-viral dsRNA to provide a double knockdown punch (69–71). Fas-mediated apoptosis is implicated in a broad spectrum of liver diseases, whereby inhibiting hepatocyte death is life-saving. Recently, dsRNA against Fas and caspase-8 in fact protected mice from fulminant hepatitis, acute liver failure and fibrosis (70, 71).

dsRNA against parasites

Parasites are lower eukaryotes and cause some of the most devastating diseases in man and livestock all over the world. Their phylogenetic closeness to mammalian hosts, immunological variability, natural reservoirs, and rapid mutations present major hurdles to antiparasitic drug development. The advent of RNA interference has offered new hope, and dsRNA drugs have indeed caused substantial inhibition of a number of parasites in the last few years. These include, but are not limited to the following (the corresponding diseases are in parenthesis): *Plasmodium* (malaria) (72–75), *Toxoplasma* (blindness, mental retardation, miscarriage) (76), *Trypanosoma brucei* (African sleeping sickness) (77–81), and *Schistosoma* or flukes (liver, kidney, and intestinal damage) (82, 83). It should be noted, however, that the exact mechanism of dsRNA action in many parasites remains to be elucidated. For example, although dsRNA shows target-specific inhibition of *Plasmodia* and *Toxoplasma*, definitive homologs of the RISC-associated proteins (e.g., Dicer) have not been reported in either genome. RNAi may not exist in *Leishmania* (84) and in *Trypanosoma cruzi* (Latin American Chagas disease) (85).

dsRNA against genetic disorders and cancer

Dominant and co-dominant mutations that underlie many non-infectious diseases are ideal targets for

dsRNA therapy. Fortunately, dsRNA does not have any major adverse effects (69, 85) and is relatively stable in serum (71). Recently, dsRNA and siRNA have proven highly effective against a number of genetic abnormalities and cancers. Protection of liver from fulminant hepatitis by dsRNA-mediated destruction of Fas has been mentioned (70): in this test, the dsRNA-treated animals survived 10 days or longer whereas the untreated control died in 3 days. In another mouse model, dsRNA targeting the vascular endothelial growth factor (VEGF) effectively inhibited choroidal neovascularization (CNV), providing a highly promising treatment for the age-related macular degeneration (AMD) and diabetic retinopathy (87, 88). While multiple pro-angiogenic proteins may be involved in CNV, both clinical and bench studies have shown that VEGF plays a critical role in the pathophysiology of this blinding condition (88). VEGF is only one of many signaling molecules that have been implicated in human diseases, and structure-based drugs have shown promise against many of them. Examples include the following growth factors/receptors and kinases (the diseases caused by their over-expression and drugs that inhibit them are shown in parenthesis): VEGF (colorectal, metastatic and non-small-cell lung cancer or NSCLC; Avastin, Neovastat, (VEGF trap); epidermal growth factor receptor (NSCLC, pancreatic and head-and-neck cancer; Erbitux, Iressa, Terceva, TheraCIM); rat sarcoma oncogene, Ras (Lung cancer; R11577, a farnesyl transferase inhibitor); cyclin-dependent kinase, CDK (solid tumor; Flavopiridol); multiple kinases (Parkinson's; CEP 1347) (89). In principle, all such molecules can be targeted by sequence-specific dsRNA drugs to cure a variety of cancers and related disorders. Cancers and tumors that are induced by viral gene products (such as E6 and E7 oncogenes of the human papilloma virus causing cervical cancer) can be treated by designing dsRNA drugs against such oncogenes. In the example in Table 1, E6 silencing induced accumulation of p53 protein and inhibited cell growth (32).

In general, mutated proteins that define specific disorders and fusion proteins generated from chromosomal translocation in cancer are ideal dsRNA targets (90, 91). Allele-specific silencing of disease genes with dsRNA can be achieved by targeting either the disease mutation directly or a linked single-nucleotide polymorphism (SNP) (92, 93). For example, nearly half of all human cancers contain point mutations in p53, and dsRNA directly silenced the mutant mRNA by exploiting the single nucleotide difference between the mutant and the wild type, inhibiting tumor growth (94). Strong and allele-specific inhibition of the Val-12 mutant of K-ras by dsRNA led to loss of anchorage-independent growth and tumorigenicity (95). Missense mutants in Tau (V337 M) and amyloid precursor protein, which play key roles in the pathogenesis of sporadic and inherited Alzheimer's disease, could be silenced by direct targeting with dsRNA, shRNA, and siRNA (93). Single nucleotide changes were also exploited to specifically silence the mutant Cu, Zn-superoxide dismutase (SOD1), characteristic of amyotrophic lateral sclerosis (ALS or Lou Gehrig's disease) (96). Expansion of trinucleotide repeats encoding an increased polyglutamine tract causes at least eight human neurodegenerative disorders including Huntington's disease, spinobulbar muscular atrophy (Kennedy's disease) and various types of autosomal dominant spinocerebellar ataxia (SCA). Because selective targeting of the expanded CAG repeats is not possible, dsRNA was generated against an associated SNP that exclusively silenced the mutant allele in SCA type 3 (Machado-Joseph disease) allele while sparing expression of the normal one (93).

Fusion proteins are quite common in cancer (90, 91). In chronic myelogenous leukemia (CML), defined by a translocation called the 'Philadelphia chromosome' (Ph), the c-abl oncogene becomes fused to the breakpoint cluster region (BCR) to produce $p210^{bcr-abl}$. Use of lentivirus-derived shRNA against $p210^{bcr-abl}$ led to complete growth inhibition of K562 (Ph+) leukemia cells (97). In Ewing's sarcoma, the transcription factor EWS is fused to a variety of other transcription factors such as FLI and Ets at various break points (91). The NPM-ALK fusion protein is found in about 75% of pediatric anaplastic large cell lymphomas (98). Specific dsRNA drugs designed against such chimeric mRNA may constitute a potent and specific form of therapy against these cancers (90, 91, 99).

Lastly, dsRNAs can also be used to generate a disease phenotype in animal models for clinical studies. Parkinson's disease, for example, can result from insufficient tyrosine hydroxylase (Th), an enzyme responsible for dopamine synthesis. In a recent study (100), shRNA genes against Th were cloned in AAV (adeno-associated virus) and delivered directly into the midbrain neurons (substantia nigra compacta) of mice. This led to a localized gene knockdown in the brain and behavioral changes in the animals, including motor disorder and impaired psychostimulant response, symptoms characteristic of the rodent model of Parkinson's disease. The usefulness of such conditional and tissue-specific knockdown of cellular genes in clinical medicine cannot be overstated.

Delivery of the dsRNA drug

The issues of delivery of a dsRNA drug are very similar to those of DNA transfection and gene

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therapy, and therefore, only the dsRNA-specific results will be briefly reviewed here. In cell culture, Oligofectamine (Life Technologies, Gaithersburg, MD, USA) and TransIT-TKO reagent (Mirus Corp., Madison, WI, USA) have been used by many laboratories with success (4, 20). In live animals, consistent delivery of sufficient quantities of dsRNA remains a challenge. In mice, 'hydrodynamic injection' through the tail vein effectively delivered the dsRNA into hepatocytes (27, 30, 70). The optimum amount was determined to be 10%–15% of the animal's body weight injected within 5–7 seconds. Delivery of a large bolus is believed to result in short-term right heart failure and in the backflow of a large volume into the liver. Tail vein injection may not transport the siRNA to all cells of the body and is an impossible procedure in human subjects.

Vector-based strategies, in contrast, produce the dsRNA from within. In one approach, the dsRNA-expressing DNA is cloned between two promoters; this results in transcription of the two strands of the dsRNA from two complementary DNA strands. In another approach, a single shRNA of 19 bp with a small loop is transcribed from a single promoter (reminiscent of miRNA; (Fig 1)); this is processed by Dicer and eventually causes RNAi. Due to their natural role in transcribing short RNA molecules, RNA polymerase III promoters have been extensively tested for intracellular expression of dsRNA. Such promoters included human and mouse U6 (101, 102), human H1 (43, 103) and human 7SK promoters (102). For delivery, these promoters were incorporated into adenoviral (103), lentiviral (104) and retroviral (95) vectors. It is also possible to achieve controlled expression in desired tissues through the use of inducible and tissue-specific promoters (102–105). While working with live animals and human patients, consideration must be given to the possibility that viral vectors may cause systemic infections and immune reactions (106). In summary, delivery of dsRNA in large primates, including humans, remains a formidable hurdle in therapeutic applications.

Duration of the dsRNA effect

Synthetic siRNA, introduced into human cells by transfection, shows its optimal effect around 24 h post-transfection. In contrast to lower eukaryotes, however, humans appear to lack an RdRP activity, and thus, the input siRNA is not amplified and the RNAi starts to wear off after 4–7 days (20). However, treatment of recalcitrant cancers, genetic disorders and chronic infections such as AIDS and hepatitis would require a stable and steady application of the dsRNA drug. This can be achieved by intracellular expression of the dsRNA or shRNA. In one example (100), AAV-cloned shRNA gene introduced in mice brain started to silence its target in 4–6 days, which agrees with the timing of AAV gene expression. The silencing phenotype (Parkinson's disease) reached its peak around two weeks and was maintained for nearly two months. Stable and nearly complete resistance to HIV was observed in T cells expressing dsRNA against Tat and Rev (36, 38). Similarly, HCV growth was highly inhibited for nearly three weeks post-infection in cells expressing shRNA or siRNA from transfected DNA clones (51, 52). If exogenously made dsRNA must be used as drug, its stability can be increased by 2′-OH modifications such as 2′-O-methylation or 2′-O-allylation (107, 108).

dsRNA versus other drugs

Comprehensive recent reviews have compared the relative merits of various sequence-specific approaches to mRNA silencing such as traditional antisense oligodeoxynucleotides (ODNs), ribozymes, DNAzymes, and dsRNA (109). The primary advantages of a dsRNA drug over traditional small molecule approach are: ease of design and synthesis, high specificity, easy target validation, multiple targets, stable gene silencing, reversible silencing if needed, and fast development and redevelopment time. Whereas the total time to bring a drug to the clinic ranges from 3–5 years for small molecule strategies, this time may be as short as 1–2 years for a dsRNA drug, which translates into substantial savings of time, cost and human lives. The cost of synthesis of dsRNA, in contrast, remains high; however, it is hoped that as the market demand grows, modified process chemistry would eventually lower the cost.

A dsRNA drug has another advantage over traditional small molecules: it may be a smarter approach against drug resistance. In diseases such as cancer, drug resistance is a major therapeutic problem and is often a result of point mutations that affect the drug-binding pocket in the target protein. In CML, as mentioned earlier, the current blockbuster drug, Gleevec (generic compound: imatinib mesylate), inhibits the tyrosine kinase activity of $p210^{bcr-abl}$ and has a 95% response rate in CML patients (110). However, relapses are common and point mutations in $p210^{bcr-abl}$ account for 80% of the resistant cases (110). In cases like these, it is technically impossible to design and synthesize new drugs for every single mutant and bring them to market in a reasonable time. A new dsRNA, in contrast, can be designed virtually in no time against the new (mutant) sequence and administered to the patient. The same argument would apply to resistant viral mutants that may appear following the first dsRNA treatment (111).

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Recently, dsRNA was called on to foil another mechanism of drug resistance. With a number of anticancer drugs such as thymidylate synthase (TS); however, translation of TS mRNA is regulated by feed-back inhibition of its own protein product such that inhibition of TS leads to a compensatory stimulation of TS translation (112), leading to cellular resistance to TS inhibitors. Sensitivity to the inhibitors was restored when TS mRNA levels were reduced by the use of dsRNA, providing a novel approach to increase the efficacy of the existing anti-TS drugs.

Designing a dsRNA /siRNA drug: a short primer

A critical review of serendipitous as well as systematic studies over the past few years, some of which were discussed above, leads to the following consensus for a rational siRNA drug design.

(a) Length. An ideal siRNA has a 19-nt long double-stranded core with a 2-nt extension at both the 3′-ends; deoxythymidines (dT) has been commonly used as extensions in the assumption that they improve stability against nucleases. Thus, the sense and antisense strands are (written 5′ to 3′): $(N)_{19}$dTdT and $(N')_{19}$dTdT, respectively, where N′ is complementary to N (14).

(b) Sequence. The following four sequence criteria should be followed (113), some of which, not surprisingly, are reminiscent of a PCR primer design: 1) Low GC content. A high-GC internal sequence can conceivably make it harder for RISC to achieve strand separation, an essential pre-requisite to engagement of the target by RISC (Fig 1); 2) An AU-rich 3′-end of the sense strand (i.e., the non-guide strand or the strand with the same sequence as the target mRNA). This criterion follows the rule of asymmetric unwinding (9, 10) described earlier (Fig 1). 3) Lack of inverted repeats. Clearly, an inverted repeat will give rise to an intramolecular hairpin, thus preventing the formation of an siRNA; 4) Internal base preferences. Stated in terms of nucleotide numbers on the sense strand (N_{19}) of the siRNA, the following bases were favored: A at 3, U at 10, any base other than G at 13, and A at 19 (G and C were bad).

(c) Mismatch. As a rule a perfect match with the target is desired for maximal specificity and efficiency of silencing (4). In fact, use of an antiviral dsRNA drug may lead to the selection of mutant viruses that became resistant to the dsRNA by mismatch (111). This is especially true with RNA viruses because of the lack of proof-reading mechanism in RNA genomes in general. This can be minimized by designing dsRNA against highly conserved regions of the mRNA containing codons with minimal degeneracy. It is mandatory to conduct a BLAST (basic local alignment search tool) search of the non-redundant nucleotide sequence to ensure sequence specificity of any siRNA drug. However, recent studies have revealed that siRNA activity can be a little more tolerant to mismatches than was originally thought. In these studies there was a gradual loss of silencing with increasing mismatch. In another study (114), analysis of transcription profiles by DNA microarray revealed siRNA-specific rather than target-specific signatures, including direct silencing of non-targeted genes containing as few as eleven contiguous nucleotides of identity to the siRNA. In some instances, mismatch tolerance may produce a beneficial effect by expanding of the target spectrum of the siRNA. For example, it may enable silencing of multiple mutational variants of a given RNA virus.

(d) position effect. The siRNA effect can be strikingly position-dependent, i.e., siRNAs directed against different regions of the same mRNA may exhibit a wide range of silencing efficiency (115). Although many positional effects can be explained by the asymmetry rule described above, exceptions are known, and it is conceivable that the mRNA secondary structure or RNA-binding proteins may play a role (20, 54, 116). It is fair to say that there is currently no single algorithm that can guarantee a perfect siRNA design, and thus, it is advisable that more than one siRNA drug be tested against a given mRNA.

The biotech, the pharma and the future of the dsRNA drug

The list of biotech companies developing dsRNA-related therapeutics is growing rapidly. The major players include (alphabetically): Acuity Pharmaceuticals, Alnylam, Avocel, Benitec, CytRx, Devgen, Intradigm, Nucleonics, International therapeutics, Intradigm, Polgen, Sequitur and Sirna therapeutics. Collectively, they target a slew of ailments including hepatitis, RSV, HIV, autoimmune diseases, asthma, AMD, diabetic retinopathy and diabetes, central nervous system disorders, obesity, ALS or Lou Gehrig's disease, and various kinds of cancer. It is obvious that dsRNA drugs hold great promise in virtually every area of therapy and are destined to serve a huge market driven by unmet medical needs. The total worldwide market for RNAi-based technology is projected to reach $185 billion (117), a large

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proportion of which is likely to derive from dsRNA drugs.

In apparent contrast, the Big Pharma has not yet stepped up to the plate, although in recent interviews representatives of Abbott Laboratories, Merck & Co, and Medtronic reportedly expressed interest in the dsRNA technology (117). The cautious optimism is understandable as the antisense ODNs of the eighties did not quite live up to the expectations. In fact, most biotech companies, exemplified by Genta, Hybridon and Novopharm discontinued a number of their antisense projects altogether (65). Enthusiasm was further dampened by the recent Phase III failure of ISIS/Eli Lilly's lung cancer antisense drug, AFFinitak. Nevertheless, the novel advantages of dsRNA drugs have rekindled excitement in the gene silencing area. Four major issues that need further scrutiny are: IFN activation, specificity, delivery and stability. A combination of short length, low concentration, avoidance of 5′-triphosphates and a customized delivery procedure for each dsRNA seems to hold the key to success. It appears that the question is not if but when all this excitement and effort will materialize into effective and reliable dsRNA drugs.

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