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Review Article

RNA interference: an exciting new approach for target validation, gene expression analysis and therapeutics

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Abstract

Recent discoveries suggest that nature employs a novel approach for the regulation of gene expression by the silencing of endogenous genes. This phenomenon referred to as RNA interference (RNAi) is a posttranscriptional gene-silencing strategy that involves the intermediacy of double-stranded RNA (dsRNA) that complex with and destroy a target messenger RNA (mRNA) (1-4). The targeting and destruction of mRNA has been demonstrated to be highly specific and more selective compared to the antisense approach. Furthermore, it has also been shown that exogenously administered 21- to 23-mer dsRNA, referred to as small interfering RNA (siRNA), is equally efficient as a gene-silencing tool compared to the much longer dsRNA. These discoveries have led to the exciting possibility of using RNAi not only as a potential tool for elucidating the function of genes and analyzing gene expression, but also as a novel therapeutic modality. This review will highlight various aspects of this rapidly advancing field.

Introduction

Since the discovery of Prontosil as an antibacterial agent in 1932, proteins and enzymes have been the favored molecular targets for the discovery and development of therapeutic agents. Examples of some enzyme- and protein-targeted drugs include ranitidine (a histamine receptor antagonist), physostigmine (a cholinesterase inhibitor) and propranolol (a β-adrenergic receptor antagonist). Many modern drugs are inhibitors of specific enzymes thus interfering with the bioconversion of substrates to products involved in the disease process. The selectivity of enzyme inhibition by drugs is due to their ability to participate in binding interactions with amino acid residues that form the crevices or grooves of the target protein or receptor. In all instances, such specific and selective binding of a drug to a receptor is facilitated by key hydrogen bonding, hydrophobic, electrostatic, polarization, charge transfer and other noncovalent interactions with complementary sites on the receptor. Specifically, hydrophobic interactions result from desolvation of nonpolar groups associated with each other and from specific binding contacts, while polar interactions could result from direct or indirect associations with the target involving charged or polar groups. The selectivity in binding is also facilitated by specific interactions between juxtapositioned groups both on the drug as well as on the receptor. By such selective binding to the target, it was anticipated that the drugs could inhibit the activity of those proteins involved in the disease process without interfering with those of others. However, as is well known, most drugs have side effects that are attributable to nonspecific interactions between the drug and other proteins.

The genomic revolution has made available a number of new protein targets for therapeutic intervention, and recently structure-based drug design has evolved into a powerful drug design tool, where the 3D structure of the target protein is utilized in designing molecules that would fit the active site, block the binding of the normal substrate at the active site and consequently interfere with the function of the enzyme or protein. Thus, protein targets continue to be a fertile ground for drug discovery.

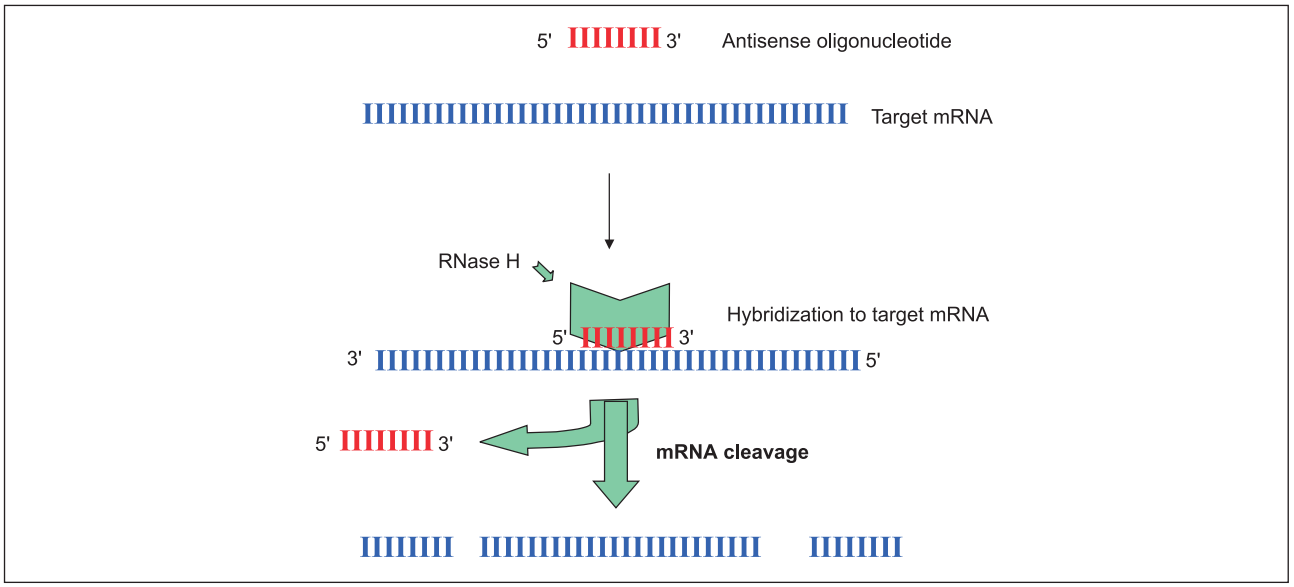

Fig. 1. The antisense approach. A synthetic oligonucleotide complementary to a target messenger RNA hybridizes with it and block its subsequent translation to proteins or can activate the intracellular enzyme RNAse H that degrades the target RNA.

RNA as a molecular target for drug discovery

In parallel with the genomic revolution in the past decade, nucleic acids have emerged as an exciting class of targets amenable to apparently simple, yet rational, drug design without the need for 3D structures. The nucleic acid-targeting drugs are designed to "hybridize" (see below) or bind to a selected site on DNA interfering with its transcription to RNA, or directly bind to RNA interfering with its subsequent translation to proteins (5-8). This opportunity to target DNA and RNA holds promise for an exciting class of better and safer drugs for the future. The feasibility of this approach is illustrated by the use of antisense oligonucleotides for targeting RNA that has received considerable attention as a therapeutic modality in the past decade and will be briefly discussed in this review. Targeting of DNA using a "triplex" strategy or aptamer approach is beyond the scope of this review and is covered in excellent reviews elsewhere (9).

Advantages of targeting RNA: antisense and ribozyme strategies

There are sharp differences in the approaches between targeting proteins and RNA as illustrated by antisense and ribozyme targeting of RNA. In fact, targeting RNA provides an opportunity to regulate gene expression in a distinctive manner. Thus, when using conventional strategies that interfere with binding interactions of ligands with proteins, only the function of a protein can be regulated but not its production. In contrast, by taking advantage of the "DNA makes RNA makes protein paradigm", and by targeting RNA, it is possible to interfere with the production of the protein itself. An example is the approach using antisense oligonucleotides (6-8), where synthetic oligonucleotides are designed to be complementary to an RNA target, bind to it and consequently block the production of the protein (Fig. 1). The underlying principle that governs recognition and binding of the target mRNA by the antisense oligonucleotide is the Watson-Crick base-pairing rules that dictate parallel-antiparallel duplex formation in nucleic acids. This is in contrast to inhibition of protein function where a combination of hydrophobic, hydrogen bonding and polar interactions between the inhibitor and structural constituents of the protein is the key to the formation of the inhibitor-protein complex. In addition, the use of antisense oligonucleotides provides the opportunity to selectively destroy the target RNA using cellular enzymes that are specifically activated in response to the formation of the RNA-oligonucleotide complex. The antisense oligonucleotide is then free to complex with another molecule of the target RNA in a catalytic cycle of complexation and target destruction. Thus, for example, DNA-like antisense oligonucleotides offer the advantage that the DNA-RNA duplex becomes a substrate for RNase H enzyme, which cleaves the mRNA strand selectively. Such a catalytic effect offers the exciting potential for the use of relatively small amounts of oligonucleotides for therapeutic intervention. It is pertinent to mention that ribonucleotide analogs and vector-encoded RNA transcripts are also capable of forming a duplex with complementary RNA and produce an antisense effect but are not intrinsically capable of invoking a catalytic effect. In fact, both unspliced RNA in the nucleus and spliced RNA in the cytoplasm can be targeted by the antisense approach. In contrast, most drugs that target proteins are reversible inhibitors and rarely inactivate or cleave the target protein.

Another attractive approach for targeting RNA is the use of synthetic oligoribonucleotides called ribozymes that cleave the target mRNA (9). The recognition of target RNA by ribozymes again involves Watson-Crick base-pairing of specific regions of the target strand by the ribozyme. The catalytic domain of the ribozyme then provokes cleavage of a complementary strand by trans-esterification followed by hydrolysis.

It should be noted, however, that RNA has complex secondary and tertiary structures. Consequently, many sequences or sites within RNA are involved in base-pairing internally to form hairpins, bulges, loops, junctions and pseudoknot structures, and may not be available for antisense or ribozyme targeting. This necessitates the screening of a number of oligonucleotides in order to select one that is most appropriate for the target.

Both antisense and ribozyme approaches have yielded an expansive body of knowledge that has unraveled the complex pharmacodynamic and pharmacokinetic aspects of oligonucleotides *in vivo*. Not surprisingly, oligonucleotides by virtue of their unique structure, molecular weight, charge and binding properties differ considerably in their absorption, distribution, metabolism and excretion attributes compared to small molecule drugs that target proteins. Remarkably, within a short span of 10 years, these studies have resulted in several oligonucleotides being evaluated in the clinic against a range of RNA targets and associated diseases. The clinical experience of compounds based on both approaches has contributed to the understanding of complex pharmacodynamics, pharmacokinetics as well as toxicity of oligonucleotides in humans. Much of this knowledge can be applied to RNAi-based therapeutics.

Novel therapeutic approach: RNA targeting by RNA interference

Regulation of gene expression by silencing of RNA was first discovered in plants in the 1990s (10). Thus, in early experiments with purple colored petunias, transgenic plants were created that carried an extra copy of the “color” gene that would enable the production of deeper coloring flowers. However, the resulting plants had white flowers instead of the anticipated deeper purple. This observation was interpreted to be the result of posttranscriptional silencing of the transgene as well as the cellular gene that encodes the gene product. The suppression of gene expression was further traced to sequence-specific degradation of the corresponding mRNA without alteration in the rate of transcription of the gene.

Thus, even though the gene was transcribed and processed into mRNA, the mRNA was destroyed in the cytoplasm as quickly as it was made. This phenomenon is referred to as RNA interference (RNAi) and has been shown to involve the intermediacy of double-stranded RNA (dsRNA) (1-4). In summary, RNA interference is a posttranscriptional gene-silencing event in which both the transgene and the homologous chromosomal loci appear to be cosuppressed by the corresponding dsRNA. RNAi appears to be present in almost all eukaryotic systems.

It is believed that the phenomenon of RNAi is utilized by nature to protect its genome from attack by mobile genetic elements such as viruses and transposons. In nature, these elements can integrate near the promoters of genes and be transcribed to dsRNA to produce an RNAi effect (1-4). In comparison to antisense-mediated down-regulation of gene expression, which mainly employs DNA-like molecules, RNA silencing can only be achieved by dsRNA. Also, a remarkable observation has been made that the RNAi effect can be passed on for several generations without alteration of the genomic DNA of the target gene (1-4, 11). Thus, from a therapeutic perspective, RNAi offers an attractive, commercially viable alternative to antisense and ribozyme strategies for downregulating the production of a protein involved in disease. As will be discussed, the simplicity in the design of compounds and their high selectivity and specificity for the target appears to be the hallmark of the RNAi approach.

Mechanism of RNA interference

Understanding the mechanism by which dsRNA triggers RNA silencing has been crucial to unraveling the potential therapeutic and diagnostic application of RNAi. Using an *in vitro* system involving *Drosophila melanogaster*, Tuschl *et al.* (12) discovered that before RNAi occurs, dsRNA is first cleaved by specific nucleases at regular intervals to generate 21- to 23-mer nucleotides (Fig. 2).

It is believed that these short oligoribonucleotides derived from dsRNA are the likely intermediates of RNAi and are termed small interfering RNAs (siRNAs). The nuclease is believed to be an ATP-dependent ribonuclease called a Dicer that belongs to an RNase III family of dsRNA-specific endonucleases. Recently, an RNAse III protein has been characterized that contains helicase, RNAse III and dsRNA motifs (1-4).

Further evidence for the intermediacy of siRNAs is based on several observations (1-4, 13, 14): (a) precursor dsRNAs of less than 38 base pairs in length are inefficient mediators of RNAi because the rate of siRNA formation appears to be significantly reduced in comparison to longer dsRNA; (b) in experiments using *D. melanogaster* embryos it was found that the siRNAs generated from dsRNA usually carried a 5’-monophosphate and a free 3’-hydroxyl group and that processing of dsRNA occurred with no apparent sequence preference; (c) the siRNAs that are 21- to 23- double-stranded ribonucleotides carry 2 nucleotide overhangs at each of the 3’-ends and RNAse III enzyme is known to cleave dsRNA to generate fragments with such 2 to 3 NT overhangs; (d) siRNAs were detected *in vivo* in *D. melanogaster* embryos and *Caenorhabditis elegans* adults when dsRNA was injected; (e) interestingly, it was observed that chemically synthesized siRNAs were also capable of effecting target RNA cleavage *in vitro*; and (vi) it has been demonstrated

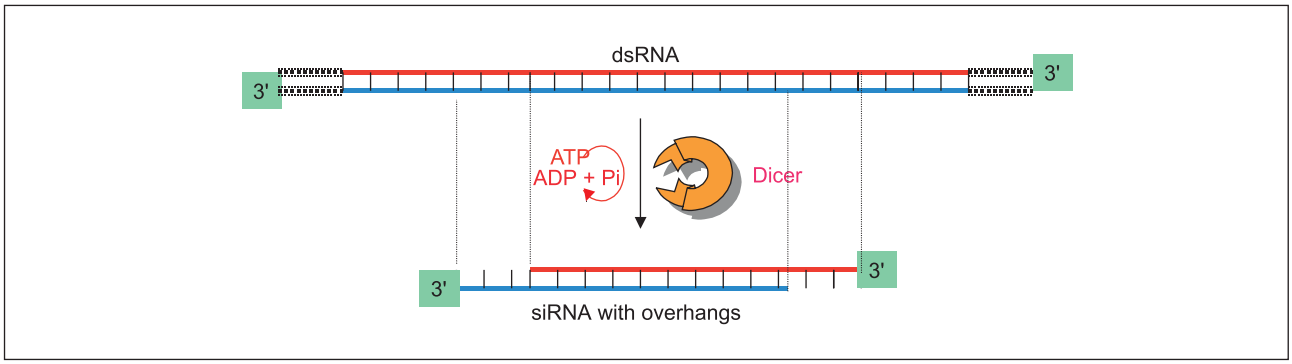

Fig. 2. Formation of small interfering RNAs (siRNAs) from double-stranded RNA (dsRNA). The initial dsRNA, that is exogeneously administered or generated intracellularly, is cut by the enzyme complex Dicer to generate 21- to 23-nucleotide pieces called siRNAs which are the key intermediates for RNA interference.

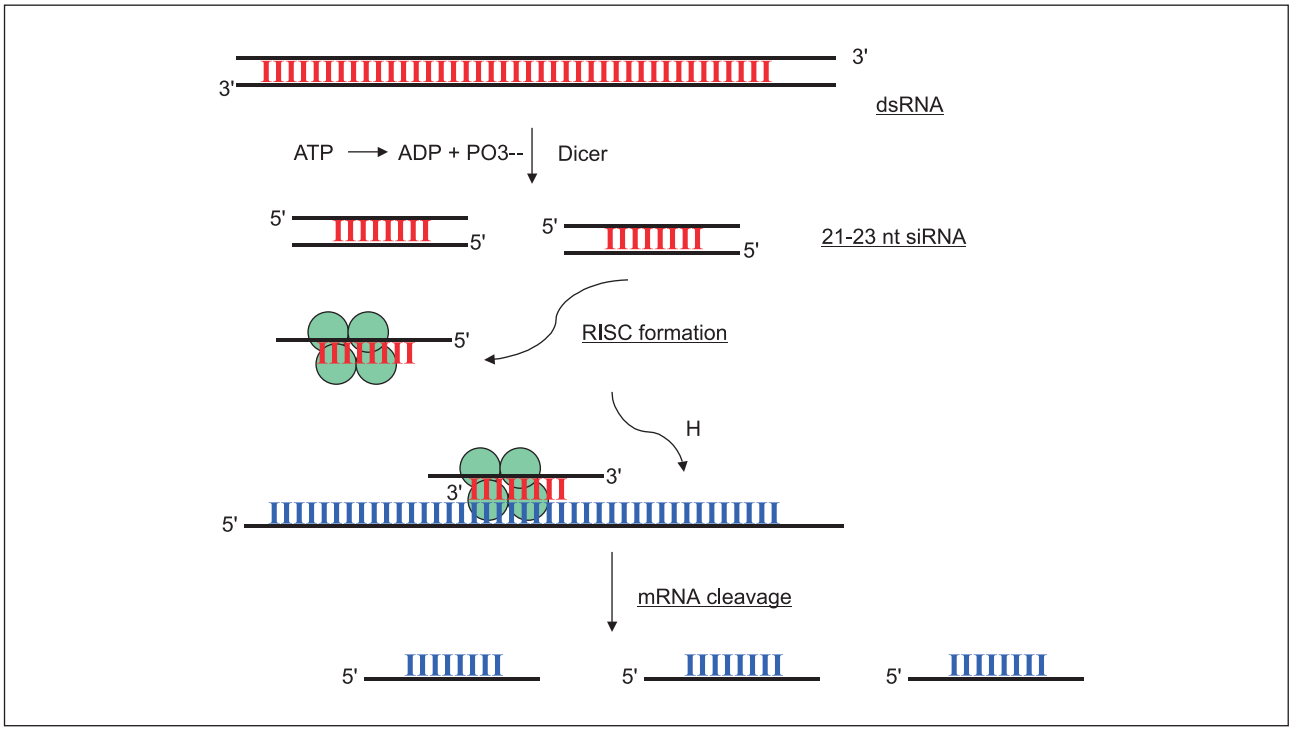

Fig. 3. Mechanism of RNA interference. The siRNA that is exogeneously administered or endogeneously generated from dsRNA complexes with a set of enzymes to form RNA-induced silencing complex (RISC). Using the antisense strand of siRNA, RISC then targets the messenger RNA for degradation.

that siRNAs induce sequence-specific RNAi in mammalian systems as well. Taken together, these observations suggest that an RNase III-like enzyme is responsible for processing of dsRNA into siRNA and that siRNA is the intermediate for RNAi.

Key role for small interfering RNAs in RNA interference

It is believed that once formed, 21- to 23-nucleotide siRNAs could serve as guide sequences in complexing with mRNA and target it for degradation (Fig. 3). The siRNA complexes with proteins to form mRNA-cleaving RNA-protein complexes, also known as small interfering ribonuclear protein particles (siRNPs) or as an RNA-induced silencing complex (RISC) that has endoribonuclease activity different from the Dicer. Using the siRNA as a guide, the RISC then cleaves the corresponding mRNA at the site that is complementary to the siRNA. The position of mRNA cleavage is within the binding segment of mRNA that is complexed with the antisense strand of the siRNA, thereby suggesting a hybridization-triggered induction of cleavage. Biochemical analysis shows that both sense and antisense strands within an

siRNA have distinct roles for the functional activity of RISC. Presumably, the antisense strand complexes with target mRNA and induces its cleavage, whereas the sense strand participates in siRNA duplex formation. It is the duplex structure that is recognized by RISC proteins which has RNase III, helicase and ATPase activities (1-4).

There appears to be specific advantages in using siRNAs for inducing RNAi effect. Thus, in early experiments with mammalian cells that used dsRNA, it was found that dsRNA induced dsRNA-dependent protein kinase (PKR) which phosphorylates and inactivates the translation factor elF2a leading to a general nonspecific suppression of protein synthesis and apoptosis. In contrast, Elbashir *et al.* (13) showed that 21- to 23-nucleotide duplexes can function as siRNAs and induce sequence-specific and selective RNAi in mammalian cell lines such as human kidney cells and HeLa cells. Thus, the use of siRNAs may overcome the problems associated with dsRNA and become the method of choice for therapeutics, diagnostics and functional genomics via the RNAi mechanism.

In some nonmammalian organisms, amplification of the siRNA is thought to take place by an RNA-dependent RNA polymerase. However, this amplification may not occur in mammalian cells since an siRNA made against one splice form of a particular gene does not silence other splicing variants (1-4).

For successful commercial realization of the potential of RNAi, siRNAs are required that have structural attributes and pharmaceutical properties (enzymatic stability, deliverability and favorable pharmacokinetic and pharmacodynamic parameters) that combine RNAi-functional competency with potency and selectivity. This would enable the downregulation of a target mRNA or protein by the use of nanomolar/picomolar concentrations of the compound and provide a commercially viable therapeutic modality for treatment.

Advantages of chemically modified siRNAs

To date, most studies on RNAi have employed unmodified siRNAs which unfortunately have the potential to be degraded by nucleases *in vivo*. Although as a duplexed structure siRNAs might in themselves have some resistance against nuclease-mediated degradation *in vitro*, little is known about their stability *in vivo* or their pharmacokinetic and bioavailability attributes. It is well known that incorporation of chemical modifications in antisense oligonucleotides confers them with enzymatic stability and pharmacokinetic advantages (5). Incorporation of such modifications in the case of siRNAs would be advantageous for realizing their therapeutic utility. However, preliminary reports on chemically modified siRNAs have been disappointing and suggest that in order to maintain RNAi-competency, the siRNA molecule only tolerates certain limited modifications at the 3'- or 5'-overhangs of the molecule (1-4) (Fig. 4). Interestingly, the antisense strand of the siRNA is less permissive for chemical modifications as compared to the sense strand.

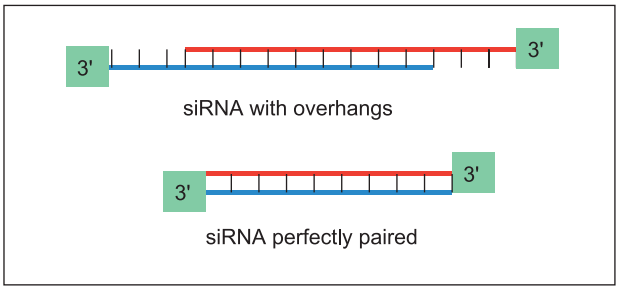

Fig. 4. An siRNA is 21- to 23-nucleotides long on each strand and often has "overhangs" at the 3'-ends of each strand. The siRNAs with overhangs are thought to be more efficient for provoking RNAi than the corresponding siRNAs without overhangs.

This may be due to reduced recognition of the siRNA by the constituent proteins of the RISC, thereby resulting in the lack of formation of stable RISC. Thus, incorporation of specific modifications coupled with appropriate design of siRNA will be the key to developing a therapeutically useful RNAi-competent siRNA molecule. In summary, the optimal design of siRNA that confers duplex stability, enzymatic stability and pharmaceutical attributes such as bioavailability and delivery, will be critical for successful commercial use of siRNAs as a therapeutic modality. Such chemically modified siRNAs may also be suitable for covalent attachment to solid matrices and may be ideal for designing siRNA chips for diagnostic applications and for gene function analysis.

Applications of RNAi

The human genome project has provided an extraordinary opportunity to study the relation and function of different genes in applications related to target validation and functional genomics. Although tools such as antisense and ribozymes continue to be useful in elucidating the role of genes, RNAi offers certain advantages as a target validation tool because of its extraordinary sequence-sensitivity, selectivity and design simplicity. For example, when using antisense and ribozyme approaches, a number of sequences need to be screened (*i.e.*, a RNA walk) to select one that can be used for target validation studies (6-8, 9). In contrast, definite rules have emerged regarding the design of siRNA as well as dsRNA and the region of mRNA that can be targeted for effecting RNAi.

It appears that RNAi may be particularly suitable in the elucidation of gene function in developing embryos and hence may be a valuable tool especially in developmental biology. For example, by using dsRNA and inducing RNAi against specific genes, the production of the corresponding proteins is inhibited (15, 16). This process is essentially similar to the production of gene knockout phenotypes. Thus, the possibility to switch genes on and off as desired may enable the study of differentiation in

developing embryos. In turn, this will enable the elucidation of the role of individual genes and the functional relationship between genes.

Early studies in functional genomics using *C. elegans* have been very useful in identifying the function of certain genes. Moreover, RNAi-induced downregulation of gene expression has been demonstrated in *Hydra* (17), *Trypanosoma* (18) and *Planaria* (19). However, experiments with zebrafish (20) and mice (21) have been only moderately successful. As mentioned previously, it is pertinent to mention that when dsRNA was used as an RNAi tool in cells, nonsequence-specific induction of interferon response was observed which in turn inhibited protein synthesis and nonspecific mRNA degradation (22). Also, in early experiments using dsRNA with mammalian cells, dsRNA was found to induce dsRNA-dependent PKR, which phosphorylates and inactivates the translation factor eIF2a leading to a general suppression of protein synthesis and apoptosis. However, as mentioned above, Tuschl *et al.* showed that 21- to 23-nucleotide duplexes can function as siRNA to induce sequence-specific RNAi in mammalian cell lines (13). Caplen *et al.* (23) have independently shown that siRNAs can induce sequence-specific inhibition of target genes both *in vitro* and *in vivo*.

From a therapeutic perspective, RNAi offers an attractive alternative to antisense and ribozyme strategies for downregulating expression of a protein involved in disease. As mentioned previously, the simplicity in the design of RNA and high selectivity and specificity for the target appears to be the hallmark of RNAi. The design principles for RNAi are potentially universally applicable across a broad spectrum of targets and disease areas. Indeed, most disease areas including cardiovascular, metabolic, cancer, CNS and infectious diseases are amenable to the RNAi approach.

Delivery of siRNAs

An siRNA is a high-molecular weight (> 12,000 MW), highly charged polynucleotide. Consequently, like other oligonucleotides, cell culture studies require the use of a transfection reagent for efficient delivery of siRNAs into cells. But the efficiency of transfection reagents differs with each type of siRNA and also varies depending on the cell line. Furthermore, other variables such as temperature, pH and the presence of factors such as serum need to be considered when comparing transfection efficiencies in different cell lines. Apparently, the transfection reagents used in the case of plasmid DNA and oligonucleotides appear to work less efficiently in the case of siRNAs. Several synthetic transfection reagents are commercially available as delivery reagents designed specifically for siRNAs (24). It should be noted that although delivery was an important issue in antisense experiments in *in vitro* cell culture experiments, most antisense compounds have been employed in *in vivo* experiments and in the clinic without the apparent need for a delivery vehicle.

Little information is available on the bioavailability of siRNAs following different routes of administration. Pharmacokinetic studies of a single-stranded chemically modified oligonucleotides demonstrate a characteristic profile in that they are rapidly absorbed following i.v. administration, are then rapidly distributed in various tissues and eliminated slowly through kidneys (6-8).

To further understand the pharmacokinetics and metabolism of siRNAs, the development of methodology to track siRNAs in cells is required. Oligonucleotides are frequently tagged with fluorescent labels in order to track their movements in and between cells. However, unlike antisense molecules, siRNAs must interact with cellular components to form RISC to elicit their action and it is not fully established whether the addition of hydrophobic bulky constituents would affect their capacity to induce RNAi. In one report, the fluorescent labels Cy3- and Cy5-carboxy-fluorescein were introduced at either or both sense and antisense strands of siRNAs directed against the *c-myc* gene in HeLa S3 cells. It was shown that both the labeled and unlabeled siRNAs were equally capable of reducing protein expression due to downregulation of *c-myc* mRNA. Thus, the attachment of hydrophobic moiety does not appear to impede the activity of siRNAs (25).

Cellular trafficking and localization of siRNAs has also been studied using fluorescent-labeled siRNAs. It appears that following treatment of cells, siRNAs were mostly localized in discrete foci on the cytoplasmic side of nuclear membrane. Fluorescent labeling has also been used to demonstrate that over a period of time, duplexed strands separate out. However, the majority of the siRNAs appear to remain in the double-stranded state during the course of experiments. Further, it has been shown that *in vitro*, siRNAs are maintained in cells for up to 10 days and are transferred to daughter cells (25).

Early studies on chemically modified siRNAs suggest that in order to retain the functional competency of RNAi, the siRNA molecule only tolerates certain limited modifications at both the 3'- or 5'-end of the molecule. Reports thus far indicate that siRNAs are not permissive to significant chemical modifications without loss of RNAi competency (1-4). Nevertheless, it should be noted that siRNAs are duplexed structures which might in themselves have sufficient stability characteristics and favorable pharmacokinetic and bioavailability attributes.

Design of siRNAs

Given a target RNA, the following rules (13) have been devised regarding the design of siRNAs: (a) start 75 bases downstream from the start codon; (b) locate the first AA dimer; (c) record the next 19 nucleotides following the AA dimer; (d) compare the target sites to the appropriate genome database and eliminate any targets with significant homology to other genes; (e) calculate the percentage of GC content of the AA-N19 21-base sequence. Ideally the G/C content is 50% but it must be less than 70% and greater than 30%; and (f) this

sequence and its complementary strand is the siRNA sequence.

Synthetic siRNAs

Although siRNAs can be produced in the cell using an appropriately engineered plasmid, only unmodified RNA can be prepared this way. The intracellular stability of unmodified exogenously administered siRNAs is not known especially *in vivo*. As mentioned above, for therapeutic utility, chemically modified and designed siRNAs may be required and therefore synthetic methods need to be used. Furthermore, for establishing structure-activity relationships and mechanism and pharmacokinetic studies, larger amounts of chemically modified siRNAs are needed that may be only accessible by chemical synthesis.

Unlike DNA, the synthesis, purification, storage and handling of unmodified RNA is a challenge. This is because RNA molecules are susceptible to both enzymatic (*e.g.*, RNAse-mediated) and chemical (*e.g.*, base-catalyzed) degradation. Notably, the presence of the 2'-OH group in RNA promotes cleavage of the internucleotidic phosphodiester linkage by neighboring group participation.

Like DNA, the chemical synthesis and assembly of oligoribonucleotides (26) is performed on a solid support using automated synthesizers in conjunction with phosphoramidite chemistry (27-30). This process also enables the synthesis of chemically modified siRNAs. The key to successful assembly and synthesis of RNA is the choice of nucleoside building blocks appropriately protected at the 2'-hydroxyl, phosphite, 5'-hydroxyl and nucleobase (28-30). For RNA synthesis, building blocks that carry nucleobase protecting groups with increased lability towards mild base are preferred (27). The preferred nucleobase protecting groups include phenoxyacetyl, 4-isopropyl phenoxyacetyl, 4-*tert*-butyl phenoxy acetyl and *N,N*-dialkyl formamidines. The 2'-OH-ribose protecting groups include *tert*-butyl dimethylsilyl and acetoxy ethyl (ACE) groups (31). The β-cyanoethyl moiety is the most favored phosphate protecting group. In the synthesis of some modified oligoribonucleotides, *p*-nitrophenylethyl or methyl group may advantageously replace the β-cyanoethyl group for phosphate protection.

Solid-phase RNA synthesis also requires a solid support that is functionalized with the nucleoside corresponding to the 3'-end of the desired oligonucleotide. Typically, the nucleoside is attached to the support via a succinate linker that is cleaved under alkaline conditions at the end of the synthesis. Controlled-pore-glass (CPG) and polystyrene are the most commonly used solid supports for the synthesis of RNA (27).

Chemical synthesis of RNA

Automated solid-phase phosphoramidite chemistry in conjunction with protected nucleoside phosphoramidite building blocks is the preferred method for RNA assembly. In this case, high step-wise coupling efficiencies (> 98%) during assembly and the easy removal of the protecting groups allow a preparative level of synthesis.

Solid-phase synthesis of RNA follows the same pathway as DNA synthesis. Each cycle for automated oligoribonucleotide synthesis consists of detritylation, coupling, capping and oxidation steps (Fig. 5). Efficient and high yields at each of these steps are critical for successful RNA synthesis. A solid support with an attached nucleoside is subjected to removal of the protecting group on the 5'-hydroxyl. The incoming amidite is then coupled to the growing chain in the presence of activator. Any unreacted 5'-hydroxyl is capped and followed up by oxidation to achieve phosphotriester linkage. This process is then repeated until an oligomer of the desired length results. In the case of RNA synthesis, because of the presence of a bulky 2'-OH protecting group, coupling reactions between ribonucleoside phosphoramidites are typically 10-30 min. Dramatic improvements in oligoribonucleotide synthesis are achieved when activators more acidic than the standard 1*H*-tetrazole (pK_a, 4.8) are used. Such activators include 5-(4-nitrophenyl)-1*H*-tetrazole (pK_a= 3.7) or 5-ethylthio-1*H*-tetrazole (pK_a, 4.28). It is believed that these tetrazole derivatives are more efficient at protonating the trivalent phosphorous for nucleophilic displacement during the coupling step.

The use of various combinations of hydrazine, ethanolamine and alcohol or more nucleophilic alkylamines has been studied for oligoribonucleotide deprotection. The use of base-labile groups pheoxyacetyl, 4-isopropyl phenoxyacetyl, 4-*tert*-butyl phenoxy acetyl-phenoxyacetyl and amidines for exocyclic amino groups allows shorter exposure to basic conditions for complete deprotection. Thus, complete deprotection of nucleobases, β-cyanoethyl phosphate protecting group and cleavage from the solid support can be achieved after a 15-min to 1-h incubation in concentrated NH_4OH:EtOH (3:1) at 65 °C. Following the removal of the base protecting groups, the 2'-TBDMS groups are removed by using *N*-tetrabutyl ammonium fluoride (TBAF) (1 M) in THF at room temperature over 24 h. The use of neat triethylamine trihydrofluoride (TEA.3HF) as desilylating reagent has also been recommended for removal of TBDMS group. Following deprotection, the crude RNA is purified by HPLC and obtained as a powder following desalting and lyophilization.

Challenges and prospects

Since the discovery of the RNAi phenomenon, a considerable amount of knowledge has emerged about the mechanism and function of siRNA. However, molecular details on the protein components of Dicer and RISC and

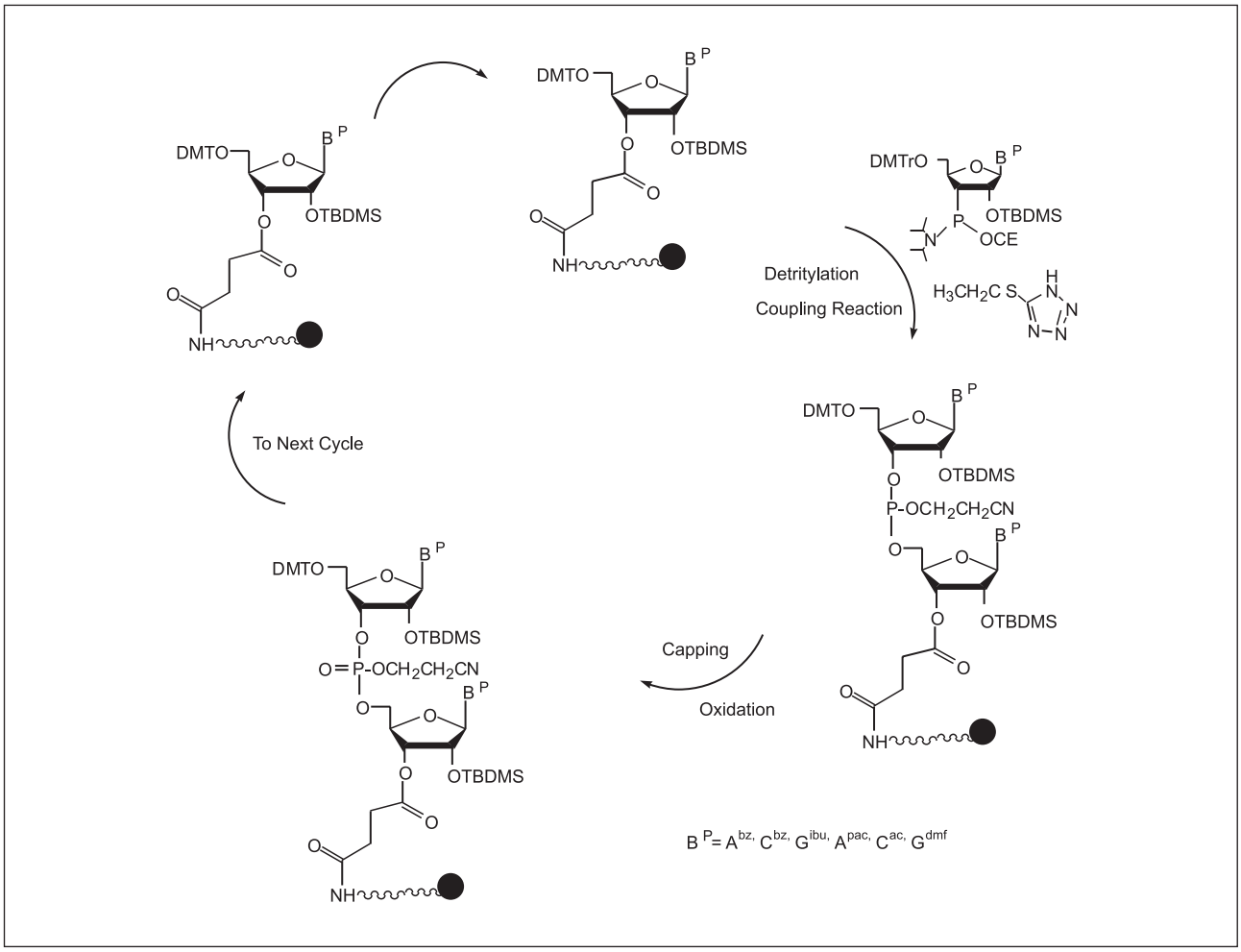

Fig. 5. Synthesis of RNA by automated solid-phase phosphoramidite chemistry approach.

their interaction with dsRNA and siRNA will be critical for gaining better understanding of the phenomenon of RNAi. Although considerable developmental and clinical data of oligonucleotides have emerged from the antisense and ribozyme approaches, little is known of the pharmacokinetics and pharmacodynamics of siRNA in animals and in man. Patient compliance in therapeutics requires the use of orally bioavailable dosage forms. Furthermore, whether siRNA can be made into an orally bioavailable form is an open question. For developmental work, large quantities of RNA will be required. Large-scale RNA synthesis is not trivial and more developmental work for the manufacture of RNA is required before siRNA can be exploited as a therapeutic modality.

Clearly, RNAi has emerged as one of the most exciting discoveries of the decade with potential for a host of applications in therapeutics, functional genomics and diagnostics. Many challenges need to be addressed and overcome before the full potential of siRNA can be unraveled.

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