Expert Opinion

Ashley Publications
www.ashley-pub.com

Modulation of gene expression by antisense and antigene oligodeoxynucleotides and small interfering RNA

Ram I Mahato†, Kun Cheng & Ramareddy V Guntaka
†University of Tennessee Health Science Center, Department of Pharmaceutical Sciences, 26 South Dunlap Street, Feurt Bldg RM 406, Memphis, TN 38163,USA

Antisense oligodeoxynucleotides, triplex-forming oligodeoxynucleotides and double-stranded small interfering RNAs have great potential for the treatment of many severe and debilitating diseases. Concerted efforts from both industry and academia have made significant progress in turning these nucleic acid drugs into therapeutics, and there is already one FDA-approved antisense drug in the clinic. Despite the success of one product and several other ongoing clinical trials, challenges still exist in their stability, cellular uptake, disposition, site-specific delivery and therapeutic efficacy. The principles, strategies and delivery consideration of these nucleic acids are reviewed. Furthermore, the ways to overcome the biological barriers are also discussed so that therapeutic concentrations at their target sites can be maintained for a desired period.

Keywords: antigene, antisense oligodeoxynucleotides, cellular uptake, nucleic acid delivery, phosphorothioates, RNA interference, siRNA, triple helix, triplex-forming oligodeoxynucleotides

Expert Opin. Drug Deliv. (2005) 2(1):3-28

1. Introduction

Modulation of aberrant gene expression by antisense oligodeoxynucleotides (ODNs), triplex forming oligodeoxynucleotides (TFOs) and double-stranded small interfering RNAs (siRNAs) offer great promise for the treatment of many severe and debilitating diseases [1-7]. ODNs that control gene expression at translation levels are known as antisense ODNs, whereas those ODNs that act at transcriptional level by forming triplexes with genomic DNA are called TFOs. Whereas the former is an antisense strategy, the latter falls under antigene strategy as it is targeting the gene. There has been tremendous progress in the understanding and application of antisense ODNs since they were first proposed in 1978 by Zamecnik and Stephenson [8]. A number of different ODNs have been in clinical trials against many diseases such as human immunodeficiency virus (HIV) infections and cytomegalovirus (CMV) ocular infections, as well as in the control of haematological disorders including Crohn's Disease [2]. Several antisense ODN-based formulations are in clinical trials and there is already one FDA-approved product, formivirsen (Vitravene®, ISIS Pharmaceuticals), for the treatment of human CMV-induced retinitis [9].

In addition to antisense and antigene ODNs, antisense RNA, ribozymes, peptide nucleic acid (PNA) and siRNA can also be used for inhibition of gene expression. Antisense RNA strategy relies on the transfection and subsequent expression of a plasmid carrying the cDNA of the gene of interest subcloned into the vector in an antisense orientation [10]. The expressed antisense RNA, which is capable of hybridising exclusively with the mRNA of the gene of interest, will thus block protein synthesis. Ribozymes, also known as catalytic RNA, are RNA molecules having

catalytic enzyme activity to cleave single-stranded RNA in a sequence-dependent manner [11]. PNA is an ODN analogue in which the deoxyribose phosphate backbone of DNA is replaced by an achiral polyamide backbone. PNAs have been demonstrated to bind to DNA or RNA with high affinity [12]. RNA interference (RNAi) is the sequence-specific gene-silencing induced by double-stranded 21 – 23 nucleotide (nt) long siRNA [13]. Although antisense RNA and ribozymes are also quite promising, these strategies will not be discussed here.

It is a challenging task to deliver specifically ODNs to target cells in various organs and tissues. The following problems associated with antisense ODNs and TFOs should be addressed before they can be used as pharmaceuticals. First, the *in vitro* and *in vivo* stability of ODNs is a problem because native phosphodiester (PO) ODNs are subject to rapid degradation by serum and cellular nucleases from the 5′ and/or 3′ terminus [14,15]. Many structural modifications have been made in their backbones to improve their stability so that they can reach their targets in intact forms [16,17]. Second, due to their polyanionic properties, nucleic acids are taken up by the cells by a combination of fluid-phase, adsorptive and receptor-mediated endocytosis [18]. However, the uptake of free ODNs into various organs in the body is still not efficient to provide therapeutic benefits. Many strategies have been proposed to improve their cellular uptake, including the use of liposomes [19] or polymeric carriers [20], or by direct conjugation with carrier molecules, such as lipids [21], hydrophilic molecules [22] and fusogenic peptides [23,24]. These delivery strategies can improve their ability to interact with cellular membranes. This review will provide an in-depth discussion of the opportunities and challenges of antisense ODNs, TFOs and siRNAs, with the main focus on their delivery issues.

2. Antisense oligodeoxynucleotides

2.1 Mechanisms of action of antisense oligodeoxynucleotides

Antisense ODNs mostly work at post-transcription levels. The length of ODNs is determined based on antisense mechanism and other practical considerations. Antisense ODNs are typically synthesised in lengths of 13 – 35 nt. The minimum ODN size needed to recognise a specific gene is 12 – 15 bases in length. It is estimated that at least 12 bp need to form a stable hybrid with a phosphodiester backbone at physiological conditions.

Normally, transcription of any given gene is carried out by RNA polymerase II (pol II) from a transcription start site in the DNA to give rise to heteronuclear RNA, which is subsequently processed by splicing and polyadenylation to mature mRNA that is trafficked to the cytoplasm where they get translated into proteins. Antisense ODNs are designed to bind to their target sense RNA sequence through the formation of reverse complementary (antisense) strands with the mRNA. The two most likely mechanisms of inhibition of gene expression by antisense ODNs appear to be: i) steric blocking in precursor to mRNA (pre-mRNA) and/or mRNA of sequences important for processing or translation; and ii) degradation of the mRNA by RNase H activated at the site of ODN binding (**Figure 1**) [25,26]. RNase H can cleave the RNA component of RNA–DNA hybrids, and it is abundant in the cytoplasm and nucleus. Nevertheless, the mechanism appears to be more complex than originally thought. Events that are triggered as a result of heteroduplex formation are dependent on the nature of the antisense molecules used for mRNA targeting. ODNs of many, but not all, types support the binding of endogenous RNase H at sites of RNA–DNA duplex formation [27,28]. Such binding is thought to be an important effector of antisense actions because, once bound, RNase H functions as an endonuclease to recognise and cleave the RNA moiety in the hybrid (**Figure 1**). Of significant interest also is the fact that the DNA comprising the duplex is undamaged by the enzymatic attack. Therefore, it is free to hybridise with multiple RNA molecules, thus leading to their destruction in a catalytic manner.

Phosphorothioate (PS) ODNs are thought to activate RNase H efficiently, whereas methylphosphonate (MP) ODNs do not support the activity of this enzyme at all. PO, PS and MP ODNs hybridise to pre-mRNA in a sequence-specific manner [26]. Duplexes formed with PO and PS ODNs are susceptible to cleavage by RNase H, whereas RNA in the duplexes formed with an MP ODNs is resistant to cleavage by RNase H. The resistance of MP ODNs to RNase H decreases with the number of methylphosphonate deoxynucleotides in the MP ODNs [26]. Eukaryotic RNase H generally requires the DNA portion of the duplex to have five or six consecutive internucleotide linkages that can be recognised by RNase H, and is highly active against duplexes with 9 – 10 consecutive internucleotide linkages [25,29,30]. Keeping this in mind, ODNs are often synthesised to contain nuclease-resistant modifications at the 3′ and 5′ ends of the oligo, and six to eight unmodified or phosphorothioate-modified linkages in the central portion. These chimeric ODNs inhibit 3′ and 5′ exonuclease degradation, while still serving as a substrate for RNase H [25,29]. However, RNase H may produce unanticipated, non-sequence-dependent effects by cleaving transiently formed duplexes, or with sites of partial complementarity. Therefore, this 'non-targeted' cleavage can compromise the sequence specificity of RNase H-competent ODNs.

Antisense ODNs containing four contiguous guanosine residues should be employed with cautions as they often lead to G-quartet formation via Hoogsteen base pairing that can decrease the available ODN concentration, and might result in undesired side effects. Modified guanosines (e.g., 7-deazaguanosine, which cannot form Hoogsteen base pairs) may be used to overcome this problem [17]. In addition, the ODN may show reduced water solubility if it contains more than seven total guanines or more than three contiguous guanines in a 25-mer ODN [31].

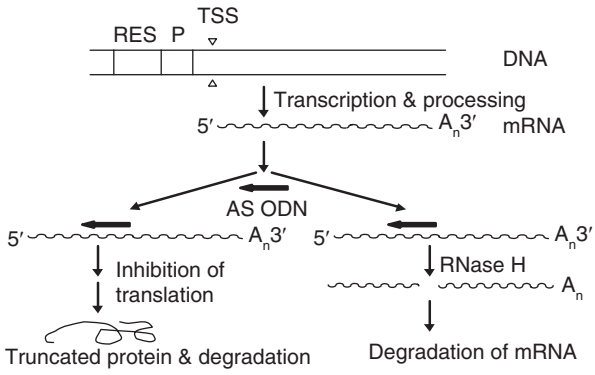

Figure 1. Mechanisms of action of antisense oligodeoxynucleotides. Translational arrest by blocking the ribosome and degradation of mRNA by RNase H cleavage.
AS ODN: Antisense oligodeoxynucleotide; P: Promoter;
RES: Regulatory elements; TSS: Transcription start site.

2.2 Structural modifications

Antisense ODNs must reach their targets intact to modulate gene expression by binding to mRNA to form DNA–RNA hybrid duplexes in the cells. However, endonucleases and exonucleases attack natural ODN molecules at the phosphodiester bridges and break them down to mononucleotides. An enormous amount of literature exists on improving the stability of these ODNs in all the relevant subunits, such as sugar, base and phosphate moieties to create selective affinity for RNA or duplex formation to enhance the ability to cleave nucleic acid targets, nuclease stability, cellular uptake and biodistribution.

2.2.1 First-generation oligodeoxynucleotides

The first-generation ODNs were designed to make the internucleotide linkages more resistant to nuclease attack. This is accomplished primarily by replacing one of the non-bridging oxygen atoms in the phosphate group with either sulfur or a methyl group. The former modified forms are called PS ODNs and the latter as MP ODNs [2]. MP ODNs are neutral in charge and lipophilic in nature. In addition to being nuclease resistant, MP ODNs are usually taken up by cells in a more efficient manner. However, these modified ODNs have not been widely used, probably because: i) they are poorly soluble in water; and ii) they do not activate RNase H-mediated cleavage of the mRNA to which the molecule may be hybridised, resulting in loss of significant antisense effect.

PS ODNs are by far the most extensively studied and significant progress has been made in this modification, which resulted in one commercial product (Vitravene, treatment of CMV retinitis in people with acquired immunodeficiency syndrome [AIDS]) [32] and several promising clinical trials [33]. PS ODNs have a half-life of ~ 9 – 10 h in human serum compared with ~ 1 h for unmodified ODNs [34,35]. In addition to nuclease resistance, PS ODNs form regular Watson–Crick base pairs, activate RNase H in the DNA–RNA hybrid duplex, and display attractive pharmacokinetic profiles [36]. However, the substitution of non-bridging oxygen by sulfur brings several limitations. PS ODNs have chiral centres at the internucleoside phosphorothioates and thus have slightly reduced affinity towards complementary RNA molecules in comparison with the corresponding PO ODNs. On the other hand, they also bind to some key structural proteins, enzymes, receptors, growth factors and transcription factors via ionic or hydrophobic interactions with their modified backbones, which leads to several side effects [37,38]. High doses of PS ODNs in rodents and non-human primates have been shown to be associated with acute haemostatic changes, complement activation and/or coagulation activities, acute renal failure and/or thrombocytopenia [7,39,40]. Low doses of PS ODNs used for clinical trials, however, were generally well tolerated.

2.2.2 Second-generation oligodeoxynucleotides

A number of strategies have been introduced to overcome the limitations of PS ODNs while preserving their useful properties. Using end-capped PS, in which both 3′ and 5′ ends are phosphorothioated, appears to be a reasonable compromise in that exonuclease stability is conferred on the molecules and side effects associated with fully phosphorothioated molecules are significantly reduced [41]. The second-generation ODNs contain nucleotides with the alkyl modification at the 2′ position of the ribose. These ODNs are less toxic than PS ODNs and have enhanced affinity towards their complementary RNAs [42]. However, 2′-*O*-alkyl RNA cannot induce RNase cleavage of the target RNA and their antisense effect can only be due to a steric block of translation. For example, expression of the intercellular adhesion molecule (ICAM)-1 could be inhibited efficiently with an RNase H-independent 2′-*O*-methoxy-ethyl-modified PS ODNs [43].

PS ODNs are often combined with other modifications, especially phosphorothioate 2′-*O*-methyl-ODNs and MP ODNs, to develop mixed-backbone oligodeoxynucleotides (MBOs) [44]. These MBOs have all the required properties for antisense activity with minimal polyanion-related effects [45]. End-modified MBOs, in which other modifications are incorporated at the 3′ end, or at both the 3′ and 5′ ends of PS ODNs, have shown improved specificity, biological activity, *in vivo* stability, and pharmacokinetic and safety profiles, lower polyanion-related effects, and reduced protein binding. Centrally modified MBOs, in which other modifications are in the centre of the PS ODNs, show increased binding affinity, increased RNase H activation and consequently rapid degradation of RNA compared with end-modified MBOs. They also show improved pharmacokinetic and safety profiles [45].

In addition to modifications to the internucleoside bridge, the 2′-deoxy-α-D-ribofuranose unit of the DNA backbone is another site for ODN modification (**Figure 2**). Various sugar modifications have been introduced into antisense ODNs to enhance binding affinity and nuclease resistance. Changing the sugar's glycosidic linkage from the naturally occurring

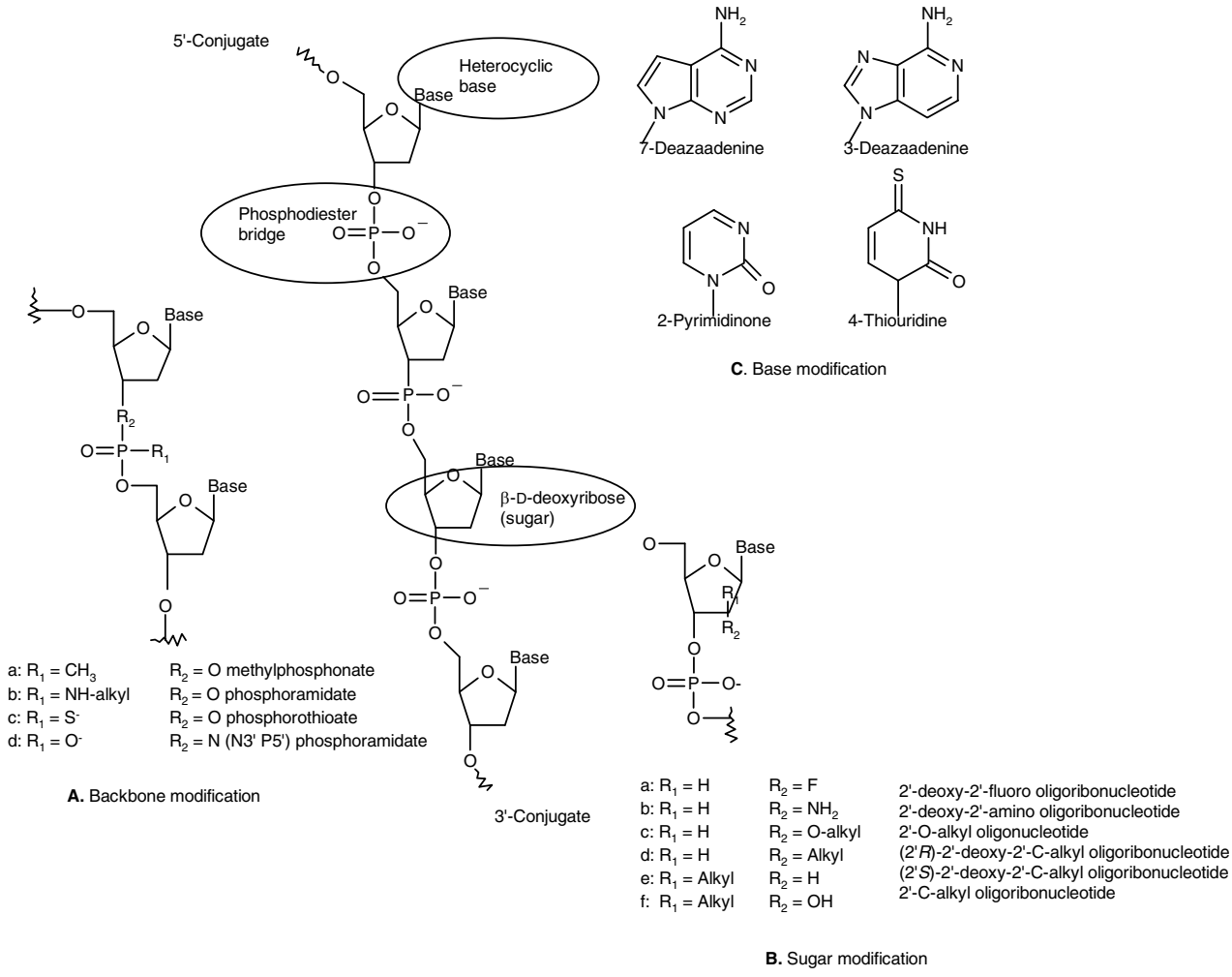

Figure 2. Chemical modification of oligodeoxynucleotides for their improved stability and activity. A) Backbone modification. **B)** Sugar modification. **C)** Base modification.

α-form to the α-anomeric form increases the nuclease stability and compromises hybridisation stability and ability to activate RNase H. Sugars are also typically modified at the 2′ position with *O*-methyl, fluoro, *O*-propyl, *O*-allyl or other groups [46,47]. These modifications have been shown to increase affinity for RNA and impart some nuclease stability. Nevertheless, these molecules do not support RNase H activity and, for this reason, do not appear to have significant antisense activity in some assays.

2.2.3 *Third-generation oligodeoxynucleotides*

DNA and RNA analogues with modified phosphate linkages or ribose's, as well as nucleotides with a completely chemical moiety substituting the furanose ring, have been developed as third-generation antisense ODNs [17]. N3′→P5′ phosphoramidates are an example of third-generation antisense ODNs, in which the 3′-hydroxyl group of the 2′-deoxyribose ring has been replaced with a 3′-amino group. It has a very high binding affinity to complementary DNA or RNA with nuclease resistance. Unlike PS ODNs, N3′→P5′ modified ODNs do not activate RNase H when bound to complementary RNA [48]. Despite inability to activate RNase H, N3′→P5′ phosphoramidate ODNs have shown more potent sequence-specific antisense activity than phosphorothioate derivatives *in vitro* [48] and *in vivo* [49]. Skorski *et al.* (1997) [49] treated severe combined immunodeficient mice with equal doses of phosphoramidate ODNs, PS ODNs and mismatched ODNs. The survival rate of mice treated with N3′→P5′ modified ODNs was significantly higher than PS ODNs and control mismatch ODNs, whereas tissue distribution of phosphoramidate ODNs was similar to that of the PS ODNs. Furthermore, N3′→P5′ phosphoramidate homopyrimidine strands form more stable triplexes with double-stranded RNA than corresponding native DNA [16,50].

2.2.4 *Morpholino oligomers*

Oligomers with morpholino nucleoside linked together by phosphorodiamidate groups are called phosphorodiamidate

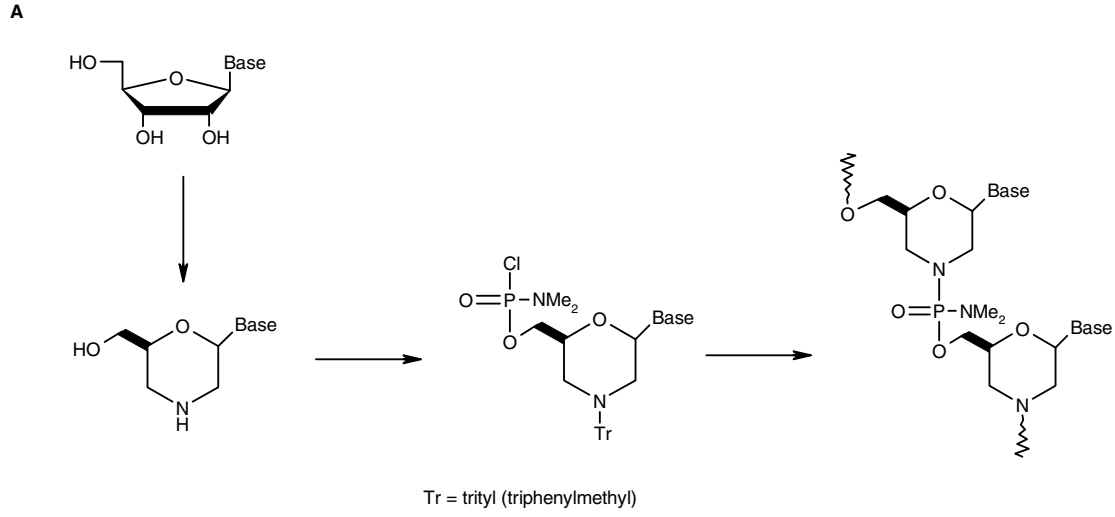

Figure 3A. Morpholino oligomers. Conversion of ribonucleoside to phosphoramidated-linked morpholino oligomers.

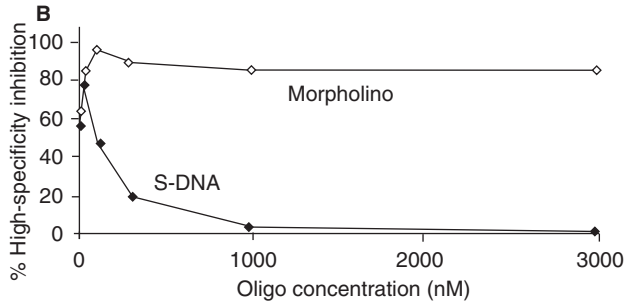

Figure 3B. Morpholino oligomers. Sequence specificity of PMOs and PS ODNs. Reproduced with permission from SUMMERTON J, STEIN D, HUANG SB *et al.*: Morpholino and phosphorothioate antisense oligomers compared in cell-free and in-cell systems. *Antisense Nucleic Acid Drug Dev.* (1997) **7**(2): 63-70 [57].

morpholino oligomers (PMOs) (**Figure 3A**), which are more efficiently assembled than many of the modified ODNs [51,52]. The chemistry of PMOs is different from DNA in that the deoxyribose sugars are replaced with a six-membered morpholine ring and the charged phosphodiester internucleoside linkages are replaced with nonionic phosphorodiamidate linkages, which renders a nonionic charged structure. PMOs have good aqueous solubility and are extremely stable to nucleases and proteases in biological fluid [53], but are sensitive to degradation after prolonged exposure to low pH. Despite possessing a chiral phosphorodiamidate linker, PMOs form heteroduplexes with complementary RNA, which are stronger than the corresponding native DNA–RNA duplexes.

The mechanism of action of PMOs involves steric blockade of the progression of the initiation complex down the 5′ cap or ribosomal assembly at AUG site. In addition, PMOs can also interfere with intron–exon splicing of pre-mRNA, which can suppress aberrant splicing pattern and restore correct splicing [54,55]. Another advantage of PMOs is the high mRNA sequence specificity. Because PMOs are designed to target the 5′ leading sequence of mRNA, which is less conserved than coding regions, the chance for PMOs to block incorrect mRNAs nonspecifically is less than for traditional ODNs. The second reason for the high specificity is the fact that PMOs cannot block the translation of mRNA if there are several mismatched nucleotides. The third reason is that PMOs do not activate RNase H, which can result in cleavage of many partially complementary sequences other than the target sequence [56,57].

PMOs have been shown to be more effective antisense agents than PS ODNs in cell-free systems and in various cultured cells. Summerton *et al.* [57] observed that although PS ODNs are largely sequestered in the nucleus, PMOs distribute more evenly throughout the cell. Transcription and processing of a pre-mRNA in the nucleus and transport of the mature mRNA to the cytosol typically occur within minutes, whereas translation of mRNA in the cytosol typically continues for hours to days. Therefore, PS ODNs can only access their target RNA in a short time between transcription and export to the cytosol, whereas PMOs can access their target RNA during the RNA's residence in both the nucleus and the cytosol. The difference in target access times might contribute to the high efficacies of PMOs compared with the low efficacies of PS ODNs. They also demonstrated that PMOs are highly effective and have specific antisense activity over a concentration range more than two orders of magnitude greater than the concentration range wherein the corresponding PS ODNs achieved reasonable efficacy and specificity (**Figure 3B**). Even in the case of their poor cellular uptake, PMOs, specific for the tumour necrosis factor-α (TNF-α) in mouse macrophages, were shown to be more effective than PS ODNs [58].

By interfering with pre-mRNA splicing, PMOs could induce missplicing. More recently, a 28-mer PMO targeted to c-*myc* pre-mRNA, overlapping the initiation codon, was shown to inhibit c-Myc protein expression completely in several human leukaemia cell lines following treatment with streptolysin O [59]. In addition to inhibiting splicing, missplicing of the c-*myc* pre-mRNA gave rise to a translatable mRNA, which resulted in a shorter, N-terminal deleted, Myc protein. In contrast, a fully modified 2′-methoxyethoxy ODN, which has high affinity for complementary RNA, did not result in inhibition of translation, splicing or induce missplicing of c-*myc* pre-mRNA in the same cell lines. PMOs can disrupt caliciviral gene function in a sequence-specific manner and are potentially effective antiviral agents [60].

The cytochrome P450 (CYP) family is the most catalytically versatile component of the phase 1 oxidation metabolic pathway and participates in the metabolism of most drugs used in clinical practice. Therefore, the inhibition of specific enzymes of this family can significantly alter the disposition and toxicity of substrate drugs by reducing and/or redirecting their metabolism. The use of PMOs in CYP inhibition has been reviewed comprehensively by Arora *et al.* [61]. The use of PMO strategies to target CYP enzymes may result in safer and more uniform therapeutic applications. Inhibition of CYP3A4 expression using AVI-4557, a 20-mer PMO, was examined in primary human hepatocytes and in Caco-2 cells [62]. AVI-4557 inhibited expression of CYP3A4 in Caco-2 cells by 64% at 24 h following administration of 2.8 μM by an assisted delivery protocol. Inhibition of CYP3A4 activity was observed in primary human hepatocytes after 24 h exposure to AVI-4557 by an average of 32 ± 11%. Furthermore, AVI-4557 exposure resulted in a sequence-dependent inhibition of cyclophosphamide-related cytotoxicity and a sequence-dependent induction of paclitaxel-related cytotoxicity in both cell types.

The PMOs also have significant advantages over other ODN analogues for oral delivery. When bioavailability of two distinct PMO sequences (AVI-4126 and AVI-4472) targeted to c-*myc* and CYP3A2, respectively, were investigated, 78.8% of the dose (3 mg/kg) of AVI-4126 was detected in plasma over 10 min through 24 h. There was a sequence-specific reduction of the target protein in the liver. AVI-4472 caused a sequence-specific reduction of approximately fivefold in the rat liver CYP3A2 protein levels after oral administration [63].

In a preclinical mouse model [64], a 20-mer PMO complementary to the hepatitis C virus internal ribosome entry site was shown to inhibit the luciferase reporter mRNA expression by > 95% for at least 6 days, and the antisense effect was specific and dose dependent. To increase the cellular uptake of PMOs, the HIV Tat peptide was attached to the PMOs [24]. Fluorescence was seen in 100% of HeLa cells treated with Tat–PMO conjugate. Most Tat–PMO conjugate was associated with cell membranes, and internalised conjugate was localised in vesicles, cytosol and nucleus. Tat–PMO conjugate targeted to c-*myc* mRNA downregulated c-*myc* reporter gene expression with an IC_{50} of 25 μM, and achieved nearly 100% inhibition. Tat–PMO conjugate targeted to a mutant splice site of α-globin pre-mRNA dose-dependently corrected splicing and upregulated the reporter gene expression. However, Tat-mediated PMO delivery required higher concentrations of PMO (> 10 μM) to cause antisense activity and caused some toxicity.

High sequence specificity, biostability, low toxicity and good solubility make PMOs good candidates for therapeutic application. Using a mouse model, Iversen *et al.* [65] investigated the morpholino oligomer AVI-4126, which inhibits c-*myc* expression, for the treatment of prostate cancer, and found a 75 – 80% reduction in tumour burden compared with the scrambled oligomer and saline control groups. Furthermore, they conducted the Phase I safety study in humans after systemic administration of AVI-4126 and showed no toxicity or serious adverse events.

2.3 Modification of mRNA splicing pathways

Some antisense compounds can be used for modification of the splicing pattern of pre-mRNA rather than for downregulation of gene expression by targeting the mRNA [66]. The shift in splicing ascertains sequence specificity, and because splicing takes place in the nucleus, the observed effects must result from nuclear antisense activity of ODNs. Schmajuk *et al.* [66] developed an application of antisense compounds that can modify the splicing pathway of α-globin mRNA by blocking aberrant pre-mRNA splice sites resulting from mutations in intron 2 of the human α-globin gene. In humans, these mutations lead to α-thalassemia: an inherited blood disorder. Blocking of the aberrant splice sites by antisense agents redirects the splicing machinery to the correct splice sites, thus restoring the proper splicing pathway and, therefore, the correct expression of the damaged gene. These authors demonstrated that morpholino oligomers were threefold more effective than 2′-*O*-methyl-oligoribonucleotides, and 6- to 9- and almost 200-fold more effective than PS and PO ODNs, respectively. Morpholino oligomers were taken up by the cells more effectively than 2′-*O*-methyl oligodeoxynucleotides. To further improve the antisense effect, Sazani *et al.* [67,68] compared the antisense activity of oligomers with 2′-*O*-methyl (2′-*O*-Me) phosphorothioates, 2′-*O*-methyloxyethyl (2′-*O*-MOE) phosphorothioate, morpholino oligomers and PNA backbones using a splicing assay in which the modified oligomers blocked aberrant and restored correct splicing of modified enhanced green fluorescent protein (EGFP) pre-mRNA, generating properly translated EGFP. In this approach, antisense activity of each oligomer was directly proportional to EGFP upregulation. The uptake and antisense efficacy of neutral morpholino derivatives and cationic PNA were much higher than that of negatively charged 2′-*O*-Me and 2′-*O*-MOE phosphorothioates. The effect of PNA oligomers was dependent on the number of L-lysine residues at the C-terminus. The PNA-4-oligomers (PNA with four lysines linked at the C-terminus) showed superior antisense activity *in vivo*, whereas PNA oligomers with only one lysine were inactive [68].

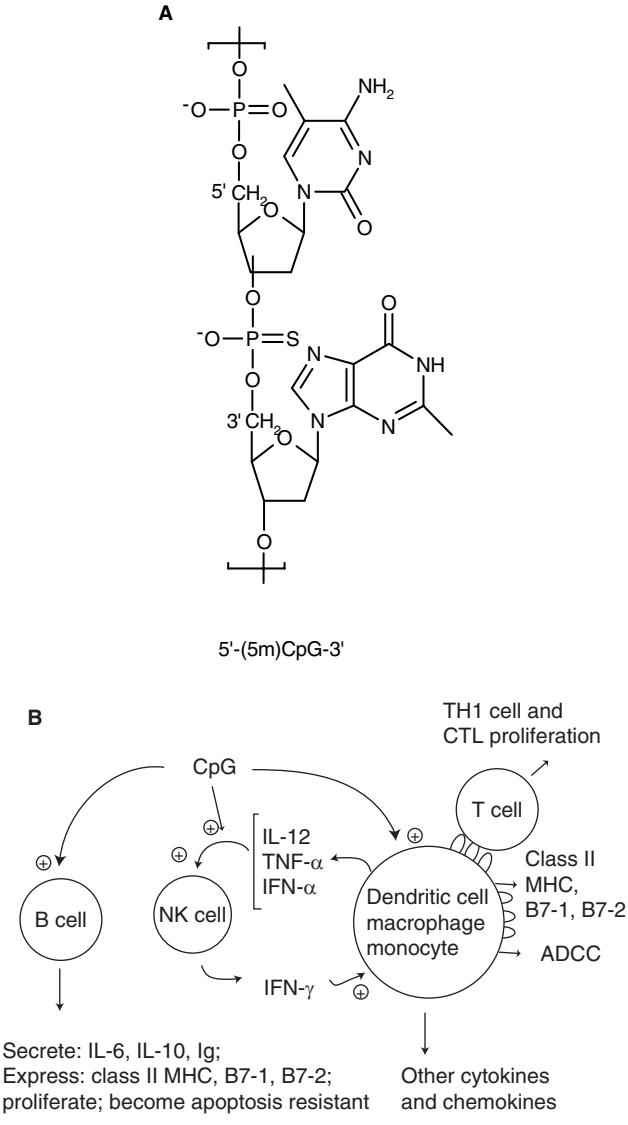

Figure 4. Structure and functions of CpG motifs. A) Structure. **B)** Role of CpG motifs in immunostimulation.
ADCC: Antibody-dependent cellular cytotoxicity; CTL: Cytotoxic T lymphocyte; MHC: Major histocompatibility complex; TH: T helper.

2.4 CpG motifs and immunostimulation

Most antisense ODNs contain dinucleotide sequence 5′-cytosine guanosine-3′, commonly known as CpG motif. These CpG motifs can elicit an innate immune reaction [69,70]. However, methylation of the cytosine in CpG dinucleotide completely abrogated any immunostimulatory properties, confirming that the immunostimulatory properties of these ODNs were dependent on the presence of the unmethylated CpG dinucleotides [71]. As discussed in detail by Krieg *et al.* [72], CpG motif-containing ODNs have been shown to induce (**Figure 4**):

- Toll-like receptor (TLR)9 to initiate signalling pathways that activate several transcription factors, including NF-κB and activator protein 1 [73].
- The direct activation of murine and human B cells, resulting in IL-6 and IL-10 secretion, major histocompatibility complex (MHC) class II and B-7 upregulation and resistance to apoptosis.
- The direct activation of macrophages, dendritic cells and antigen-presenting cells, resulting in the release of IFN-γ and IL-12.
- The activation of NK cells, resulting in the rapid induction of IFN-γ.
- The indirect influence on the activation of CD4 and CD8 T cells.

Therefore, CpG motif-containing ODNs are being explored as immunomodulators in antiviral, antibacterial, anticancer and anti-inflammatory therapies [74]. Following intratumoural injection, CpG-containing ODNs abolish the immune privilege of tumours by recruiting and activating local dendritic cells and inducing IL-12 production. Therefore, CpG-containing ODNs can be used for cancer treatment [75]. Several studies have demonstrated the potential effectiveness of CpG-containing ODNs as therapies in animal models, and a number of CpG-containing ODNs are currently being tested in clinical trials either alone or in combination with antibodies, vaccines and allergens for the treatment of cancers, allergies and asthma [76,77].

The 5′ and 3′ ends of CpG DNA have different effects on its immunostimulatory activity. Kandimalla *et al.* [78] conjugated a CpG DNA through their 5′ ends (5′–5′-linked DNA) and 3′ ends (3′–3′-linked DNA) with an ODN. 5′–5′ linkage significantly reduces the immunostimulatory activity, whereas 3′–3′ has an insignificant effect. Cellular uptake experiments indicated that the difference between 5′- and 3′-end conjugation is not due to the differences in their cellular uptake properties. The reduction of immunostimulatory activity of 5′-linked CpG DNA depends on the conjugated ODN size. Therefore, to achieve optimal immunostimulatory activity, ligands should not be conjugated to the 5′ end of CpG DNA. On the contrary, blocking the 3′ end with some chemical modifications or conjugating the 3′ end with ODNs can increase the stability of CpG DNA without comprising the immunostimulatory activity. Yu *et al.* [79] reported 'PO-immunomers' having two PO–CpG DNA joined through their 3′ ends. Compared with CpG DNA, PO-immunomers are more stable to nucleases in culture medium containing 10% fetal bovine serum. With improved stability *in vivo*, PO-immunomers exhibited potent antitumour activity in nude mice bearing human breast (MCF-7) and prostate (DU145) cancer xenografts.

3. Antigene oligodeoxynucleotides

3.1 Mechanisms of action of antigene oligodeoxynucleotides

In contrast to antisense ODNs, TFOs inhibit gene transcription by forming DNA triple helices in a sequence-specific manner on polypurine–polypyrimidine tracts [5].

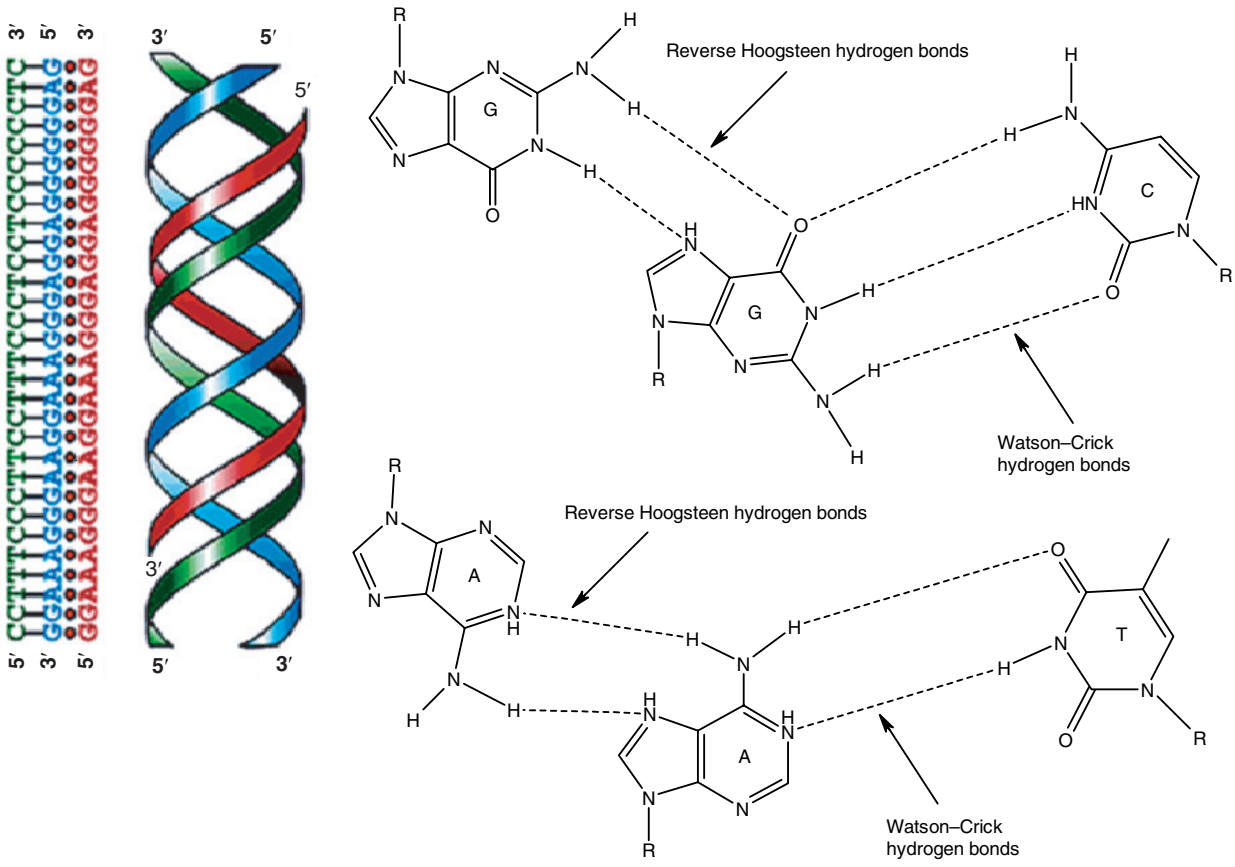

Figure 5. Pyrimidine and purine motifs for triple helix formation.

Targeting ODNs to the gene itself presents several advantages compared with antisense ODNs, which are directed to mRNAs [80,81]. There are only two copies (two alleles) of the targeted gene, whereas there are thousands of copies of an mRNA. Blocking mRNA translation even by inducing sequence-targeted cleavage of the RNA does not prevent the corresponding gene from being transcribed, thereby repopulating the RNA pool. In contrast, prevention of gene transcription is expected to bring down the mRNA concentration in a more efficient and long-lasting way.

Inhibition of gene transcription depends on the residence time of the TFO on its target sequence, as well as its nuclease sensitivity. One of the difficulties in designing a TFO is its accessibility to the target sequence in the chromatin structure of the cell nuclei.

DNA normally exists in a duplex form, but under some circumstances DNA can assume triple helical (triplex) structures, which are either intramolecular or intermolecular. Intermolecular triplexes are formed by the addition of a sequence-specific third strand to the major groove of the duplex DNA [82]. Triplex formation may then prevent the interaction of various protein factors required for transcription, or it may physically block the initiation or elongation of the transcription complex.

TFOs are of 10 – 30 nt in length and require runs of purines on one strand and pyrimidines on the other for stable hybridisation. TFOs may consist of either pyrimidine bases to form the PyPuPy triplex or predominantly purine bases to form PyPuPu triplexes, depending on the nature of the target sequences (**Figure 5**). TFO bases form reverse Hoogsteen hydrogen bonds only with adenines and guanines, thus forming base triads. An A or a T in the TFO can bond with the A of an A•T pair in the DNA duplex, whereas G can bond with the G of a G•C pair. C can also bond with the G of a G•C pair after protonation at the N3-position in slightly acidic media (pH < 6). Depending on the relative orientation of the third strand (R), two types of triplex structures can be formed. In the pyrimidine (or Y:R:Y) motif, a homopyrimidine oligodeoxynucleotide binds in a direction parallel to the purine strand by Hoogsteen hydrogen bond, with canonical base triplets of T:A:T and C:G:C. In the alternate purine motif (R-R-Y), a homopurine strand binds antiparallel to the purine strand by reverse Hoogsteen hydrogen bonds, with base triplets of A:A:T and G:G:C [5,83].

A (G,T)-motif TFO is also permitted, whose orientation depends on both the number of GpT or TpG steps, and the length of G and T tracts. Some TFOs have been designed to

'switch' strands when blocks of homopurine sequences alternate between strands at the duplex DNA target sites. Based on the studies of TFOs performed so far, it appears that in the absence of chemical modification a TFO sequence should contain at least 20 bases in order to bind its target site with sufficient affinity so as to achieve biochemical effects.

Guanine-rich TFOs can form intra- or intermolecular four-stranded structures involving G-quartets; these structures are favoured when the sequences containing repeats of consecutive guanines. (G,A)-TFO can form other intermolecular structures, such as parallel homoduplexes involving A•A, G•G and G•A base pairs. Monovalent cations, such as K^+ present under physiological conditions, enhance the formation of quadruplexes but decrease the formation of a parallel homoduplex in the presence of divalent cations [84]. To overcome this K^+ effect several strategies have been employed. In one, some of the G residues within a G-rich TFO were replaced with 6-thioguanine. Although this modification reduces G-quartet formation, it also lessens the overall binding affinity of the third strand. Similarly, the replacement of the N-7 of guanine with carbon, creating 7-deazaguanine, eliminates the ability of the TFO to form G-quartets, but also decreases the capacity of the ODNs to form triple helices [85]. Cytosine-rich TFO can form, under acidic pH conditions, four-stranded structures called iDNA, which involve hydrogen bonded pairs between cytosine and protonated cytosine ($C•C^+$).

3.2 Stability of triplex DNA

Triplex formation involves the binding of a negatively charged third strand to a double negatively charged duplex, and cations have been proved to play an important role in triplex formation. For phosphodiester ODNs, magnesium is generally required and neutralisation of charge repulsion is typically provided experimentally by levels of Mg^{2+} ions (5 – 10 mM) that are much higher than what is thought to be available in cells (~ 0.8 mM) [5,86]. In contrast, monovalent cations (Na^+, K^+) at physiological concentrations (140 mM) inhibit triplex formation and favour G-quartet formation. To overcome the limited ability of phosphodiester TFOs to form triplex formation under physiological conditions with low magnesium and high potassium concentration, Lacroix *et al.* used morpholino TFOs to demonstrate triplex formation in the absence of magnesium [87]. Results indicated that the pyrimidine motif is the preferred motif for morpholino TFOs to form triplexes.

Triplex formation involves conformational changes on the part of the third strand, and some distortion of the underlying duplex. Pyrimidine motif triplexes are unstable at physiological pH because of the requirement for cytosine protonation that occurs at relatively acidic pH (pK_a = 4.5). Pyrimidine motif triplexes containing adjacent cytosines are often less stable than those with isolated cytosines. In addition, purine motif third strands (which are G rich) may form G tetrads in physiological levels of K^+, which inhibit triplex formation. All these factors impose kinetic barriers on triplex formation and reduce the stability of triplexes once formed [5].

Binding constants in the order of 10 – 100 nM are likely to be required for efficient TFO activity in cultured cells and *in vivo*. Considerable research efforts have been devoted to increase the stability of triple helices under physiological conditions. These include overcoming the pH dependence in C-containing TFO, substitution of thymines in TFOs to avoid the less stable T:A*T triplet, and minimisation of self-associated structures, which compete with triplex formation, especially in the case of G-rich or C-rich TFOs.

Neutralisation of electrostatic repulsion among DNA strands associated in triplex may increase triplex forming efficiency. In this regard, Ferdous *et al.* introduced comb-type copolymers of poly (L-lysine) with polysaccharide to stabilise the triplex [88], which were shown to form reversible complexes with TFO [89]. The T_m value of DNA triplex was increased up to 15°C in the presence of this comb-type copolymer depending on the grafting degree of dextran chains; and the negative effect of K^+ on triplex formation was almost completely abrogated [90].

Several intercalating agents, such as acridine, psoralen and anthracycline derivatives, have been developed to increase the half-life of the triplex in physiological conditions without compromising the specificity of the TFO. Non-sequence-specific intercalating agents are often directly conjugated to the 5′ or 3′ end or to internal positions to stabilise triplexes, especially those containing one or two base interruptions in the purine-rich TFOs. Carbone *et al.* [91] have investigated a TFO modified with an anthracycline derivative, daunomycin, which is a potent DNA-intercalating agent. Binding of daunomycin resulted in increased triplex stability and biological activity of the TFO.

Some chemical agents can be attached to TFOs to introduce irreversible modifications on the DNA targets. For example, psoralen derivatives are remarkable by their *in vitro* efficacy at inducing triplex-mediated crosslinking and cleavage reactions, respectively, on a duplex target, such that a durable biological effect can be obtained [80].

Several modified bases have been substituted for cytosine in order to extend formation of homopyrimidine ODN-directed triplexes under the physiological pH ranges. Some modifications intended to increase ODN nuclease stability such as changing the anomeric configuration of glycosidic bond from β to α do not disturb the triplex stability. Substitution of deoxyribose for ribose in the TFO can stabilise triplexes for the (C,U)- or (G,U)-motif in a parallel orientation with respect to the purine target sequence in duplex DNA. The stability of triplexes involving 2′-*O*-alkyl analogues is even higher than that of PS ODNs. Backbone modifications, such as phosphoramidates and PNAs have also been exploited. Analogues containing N3′→P5′ phosphoramidate linkages highly stabilise triplexes formed with (T,C)- and (T,G)-motif TFOs [92]. However, triplex stability is greatly reduced in the

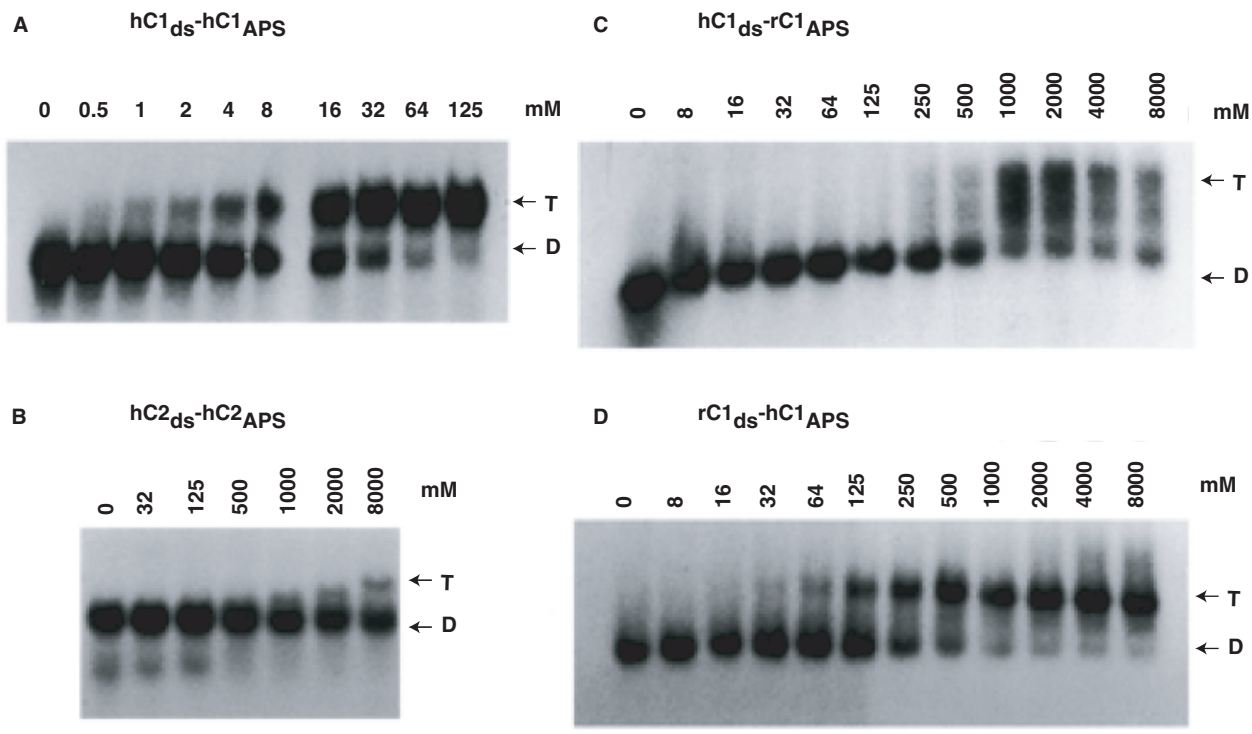

Figure 6. Electrophoretic mobility shift assays demonstrating triplex formation with human and rat antiparallel phosphorothioate triplex-forming oligodeoxynucleotides. Either human [(A and C) hC1ds;(B) hC2ds] or rat [(D) rC1ds] double-stranded oligodeoxynucleotides were radiolabelled and aliquots were incubated with human (hC1 APS) or rat (rC1 APS) TFOs and analysed on polyacrylamide gels. Reproduced from NAKANISHI *et al. Nucleic Acid Res.* (1998) **26**:5218-5222, by permission of Oxford University Press.

APS TFO: Antiparallel phosphorothioate triplex-forming oligodeoxynucleotide; D: Duplex; T: Triplex.

pyrimidine motif using phosphorothioates, whereas purine motif by this modification appears as stable as PO.

3.3 Transcriptional inhibition by triplex formation

TFOs have to reach the nucleus and compete with all proteins binding to DNA. Accessibility of target sites in the context of chromatin is crucial for the development of the antigene strategy. Transcriptional active genes might be accessible to triplex-mediated regulation, especially when the target sequences for ODNs are located in the same DNA domain as transcription factor binding sites. TFOs bind efficiently to the PyPu tract. If the PyPu tracts are localised in the coding regions of the gene, then TFO binding presents a physical block to the progressing RNA polymerase that cannot unwind the triplex. This results in the inhibition of mRNA synthesis and, therefore, synthesis of the functional protein. If a TFO binds in the promoter region, several events may occur to inhibit or activate gene expression. Other enzymes involved in transcription, replication, recombination and repair processes, such as helicases and topoisomerases, can also be disturbed.

Sequence-specific effects can be obtained by mechanisms other than the expected one. This is easily explained by the fact that some TFOs adopt highly structured conformations that can be recognised by specific proteins; G-rich TFOs are particularly prone to such effects. In most of the reports, control TFOs of different length or composition, or with disrupted tracts of $(G)_n$ are not satisfactory and definitive demonstration of a triplex-mediated mechanism is generally lacking. The best control experiment actually uses the same TFO, but a mutated target sequence that affects triplex stability but not gene expression. However, this is easy to achieve when the target gene is carried by a plasmid, but more difficult with endogenous genes. For example, the c-*myc* oncogene was first shown to be modulated by a TFO forming triplexes in the promoter region. However, a mechanism other than triplex formation was subsequently proposed: titration of one of the transcription activators by the purine-rich TFO could fully account for the observed decrease of c-*myc* RNA transcription. Recently, a covalent triplex was reported to enhance recombination processes between two tandem *sup*F genes present on a plasmid construct, in mammalian cells.

Although TFO has been proven to modify gene expression successfully *in vitro*, to provide a therapeutic benefit, TFO must be able to bind specifically to chromosomal target in an intact animal. Vasquez *et al.* [93] tested AG30 (specific TFOs bind to polypurine site in the *sup*FG gene) and controlled TFO (SCR30) in mice for 5 consecutive days via intraperitoneal injection. There was substantial induction of mutagenesis in many

tissues in transgenic animals for AG30, whereas no induced mutagenesis was detected in any of the tissues for SCR30. These findings provided the initial evidence that sequence-specific TFO can be useful in intact animals for therapeutic purposes.

3.4 Regulation of transcription of Type I collagen gene for treatment of fibrosis

The major structural protein of the extracellular matrix is Type I collagen, which is the heterotrimer product of two α1(I) and one α2(I) polypeptide chains encoded by the α1(I) genes found on human chromosomes 17 and 7, respectively [94-96]. The entire region spanning from -140 to -200 bp exists as an asymmetrical polypurine–polypyrimidine tract, in which the polypyrimidine sequence at -141 to -170 is called the C1 region, which is localised on the non-coding strand, and the adjacent polypurine sequence from -171 to -200 is called the C2 region, which is present on the coding strand [97]. The *cis*-acting elements in the C1 and C2 regions play a key role in collagen transcription, and hence make it an ideal target for developing antigene-based antifibrotic agents [98,99].

Using parallel and antiparallel TFOs of 18 – 30 nt in length corresponding to the C1 sequence, the authors found that TFOs specific for C1 form triplexes much more efficiently than C2, and that the antiparallel polypurine TFOs form triplexes 100- to 1000-times more efficiently than parallel TFOs. The antiparallel 18-mer TFOs (-158 to -141 or -164 to -147) form triplexes at a K_d similar to the 30-mer TFOs, and the effective K_d is at least 100 times lower for antiparallel compared with parallel TFOs. In contrast to the polypurine TFOs, there was no appreciable triplex formation for 18-mer polypyrimidine TFOs at physiological or slightly acidic pH in the presence of polyvalent cations. The authors have also found that the efficiency of triplex formation with phosphorothioate TFOs is comparable with that with phosphodiester TFOs. Of the three TFOs used corresponding to the C1 region, 158 antiparallel phosphorothioate (APS) appears to form a triplex at a lower concentration than 164 APS, whereas 170 APS formed a triplex at a slightly higher concentration than 164 APS. The physiological milieu that exists within a cell has been reported to be unfavourable for purine motif triplex formation, possibly due to the high potassium concentration (140 mM), which promotes aggregation of guanine-rich ODNs into guanine quartets [84,100]. Hence, the ability of 158 APS to form triplex under conditions closely simulating those in the living cells was tested [97]. C1 TFOs formed stable complexes even under physiological conditions (1 – 5 mM Mg^{2+}, 140 mM KCl).

It is important to determine whether the TFO is specific for C1 region of Type I collagen α1(I) promoter and not for other sequences present in the rodent and human genomes. The overall sequence organisation of C1 and C2 regions of the human α1(I) procollagen promoter is similar to that of rat and mouse promoters; however, there are eight nucleotide changes in the human α1(I) sequence compared with rat or mouse. These nucleotides are -141, -142, -145, -150, -153 to -155, and -165. Of these eight nucleotides, alteration of nucleotides -141 to -143 resulted in the association constant as evidenced by C1 antiparallel or C1 APS. Most of the nucleotide differences in the human C1 sequence are A $\rightarrow$ G, which could be responsible for triplex formation with higher efficiency. Antiparallel TFO to rat C1 forms stable triplexes with a K_d of ~ 100 nM, and TFOs as short as 18 nt could form stable triplexes. Similar analysis with human C1 indicated the formation of triplexes with a K_d of ~ 10 – 20 nM (**Figure 6A**). Antiparallel TFO to human C2 formed triplexes, but very inefficiently at extremely high concentrations (> 8000 nM) (**Figure 6B**). Because the human C1 sequence is different from the rat or mouse sequence, the formation of triplexes by these heterologous TFOs was tested. The human C1 double-stranded probe was incubated with rat C1 TFO and vice versa. Results indicate that although rat TFO formed less stable triplexes at very high concentrations (> 1000 nM) (**Figure 6C**), human C1 APS formed stable triplexes with rat C1 duplex DNA with a K_d of ~ 200 nM (**Figure 6D**), suggesting that the TFO rather than the duplex DNA is important to drive stable triplex formation. Therefore, the sequence organisation and composition are very important in determining the specificity of triplex formation [101].

C1-specific (-141 to -170) antiparallel TFOs in the range of 18 – 30 nt in length significantly inhibited transcription of the α1(I) collagen promoter activity in *in vitro* cell-free transcription systems as well as in rat-transfected fibroblasts in culture. pCOL-CAT 220, containing the reporter gene chloramphenicol acetyltransferase (CAT) driven by the rat α1(I) collagen promoter from -220 to +100, was transfected into rat $2tk^-$ fibroblast cells, which express high levels of Type I collagen [97]. This was followed by transfection with specific APS TFO, and cellular extracts were assayed for CAT activity. The results clearly indicated that both 158 and 164 APS TFOs form triplexes and significantly inhibit transcription in rat cells (~ 50% of control) at a TFO-to-plasmid ratio of 125:1. 164 APS TFO showed a more pronounced effect. CAT activity was reduced by 70% compared with the control ODN (5′-CAT GGA GCC ACA TTC ATG-3′) at a TFO-to-plasmid ratio of 125:1, with a further reduction in activity (80%) on increasing the ratio to 250:1. These results clearly demonstrate significant inhibition of collagen promoter function on triplex formation of the target region with 158 APS and 164 APS, with 164 APS showing a more dramatic effect [97].

4. RNA interference

RNAi is the phenomenon in which siRNA of 21 – 23 nt in length silence a target gene by binding to its complementary mRNA and triggering its elimination. It was initially discovered by Fire *et al.* in *Caenorhabditis elegans* in 1998 [102] that small double-stranded RNAs can lead to potent targeted degradation of complementary mRNA. This finding generated huge interest in the application of siRNA for biomedical

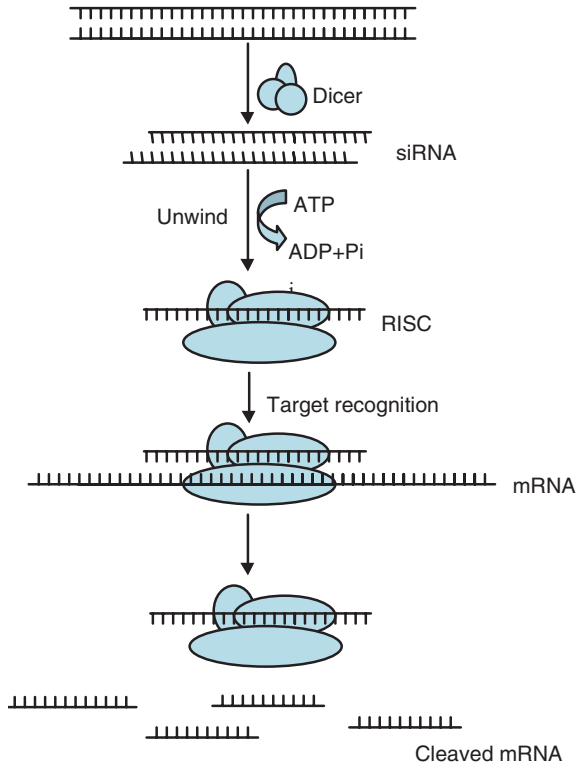

Figure 7. Mechanism of RNA interference. Long double-stranded RNA is cleaved by Dicer into fragments of 21 – 23 nucleotide siRNAs. ATP-dependent helicase unwind the duplex siRNA and one strand of siRNA is incorporated into a multi-protein RISC. Subsequently, the incorporated siRNA strand guide RISC to its homologous target mRNA for endonucleolytic cleavage.
RISC: RNA-induced silencing complex; siRNA: Small interfering RNA.

research [103]. Potent knockdown of the targeted gene with high sequence specificity makes RNAi a powerful tool for studying gene function and a promising therapeutic strategy for some diseases.

4.1 Mechanism and strategies of RNAi

RNAi has most likely evolved as a mechanism for cells to eliminate unwanted foreign genes, and its physiological function lies in viral defence, transposon silencing and regulation of developmental pathways [104,105]. Similar RNAi exists in plants, fungi and throughout eukaryotic organisms: from worms to humans [106]. Extensive attempts have been made to provide better understanding of the mechanism of RNAi.

The silencing process by siRNA occurs post-transcriptionally in the cytoplasm [107] and it is an ATP-dependent and translation-independent event. The current RNA-induced silencing complex (RISC) model is shown in **Figure** 7. The first step is the production of double-stranded RNA directed against an mRNA [108]. The second step is the cleavage of the double-stranded RNA by the endogenous enzyme Dicer into fragments of 21 – 23 nt siRNAs. The third step is the unwinding of the duplex siRNA using an ATP-dependent helicase. One strand of siRNA is then incorporated into a multi-protein RISC, which is the key step in RNAi [109,110]. Subsequently, the incorporated siRNA strand guides RISC to its homologous target mRNA for endonucleolytic cleavage [111]. One confusion about RNAi's mechanism is that only one of the strands from siRNA is incorporated into RISC, whereas biologists previously believed that both of the siRNA strands were incorporated. The RISC assembly is governed by an enzyme that selects which strand of an siRNA is loaded into RISC. The stability at the 5′ ends of the two siRNA strands determines which one enters RISC [112], and the strand with a less stable 5′ end is more likely to be incorporated. To be functional against target mRNA, the antisense strand must be the one incorporated into RISC, whereas the sense siRNA strand directs cleavage of an antisense target [112].

There are several strategies to introduce siRNA into mammalian cells for the application of RNAi in gene knockdown and therapeutic uses. The most commonly used strategy is to chemically synthesise double-strand siRNAs of 21 – 23 nt for transfection into cells. There are five advantage of synthesised siRNAs: i) it provides precise control of the amount and purity of siRNA used in the experiment; ii) it can be characterised; iii) modifications can be introduced into the siRNA to enhance its target specificity; iv) high transfection efficiency into the cells of interest; and v) the initiation of siRNA-transfected silencing is immediate [111,113]. With the help of rapidly evolved siRNA synthetic chemistry and siRNA sequence design software, most current protocols for RNAi in mammalian systems use chemically synthesised siRNAs.

Although RNAi provides a powerful tool for inhibition of targeted genes, there are several concerns and limitations in the practical application of this technology [114]. The transfection methods used for ODNs and plasmids can also be applied for siRNA delivery. However, the transfection conditions should be optimised for different cell types to achieve maximum gene silencing. Milhavet *et al.* [105] summarised several parameters that could affect transfection and gene silencing efficacy: i) cell culture conditions, cell density and medium composition; ii) amount and type of transfection agent; iii) quality and amount of siRNA; and iv) exposure time of the cells to siRNA.

On the other hand, the use of chemically synthesised siRNA is limited by the fact that different sequences have significantly different inhibitory abilities [115]. Consequently, many different synthetic siRNAs have to be screened for their silencing ability for a targeted gene, which is laborious and costly [116]. An alternative approach is to use long double-stranded RNAs that have either been partially digested with *Escherichia coli* RNaseIII to give a mixture of short siRNAs with lengths of 18 – 30 nt (endoribonuclease-prepared siRNAs: esiRNAs) [117], or digested by Dicer *in vitro* [118]. The advantage of esiRNAs is that digestion of long double-stranded RNAs results in cleavage of a larger portion of the mRNA, which increases the chance for sequence-specific gene

silencing. However, esiRNAs prepared from long double-stranded RNAs requires not only each potential siRNA to be analysed for its specificity for the target gene, but possible cross-silencing with other genes must also be avoided.

As there is no indication of siRNA replication in mammals [107], the transduction of siRNA into cells leads to only a transient knockdown of the target gene, which limits its application in *Drosophila* and mammals [111]. Consequently, plasmid DNA and viral vectors have been developed to express double-stranded RNA that can be converted into siRNA *in vivo*. Compared with synthesised siRNAs, expression vectors can produce siRNAs continuously in cells. In addition, viral vectors have been shown to have higher transfection efficiency, especially in non-dividing cells [119].

In the plasmid-based expression vector, hairpin RNA can be expressed in cells under the control of an RNA pol II (CMV) [120] or polymerase III (pol III) (U6 or H1) [121,122]. Although Pol II promoters can induce tissue-specific RNA expression, pol II expresses long hairpin RNA that can induce the IFN response in many mammalian cells [111]. Therefore, the application of the pol II promoter was limited and most of the current plasmid-based expression systems use a pol III promoter. Pol III expresses short hairpin RNA species and does not induce IFN responses like pol II. Brummelkamp *et al.* [121] designed a mammalian expression vector (pSUPER: suppression of endogenous RNA) using a pol III H1-RNA promoter. It produces a small RNA transcript lacking a polyadenosine tail and has a well-defined transcription start and termination signal. The cleavage of the transcript after the second uridine at the termination site resulted in a transcript resembling the ends of synthetic siRNAs, which contain two 3′ overhanging U or T nucleotides. Northern blot analysis indicated the stem-loop precursor transcript (49 nt) was generated and rapidly cleaved into functional siRNAs after transfection with pSUPER vector. The vector showed efficient specific downregulation of target gene and the suppression of gene was persistent. Typically, a single promoter is used to express a hairpin precursor transcript. However, the hairpin vectors have some limitations: i) hairpins are hard to synthesise in bacteria; ii) sequencing is difficult; and iii) length and sequence of hairpin can affect the gene silencing ability of siRNA [123]. To overcome these limitations of hairpin vectors, dual promoter siRNA expression vectors have been developed [123,124]. Kaykas *et al.* [123] constructed a convergent opposing siRNA expression system (pHippy), which contains two opposing pol III (H1 and U6), to express both strands of a template DNA inserted in between the promoters and form a double-stranded RNA with two 5′ uridine overhangs that mimic naturally occurring siRNA produced by Dicer. Results indicated that the dual-promoter vector can generate functional siRNAs in mammalian cells to knockdown the target gene.

Although plasmid-based siRNA expression vectors have been successfully used in different cell types, these vectors usually have low transfection efficiency in non-dividing and primary cells [111]. To overcome the problem of poor transfection efficiency of plasmid-based siRNA expression vectors, viral vectors for siRNA delivery are being developed. Adeno-associated viral (AAV) vectors [125], adenoviral vectors [126], retroviral vectors [127] and lentiviral vectors [119,128-130] have been reported for siRNA delivery. Tomar *et al.* [125] modified a commercially available AAV vector for p53 siRNA delivery under the control of an H1 or U6 RNA promoter. Results showed a significant downregulation of p53, but not caspase 8, for H1 promoter, whereas U6 promoter showed downregulation in both p53 and caspase 8. Results suggested the potential of AAV vector for the therapeutic use of siRNA. Zhao *et al.* [126] used an adenovirus-based vector to express siRNA targeting p53 of VprBP/KIAA0800 (a cellular protein that interacts with the HIV auxiliary protein Vpr). Specific reduction in the targeted protein concentration was demonstrated after transfection and it correlated with the specific reduction in the mRNA concentration. Lentiviral vector can express integrated siRNA efficiently in a wide variety of actively dividing, non-dividing and primary cells, both *in vitro* and *in vitro*. An *et al.* [119] reported a lentiviral vector to express siRNA stably in human cells. They inserted a U6 pol III transcriptional unit into a self-inactivating lentiviral vector and results indicated a 16- to 43-fold reduction of gene expression in infected cells. Matta *et al.* [128] modified a self-inactivating lentiviral vector for siRNA delivery and showed efficient transfection of this modified vector into HeLa S3 cells and downregulation of p53 gene expression.

4.2 Sequence design of siRNA

The selection of siRNA for a target gene is a crucial step for its application. It should, however, be mentioned that the selection of siRNA is an empirical process because the rules that govern efficient siRNA silencing are still unknown [111]. Nevertheless, Ambion (Houston, TX) recommended four guidelines for the design of siRNAs: i) beginning with the AUG of the target gene transcript, search downstream for AA dinucleotide sequences, each AA and the 3′ adjacent 19 nt are potential siRNAs; ii) blast the potential sequences against the sequences in the species-appropriate genome database to eliminate cross-silence phenomenon with non-target genes; iii) select three to four target sequences along the gene for production of siRNAs; and iv) for all siRNA experiments, negative control siRNAs with the same nucleotide, but a scrambled sequence [105]. Ui-Tei *et al.* [131] proposed another four practical guidelines for sequence design of siRNA in mammalian cells: (i) A/U at the 5′ end of the antisense strand; (ii) G/C at the 5′ end of the sense strand; (iii) at least five A/U residues in the 5′ terminal third of the antisense strand; and (iv) the absence of any GC stretch of > 9 nt in length (**Figure 8**). These guidelines closely related to the molecular mechanism of RISC assembly.

Using web-based software and bioinformatics is the current method for scientists to design siRNAs. For example, based on the above guidelines, Naito *et al.* [132] presented a web-based software, siDirect [201], to compute highly effective

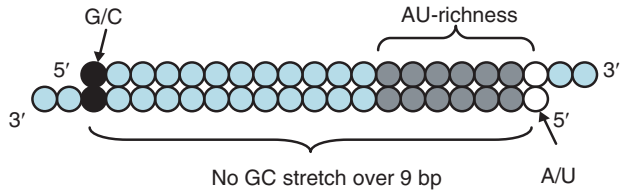

Figure 8. Guideline to design an effective siRNA. (i) A/U at the 5′ end of the antisense strand; (ii) G/C at the 5′ end of the sense strand; (iii) at least five A/U residues in the 5′ terminal; (iv) no more than 9 nt for GC length. Modified from NAITO Y, YAMADA T, UI-TEI K *et al.*: siDirect: highly effective, target-specific siRNA design software for mammalian RNA interference. *Nucleic Acids Res.* (2004) **32**(Web Server issue): W124-129, by permission of Oxford University Press [132].
siRNA: Small interfering RNA.

siRNA sequence with maximum specificity. The siDirect algorithm incorporated the four guidelines to favour efficient mammalian RNAi with a high success rate. In addition, it investigates all the potential cross-hybridisation to avoid off-target gene silencing effects.

Bioinformatics approaches have also been used to help select optimal siRNAs. Yuan *et al.* [133] built a web-based tool [202] that implements several algorithms to identify siRNAs with a high probability of silencing the target gene. One advantage of the server is that they keep incorporating rules from new results, which provides the biologist access to the newest design features.

To design esiRNAs, Henschel *et al.* [116] developed a web-based tool, DEQOR [203]. The program mimics esiRNAs by fragmenting the input sequence into small pieces (16 – 25 nt), whereby the sequence window is shifted along the input query by 1 nt at each iteration step of the algorithm. Each simulated siRNA will be: i) analysed using a scoring system based on the state-of-the-art parameters for its ability to induce specific gene silencing; and ii) analysed for its ability to cross-silence genes different from the target by performing BLAST searches against the transcriptomer or genome of the studied organism.

4.3 Therapeutic applications of RNAi

RNAi has emerged as a powerful tool in gene knockdown experiments to understand gene function and it is currently being investigated for therapeutic purposes because of its ability to specifically silence a disease-related gene [13,134,135]. In mammalian cells, double-stranded RNA > 30 nt activates antiviral cellular pathways that turn off protein synthesis and activate proapoptotic proteins and ultimately kill the infected cells [134]. To overcome the non-gene-specific effects, chemically synthesised siRNA should be used for mammalian cells.

The application of siRNA for cancer therapy is to silence the cell cycle gene expression and/or the antiapoptotic gene to eliminate cancer cells [105]. siRNAs have been used to silence the epidermal growth factor receptor expression in cultured cells and reduced proliferation and apoptosis. Brummelkamp *et al.* [136] utilised a retroviral siRNA expression vector to suppress the oncogenic K-*RAS*^{V12} allele in human tumour cells. K-*RAS*^{V12} has a single base mutation from the wild-type K-*RAS* allele. Results showed the siRNAs specifically silence the mutant K-*RAS*^{V12} in human pancreatic carcinoma without affecting the wild-type K-*RAS*. Inhibition of K-*RAS*^{V12} expression leads to loss of anchorage-independent growth and tumorigenicity. The study suggests that siRNAs can be applied for tumour-specific gene therapy to reverse the oncogenic phenotype.

Song *et al.* [3] investigated the *in vivo* silencing effect of siRNA targeting the gene *fas* (encoding the Fas receptor) to protect mice from liver failure and fibrosis. The synthetic siRNA was injected into mouse by hydrodynamic tail vein injection and 88% of hepatocytes took up the siRNA after 24 h. Both *fas* mRNA expression level and Fas protein expression in mouse hepatocytes were reduced and the effects persisted for 10 days without diminution. Furthermore, the treatment of *Fas* siRNA protected mice from fulminant hepatitis and hepatic fibrosis in two models (**Figure 9**). **Figure 9A** shows there was no hepatic fibrosis or necrosis in the siRNA-treated group, whereas the controlled group exhibited bridging fibrosis in the liver parenchyma. Both hepatic hydroxyproline (chemical indicator of active fibrosis) and serum procollagen Type III of siRNA-treated groups were reduced significantly compared with the controlled group (**Figure 9B**). The results suggested the therapeutic promise of siRNA to prevent liver injury by protecting hepatocytes from cytotoxicity.

4.4 Off-target activity of siRNA

In spite of the rapid adoption of siRNA for gene function analysis and therapeutic application, challenges still exist. siRNA is reported to induce IFN and sequence-independent suppression of both endogenous and exogenous gene expression. There is increase in IFN-α and TNF-α gene expression in macrophages and dendritic cells. TLR3 responds to siRNA with the production of Type I IFN, IL-8, TNF-α and activation of NF-κB promoters, and cytokine production is increased when HEK293 cells overexpress TLR3 [137].

siRNA was assumed to be highly specific for target mRNA and even a single base mismatch was believed to protect the mRNA from degradation [138]. However, the specificity of siRNAs in mammalian cells has not been comprehensively investigated and several off-target effects of siRNAs have been reported recently. For example, Jackson *et al.* [139] determined the efficacy and specificity of siRNA-induced silencing of two genes involved in signal transduction: the insulin-like growth factor 1 receptor (IGF1R) and eight siRNAs to target mitogen-activated protein kinase 14 (MAPK14), by designing 16 siRNAs to target the coding region of IGF1R and eight siRNAs to target MAPK14. The transcript expression patterns were found to be siRNA specific rather than target specific. An siRNA targeted to luciferase regulated the expression of several genes without homologous target in human genome. Further concentration and kinetic analysis indicated

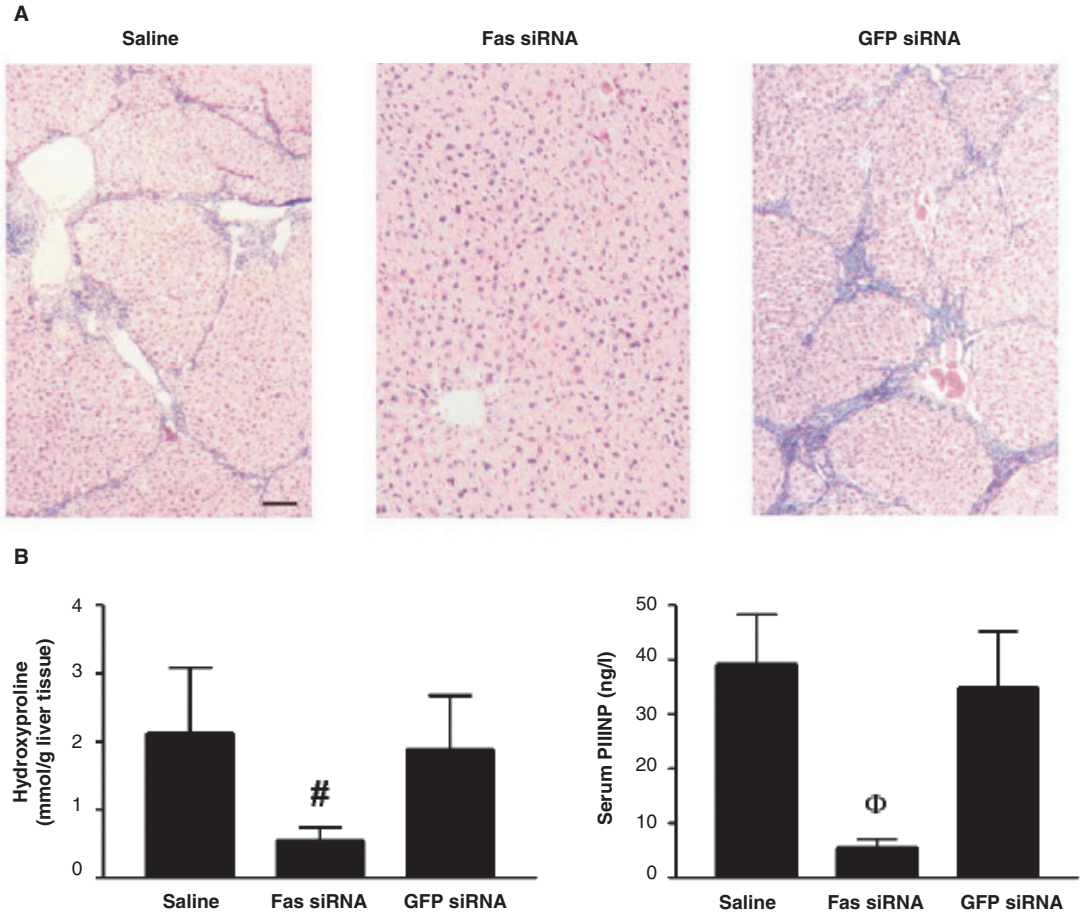

Figure 9. *Fas* gene silencing protects mice from fulminant hepatitis and hepatic fibrosis. Liver histology of mice after six weekly injections of ConA and treatment of Fas siRNA or control solution. Fas siRNA-treated group protect mice from fibrotic damage whereas control treatment did not. Used with permission from SONG E, LEE SK, WANG J *et al.*: RNA interference targeting Fas protects mice from fulminant hepatitis. *Nat. Med.* (2003) **9**(3): 347-351, copyright © Nature Publishing Group [3].
GFP: Green fluorescent protein; siRNA: Small interfering RNA.

the off-target effect is not an artifact of high siRNA concentration. Scacheri *et al.* [140] investigated the specificity of siRNA-mediated gene silencing by transfecting 10 different siRNAs corresponding to a single gene (multiple endocrine neoplasia Type I [*MEN1*]) by examining the expression of two additional genes, *TP53(p53)* and *CDKN1A(p21)*, in four different human cell lines. Whereas *MEN1* encodes a tumour suppressor of menin, *p53* and *p21* are considered functionally unrelated to menin, but function in a vast number of related and unrelated cellular pathways. These proteins were used as sensitive detectors for off-target effects mediated by siRNA in this study. The authors observed dramatic and significant changes in protein levels of p53 and p21 that are unrelated to the silencing of target gene. This result suggests that siRNAs can induce nonspecific but sequence-dependent effects, which are presumably caused by acting on other unknown targets.

Snove *et al.* [141] investigated 359 published siRNA sequences and found that about 75% of them have risk of eliciting nonspecific effects. Use of the popular BLAST search engine for the design of siRNA could account for this problem as BLAST is not appropriate for short oligos such as siRNAs. With the help of a new special purpose processor, the Pattern Matching Chip (Interagon; Trondheim, Norway), they showed that many unique siRNAs existed per target, and argued that the risk of off-target effects is unnecessary and can be avoided during the siRNA design. However, their *in silico* study was not proved by direct experiment testing. The off-target effects emphasise the need to spend more effort in the design and careful validation of siRNAs before the experiment.

5. Pharmacokinetics of oligodeoxynucleotides

The biodistribution of ODNs has been studied intensively in rodents and non-human primates. For their action at a target site within the body, there are many biological barriers to be overcome: moving from the bloodstream into tissues, from the extracellular space, across the plasma membrane and into

the cytoplasm, and from the cytoplasm into the nucleus. To construct strategies for establishing efficient and safe delivery systems for ODNs, a thorough understanding of their *in vivo* disposition characteristics is necessary.

Unmodified PO ODNs are rapidly degraded by serum and cellular nucleases. Following intravenous administration, ODNs are rapidly eliminated from the circulation and are widely distributed to most peripheral tissues, with the liver, kidney, bone marrow and muscle accumulating the most [142-144]. PS ODNs, possibly due to the extensive binding to the plasma proteins, accumulate much less in the kidney compared with PO ODNs. Takakura *et al.* examined the hepatic disposition characteristics of 20-mer PO ODNs and their partially (PS_3 ODNs, in which three internucleotide linkages at the 3′ end are phosphorothioated) and fully phosphorothioated derivatives in the isolated rat liver perfusion system after bolus injection into the portal vein. The magnitude of the hepatic interaction of ODNs increased as the extent of PS modification in the molecules increased: about 20, 36 and 52 of the injected dose was taken up by the liver during a single passage after bolus injection of PO, PS_3 and PS, respectively [144-146]. PS ODNs are mainly taken up by the liver endothelial cells, and to some extent by hepatocyte and Kupffer cells [147,148].

Sawai *et al.* determined the renal disposition characteristics of ^{32}P-labelled 20-mer ODNs using the isolated rat kidney perfusion experimental system [142]. Binding of the ODNs to bovine serum albumin (BSA) in the perfusate prior to kidney perfusion was in the following order of magnitudes: PS > PS_3 > PO. ODNs showed a sulfur-atom-dependent interaction with renal vasculature; hence, the volume of distribution (V_d) increased as the extent of PS modification increased. A significant amount of the ODNs filtered through glomeruli underwent tubular reabsorption, which might be mediated by the interactions with specific proteins in the brush border membrane. PS ODNs are accumulated in a non-filtering kidney, suggesting that there is also uptake from the basal side [149].

Gross tissue distribution of PS ODNs is independent of sequence. Plasma clearance rates are largely species independent in rat, rabbit, dog and monkey, and have been estimated to be 1 – 3 ml/min/kg [150,151]. DeLong *et al.* compared the pharmacokinetics and tissue distribution of PS, phosphorodithioate (PS_2) and MP ODN analogues, which were 15 nt ODN complimentary to the AUG region of k-ras [152]. These ODNs were injected intravenously as a single dose used in nude mice bearing a K-ras-dependent human pancreatic tumour. There was a rapid distribution phase with half-life α-values of 1 min and an elimination phase with average half-life α-values of 24 or 35 min. V_d values were 3.2, 4.8 and 6.3 ml for PS_2, MP and PS, respectively, compared with 3.6 ml for sucrose: a fluid-phase marker. In general agreement with previous studies, relative tissue drug levels obtained at 1 and 24 h after administration were kidney > liver > spleen > tumour > muscle. Total kidney and liver ODN accumulation was ~ 7 – 15% of the initial dose, with tumour accumulating 2 – 3%. Intact compound was recovered from all tissues, including tumour. Importantly, integrity of the ODNs ranged from 73% in blood to 43% to 46% in kidney and liver, which appear to be the primary sites of metabolism.

Studies have been also performed on patients with leukaemia and AIDS. In six HIV patients, after 2-h intravenous infusions (0.1 mg/kg) of PS ODNs, plasma disappearance curves could be described by the sum of two exponential, with half-life values of 0.18 ± 0.04 and 26.71 ± 1.67 h. Urinary excretion represented the major pathway of elimination, with ~ 50% of the administered dose excreted within 24 h and ~ 70% eliminated > 96 h after dosing. Intact and degraded material was found in the urine. The half-lifes of the ODNs were shorter than those observed in experimental animals.

6. Cellular uptake of oligodeoxynucleotides

Cellular uptake of ODNs appears to be an active process dependent on cell type, time, concentration, energy, temperature, saturation, and sequence and type of ODNs [1,153,154]. ODNs need to cross the plasma membrane, be released from endosomes after endocytosis, migrate to nucleus and pass through the nuclear membrane. Cellular uptake of ODNs is highly variable and dependent on cell type and cell cycle [155]. MP ODNs are uncharged molecules that enter cells via passive diffusion [156], although the rate of diffusion across the lipid bilayer membrane is extremely slow [157]. In contrast, PO and PS ODNs are taken up by the cells via receptor-mediated endocytosis at lower concentrations, but via a fluid-phase endocytosis at higher concentrations.

As noted, endosomal release is another barrier for the cellular uptake of ODNs. Microinjection of fluorescent-labelled ODNs into the cytoplasm has been shown to result in their rapid accumulation in the nucleus. In contrast, when cells were incubated with fluorescent-labelled ODNs, these labelled molecules were seen to accumulate mainly in the endosomal/lysosomal vacuoles, with a small percentage in the perinuclear membranes, but little inside the nucleus. This indicates that the release of ODNs in the cytoplasm and their translocation to the nucleus is an inefficient process.

Several synthetic carriers have been developed to enhance the cellular uptake of ODNs to target cells. For example, cationic liposomes can form complexes with ODNs, and condense and impart an overall positive charge that facilitates adsorption to the cell membrane, whereas the lipid tails enhance subsequent passage through the lipid bilayer of the cell membrane [158,159]. Cationic liposomes/ODN complexes are taken up by endocytosis and ODNs are released from the liposomes into the cytoplasm due to the protonation of the lipids [160]. Once free in the cytoplasm ODNs diffuse into the nucleus by passive diffusion through the nuclear membranes, and the diffusion rate is length dependent [161].

In addition to modification of their backbone to enhance their cellular uptake, ODNs are either conjugated to a ligand specific for a receptor on the target cell or complexed with a carrier molecule containing a targeting ligand. For example, ODNs have been delivered to haematopoietic cells, pulmonary alveolar, hepatocytes, and a variety of tumour cells via receptor-mediated endocytosis, including folate, mannose, asialoglycoproteins, and tumour-specific antibodies [162-166]. Although such modifications have been reported to be effective in certain controlled circumstances, the general applicability of this approach remains to be determined. To make this approach practically successful, several obstacles need to be overcome, including: (i) the inefficient dissociation of ODNs from the carrier after endocytosis; and (ii) uptake by reticuloendothelial and other non-target cells.

Conjugation with peptide or polymer is another promising strategy to enhance the cellular uptake and nuclear translocation of ODNs. Among them, TAT is a peptide derived from HIV-1 TAT protein that can mediate cytoplasmic transport of other compounds to which they are tethered. Nori *et al.* used a TAT-conjugated *N*-(2-hydroxypropylmethacrylamide) (HPMA) copolymer for ODN delivery and demonstrated very high concentrations of ODNs in the nucleus [23].

7. Delivery strategies of oligodeoxynucleotides and siRNA

To enhance the stability, control the pharmacokinetic profiles and facilitate site-specific delivery of ODNs, both macromolecular conjugates and particulate systems are being developed. ODNs can be linked directly to a carrier protein or targeting ligand via a covalent bond or non-covalently via polycation–carrier conjugates. The choice of a carrier protein is dependent on its known ability to bind to specific cell membrane receptors and accumulate in the cell via endocytosis.

To develop a receptor-mediated ODN delivery to different liver cells, the authors first determined the disposition characteristics of ^{35}S-labelled ODNs and their glycosylated poly(L-lysine) (PLL) complexes in mice in relation to their physicochemical properties [135]. Both hepatocytes and Kupffer cells possess receptors on their plasma membranes that specifically bind and internalise D-galactose-containing materials in a size-dependent manner: small particles are efficiently taken up by hepatocytes, whereas large particles are taken up by Kupffer cells [167]. Complex formation with galactosylated poly(L-lysine) (Gal-PLL) enhanced the hepatic uptake of ODNs. Although the uptake of PS/Gal-PLL complexes by hepatocytes was significantly higher than that of naked PS ODNs, the difference between their intracellular distributions was only moderate [135]. The hepatic uptake of PS ODNs/Gal-PLL was partially inhibited by prior intravenous administration of excess Gal-BSA, suggesting that the complexes were taken up by the hepatocytes via galactose receptor-mediated endocytosis. Due to the negative zeta potential (-30 – 40 mV) and wide particle size distribution (150 ± 70 nm), a part of the complexes is likely to be recognised by the galactose receptor of Kupffer cells, by scavenger receptors of the endothelial cells and Kupffer cells as polyanions [168] and/or being phagocytosed by Kupffer cells. Moreover, sulfur atoms present in the PS ODN also influences the nonspecific hepatic uptake of its complexes. In conclusion, the hepatic uptake of ODNs/glycosylated PLL is greatly influenced by the particle size, zeta potential, sugar substitution level, molecular weights of both polycations and ODNs, and types of ODNs [14,135]. The particle size of ODN delivery systems must be very small so that they can easily pass through the sinusoidal gaps for efficient delivery to hepatocytes and hepatic stellate cells. Moreover, the formulated ODN particles should not carry excess positive charge on their surface in order to avoid being taken up by Kupffer cells before reaching hepatocytes and stellate cells [135]. To minimise nonspecific ionic interaction with plasma proteins and cytotoxicity, poly(ethylene glycol) (PEG) can be used as a spacer between polycations and the targeting ligands.

To avoid the use of polycations by direct conjugation with the ligands, Rajur *et al.* covalently conjugated ODNs to asialoglycoprotein by a stable disulfide linkage [169]. ODNs were also covalently conjugated to carbohydrate cluster for specific delivery to the hepatocytes [170]. This conjugation strategy can also improve the stability of ODNs by introducing ligands and appropriate cationic tethers, which can interfere with interactions between nucleolytic enzymes and ODNs. Consequently, this conjugation approach will have significant influence on the bioactivity of ODNs.

7.1 Conjugation of lipophilic molecules

The hydrophilic character and anionic backbone of the ODNs reduces their uptake by the cells. Therefore, various lipophilic molecules have been conjugated to ODNs [171]. Among them, cholesterol is perhaps the best characterised. Biophysical and pharmacokinetic properties of 20-mer PS ODNs and its cholesterol analogues have been evaluated. As shown in **Figure 10**, cholesterol conjugation to PS ODNs resulted in increased retention in plasma as well as accumulation in various liver cell types, with up to fivefold increase in the uptake by Kupffer cells [172,173]. Cholesterol-conjugated PS ODNs (ISIS-8005) showed greater affinity to serum proteins than free PS ODNs (ISIS-3082). This difference in serum protein binding is responsible for the difference in pharmacokinetics. The concentration in liver was correlated with the therapeutic effect of cholesterol-conjugated PS ODNs as measured by ICAM-1 mRNA levels in mouse liver *in vivo*, whereas free PS ODNs showed little effect [174]. Moreover, the presence of cholesterol moiety at the 3′ end of an ODN did not affect hybridisation with target sequences. However, this 5′-cholesterol conjugate of PS ODNs (ISIS-8005) did not change the stability of ODNs significantly, whereas the 3′-cholesterol conjugate (ISIS-9388) of ISIS-3082 was much more stable [173]. This is because the 3′-hydroxyl group, which is involved in the nucleophilic attack of the adjacent phosphate bond when the

Comparison of hepatic cellular distribution of 20-mer phosphorothioate oligodeoxynucleotides with and without lipid conjugation.

Cell type	Contribution to liver uptake (% of total)		
	ISIS-3082	***ISIS-9388***	***ISIS-9388/ LacLDL***
Kupffer cells	8.3 ± 3.2	14.5 ± 3.1	43.9 ± 5.4
Endothelial cells	62.5 ± 6.2	55.9 ± 7.2	39.4 ± 3.8
Parenchymal cells	29.1 ± 6.3	29.6 ± 8.1	16.7 ± 6.8

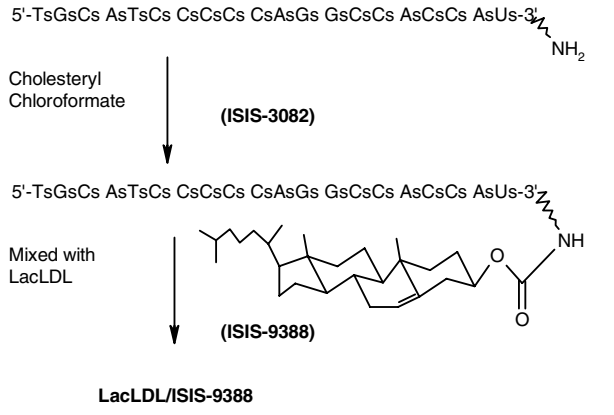

Figure 10. Sequence and hepatic cellular distribution of 20-mer phosphorothioate oligodeoxynucleotides with and without lipid conjugation. Reprinted with some modifications from *Biochem. Pharmacol.*, **62**(5), BIJSTERBOSCH MK, MANOHARAN M, DORLAND R *et al.*, Delivery of cholesteryl-conjugated phosphorothioate oligodeoxynucleotides to Kupffer cells by lactosylated low-density lipoprotein, 627-633. Copyright (2001), with permission from Elsevier [174].

exonuclease enzyme makes a complex with the nucleic acid, is unavailable in ISIS-9388.

Many other studies [21,175] have shown similar effects in increasing the cellular uptake of ODNs when cholesterol was conjugated with ODNs. Cellular uptake of 3′-cholesterol-conjugated ODNs has been examined with a real-time confocal laser microscopy. Cytosolic uptake of cholesterol conjugate was five times as rapid as that of PS ODNs and nuclear uptake of cholesterol conjugate was twice as fast as that of unmodified PS ODN.

The effects of conjugating cholesterol to either or both ends of a PS ODN were compared in terms of cellular uptake and antisense efficacy against the p75 nerve growth factor receptor (p75) in differentiated PC12 cells [176]. The addition of a single cholesterol group to the 5′ end significantly increased cellular uptake and improved p75 mRNA downregulation compared with the unmodified ODN. Although the 3′-choloesterol analogue was still active, *bis*-cholesteryl (5′- and 3′-) conjugated ODN was even more potent. LeDoan *el al.* [177] reported on the interactions of PO ODNs linked to the cholesterol group at internal position. The conjugates were assessed for their capacity to bind, penetrate and partition in the cytoplasmic compartment of murine macrophages and showed similar effects.

In addition to effects on the cellular uptake, cholesterol modulates ODN–mRNA hybrid stability via hydrophobic interactions [178]. An increase in the T_m of up to 13.3°C was observed. Stabilisation of triplexes by up to 30°C due to intercholesteryl interaction using 5′,3′-*bis*-cholesterol-containing ODNs was also observed.

7.2 Conjugation of vitamins

Cells and tissues have specific transport systems for vitamins. Hence, the conjugation of antisense ODNs to vitamins is expected to improve their transport into cells, and a number of vitamins, including folic acid, biotin, retinoic acid and vitamin E, have been investigated [179]. Among them, folic acid is paid most extensive attention. Folate receptor is overexpressed on many cancer cell surfaces, but is highly restricted in most normal tissues. Direct conjugation of folic acid to the 3′ terminus of an anti-c-fos ODN has been shown to increase the ODNs uptake by FD2008 cells that over express folate receptors by eightfold. In contrast, there was no increase in CHO cells that do not express folate receptors [179]. In addition, the inhibitory effect of the ODNs on the growth of FD2008 cells was significantly increased when ODNs were conjugated to folic acid compared with unmodified ODNs.

7.3 Pegylation of oligodeoxynucleotides

Pegylation of ODNs plays an important role in the uptake and biological properties of ODNs. Manoharan *et al.* showed that when an ODN that targets human ICAM-1 has been conjugated to a series of PEG esters of average molecular weight 550, 2200 and 5000, the cellular permeation of ODNs *in vitro* can be interfered with [173]. The effect of the different structures of high molecular weight PEG chains on the biological properties of the conjugated antisense ODNs have been investigated by Bonora *et al.* [180]. Two different conjugates of an anti-HIV 12-mer ODN have been tested for antisense activity in MT-4 cells. Only the ODN conjugated to the linear monomethoxy PEG showed anti-HIV activity. A 20-mer ODN targeting mouse α-globin mRNA has been conjugated at the 5′ terminus to *bis*-aminoalkyl PEG. At 15 μM the conjugate selectively inhibited Hb synthesis in cultured Friend murine erythroleukaemia cells by 95%. Bonora *et al.* [180] conjugated PEG to ODNs at the 5′, 3′, or both 5′ and 3′ termini. The number and attachment sites (3′ terminal, 5′ terminal and internal positions) of coupled ethylene glycol units had great influence on the hydrophobicity and electrophoretic mobility of the conjugates. Pegylation had little effect on the hybridisation behaviour of ODNs. Conjugates with PEG coupled to both 3′ and 5′ terminal positions showed a > 10-fold increase in exonuclease stability of the ODNs.

PLL–PEG block copolymer [181] and PEG–polyethylenimine (PEI) copolymer [182] can be used to form soluble

polyion complexes with ODN and the complex is self assembled through electrostatic interaction. The ODN is entrapped in the core of the complex, the serum protein binding is prevented and the nuclease resistance is improved. Furthermore, complement activation decreases with increase in the number and chain length of PEG [183]. On the other hand, Jeong *et al.* [184,185] conjugated ODN to PEG via a noncleavable amide linkage or acid-cleavage linkage, which can be cleaved in the acidic endosome, then form polyion complex micelles with various cationic peptides (fusogenic KALA peptide) and polymers such as PLL, PEI and protamine. The ODN is complexed with cationic polymers to form an inner core surrounded by PEG on the surface of the complexes. The hydrated PEG chains on the surface minimised the adsorption of serum proteins and prolonged the circulation in the blood. In one study, therapeutic antisense c-*raf* ODN was conjugated to PEG and formed complex micelles with PEI. The complex showed a higher antiproliferative activity against ovarian cancer cells both *in vitro* and *in vivo* [184].

7.4 Multivalent carbohydrate clusters

Carbohydrate cluster conjugates may be used for targeted delivery of ODNs to different liver cells. When an MP ODN was conjugated to the triantennary, *N*-acetylgalactosamine neoglycopeptide, Tyr-Glu-Glu-(aminohexyl GalNAc)$_3$, YEE(ahGalNAc)$_3$, there was a 20- to 40-fold enhancement in the uptake of the ODNs by HepG2 cells [186]. These carbohydrate clusters are known to bind to Gal/GalNAc receptor sites on hepatocytes with high affinity ($K_d \sim 7$ nM). Similarly, when a PS oligomer was conjugated to this glycotripeptide, there was sequence-specific suppression of the integrated hepatitis B viral expression in hepatoma cells (> 90% inhibition) in a dose-dependent concentration range of 1 – 20 μM of ODNs [187].

Galactose is the ligand for the asialoglycoprotein receptor, which is expressed on the surface of liver parenchymal cells. Biessen *et al.* (1999 and 2000) [162,188] constructed four molecules of galactoses on a oligolysine (*N*2-(*N*2-(*N*2,*N*6-*bis*[*N*-[p-(a-d-galactopyranosyloxy)-anilino]thiocarbamyl]-L-lysyl)-*N*6-(*N*-[p-(a-d-galactopyranosyloxy)-anilino]thiocarbamyl]-L-lysyl)-*N*6-(*N*-[p-(a-d-galactopyranosyloxy)-anilino]thiocarbamyl)]-L-lysine, (L3G4)), and attached it to a 20-mer PO ODN 3′-capped with an amine. The ligand has a K_d of 6.5 nM. This L3G4-conjugated ODN was far more efficiently taken up by parenchymal liver cells than underivatised ODN. Studies in rats showed that hepatic uptake was greatly enhanced from 19% for unconjugated ODN to 77% of the injected does after glycoconjugation. Accumulation into liver parenchymal cells was increased by 60-fold.

7.5 Conjugation of polyamines, cationic groups and polypeptides

Polyamine-conjugated ODNs form amphipathic molecules and reduce the net negative charge on ODNs. As these modified ODNs may serve as ligands for polyamine receptors present on certain cells, polyamine conjugation may improve the antisense activity of ODNs. Because the monoalkylamines and at least some of the amino groups of the polyamines are protonated at the physiological conditions, they can also improve the hybridisation rates of antisense ODNs to the target RNA. Corey [189] demonstrated 48,000-fold acceleration of hybridisation by ODNs conjugated to cationic peptides derived from lysines. Cationic polypeptides (polylysine, polyornithine, polyhistidine and polyarginine), due to their positive charges, when conjugated with ODNs, which are negatively charged, can enhance hybridisation of ODNs.

A fusogenic peptide derived from the influenza haemagglutinin envelope protein has been conjugated to antisense ODNs. This peptide changes its conformation at acidic pH and destabilised the endosomal membrane, resulting in an increased cytoplasmic delivery of the antisense ODNs. A similar fusogenic peptide conjugated to an antisense ODN via a disulfide or thioether bond resulted in 5- to 10-fold improvement of the anti-HIV activity on *de novo* infected CEM-SS lymphocytes in serum-free media. However, no sequence specificity was observed and the fusogenic peptide also possessed some antiviral activities on its own.

Although the sequence specificity, biostability and low toxicity of PMOs make them good antisense agents, they are poorly taken up by the cells. Conjugation to positively charged arginine-rich TAT-like peptide has been shown to enhance the cellular uptake of the PMOs significantly. PMO delivery to the cell nucleus and cytosol required conjugation rather than complex formation of peptides to the PMO. Conjugation of NH_2-RRR RRR RRR-FFC-$CONH_2$, designated R9F2 peptide, to the PMO resulted in the strongest antisense effect. This particular peptide-conjugated PMO was more effective than the PMO conjugated to the transmembrane transport peptides of HIV-1 TAT protein [24]. Moreover, antiviral effect of PMO was significantly increased following conjugation to the R9F2 peptide. In contrast, peptide conjugate did not result in antiviral function of the nonsense PMOs [190]. However, R9F2–PMO conjugate was ineffective in the presence of culture medium containing 10% fetal bovine serum, raising a question about the utility of intravenous or orally administered R9F2–PMO conjugate *in vivo* [190].

siRNAs have also been conjugated to peptide for efficient delivery into mammalian cells. Muratovska *et al.* [191] coupled siRNAs to the membrane permeant peptides (MPP) penetratin and transportan via a disulfide bond that is labile in the reducing environment of the cytoplasm. The MPP–siRNAs reduced transient and stable expression of target gene in several mammalian cell types with equivalent or better delivery characteristics than cationic liposomes. They summarised three advantages of the MPP–siRNAs system: i) MPP–siRNAs are freely translocated into the cytoplasm; ii) the disulfide bond is reduced in the cytoplasm, releasing the siRNA to induce target gene silencing; and iii) the uptake of the conjugate is rapid and occurs directly through the cell

membrane without the help of receptor-mediated uptake, endocytosis or pinocytosis.

8. Concluding remarks and expert opinion

Despite many early problems, nucleic acid-based strategies for modulation of aberrant gene expression offers great hope in the therapy of many severe and debilitating diseases. Whereas antisense ODNs are used for inhibiting translation of mRNA into proteins, TFOs are used for inhibiting gene expression via site-specific gene targeting, and siRNA are used to silence target mRNA. Antisense compounds can also be used for modulation of the splicing pathways by blocking the aberrant pre-mRNA splice resulting from mutations. Although major improvements have been achieved by modifying the backbone, sugar and base of the ODNs to provide high target affinity, enhanced stability and low toxicity, still there are many hurdles in making these ODNs more effective. These include nonspecific protein binding, delivery to the appropriate compartment of the cell where the target is localised, and delivery to the appropriate cell and tissue in the body. Although thousands of papers have been published and several antisense ODNs are in various stages of clinical trials, the outcome is not absolute. This is largely due to the abundance of mRNA, which varies from target to target, half-life of these mRNAs, and other delivery and stability problems.

Gene expression can be modulated at transcription or translation level by site-specific gene targeting. In the antigene approach, ODNs are targeted to the target DNA sequence. Specific regulatory or coding sequences in certain genes have the potential to form triple helices on addition of exogenous TFOs. Depending on the target, these TFOs can cause inhibition of gene expression by inhibiting transcription or introducing mutations at the site of triplex formation. However, the use of TFOs appears to have major limitations, which preclude their use for modulating gene expression of a wide variety of genes. Potential triplex-forming sites are limited in the genomes and target sites in chromosomes may not be accessible to the TFO due to histones and other proteins binding to these sequences. However, for some genes discussed in this review this antigene approach appears to be most promising for modulating gene expression. For example, results from studies of TFO delivery to control liver fibrosis by inhibiting collagen synthesis are quite promising [101].

Compared with antisense ODNs, the effect of siRNA has been shown to be higher and longer [192]. Some of the above mentioned problems have been resolved in the case of ODNs but not for siRNAs. In order to use siRNAs as therapeutic molecules several problems have to be overcome. These include: i) the selection of the best sequence-specific siRNA for the gene to be targeted; ii) the ability to minimise degradation in the body fluids and tissues; and iii) if pol III-mediated vectors are used to generate intracellular siRNAs, then delivery of these vectors to the target tissues poses another hurdle. In spite of these hurdles, there is a great potential for siRNAs in modulating gene expression. As the field is still in its infancy we are confident that in the near future techniques to abate some of these hurdles will be developed.

One of the major obstacles in nucleic acid-based therapeutics is the limited ability to deliver these macromolecules into target cells. Without this, even an appropriately targeted sequence is not likely to be efficient. Nevertheless, these nucleic acids can escape from the endosomal/lysomal compartment, enter the cytoplasm and then diffuse into the nucleus. Macromolecular and particulate delivery systems are being developed for their site-specific delivery and enhanced cellular uptake for transporting these molecules to specific disease targets. Delivery technologies continue to improve, so it is likely that present and/or evolving technologies will be used successfully to deliver optimised nucleic acids to their cellular targets [193]. A thorough understanding of the relationship among their backbone structures, pharmacokinetic profiles, cellular uptake mechanisms and therapeutic activity of these nucleic acids with or without a delivery system will definitely speed up the process of turning these macromolecules into therapeutics.

Acknowledgements

This work was supported by a grant RO1 DK 064366 from the NIH and USPHS grant AR 47379.

Bibliography

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. CROOKE ST: Progress in antisense technology. *Ann. Rev Med.* (2004) **55**:61-95.

•• **Comprehensive review on backbone modification, cellular uptake and biodistribution of ODNs.**

2. OPALINSKA JB, GEWIRTZ AM: Nucleic-acid therapeutics: basic principles and recent applications. *Nat. Rev. Drug Discov.* (2002) **1**(7):503-514.

•• **Excellent review on different types of nucleic acids used for blocking abnormal gene expression.**

3. SONG E, LEE SK, WANG J *et al.*: RNA interference targeting Fas protects mice from fulminant hepatitis. *Nat. Med.* (2003) **9**(3):347-351.

• **An important study demonstrating the therapeutic usefulness of siRNA in hepatitis mouse model.**

4. GUNTAKA RV, VARMA BR, WEBER KT: Triplex-forming oligonucleotides as modulators of gene expression. *Int. J. Biochem. Cell Biol.* (2003) **35**(1):22-31.

5. SEIDMAN MM, GLAZER PM: The potential for gene repair via triple helix formation. *J. Clin. Invest.* (2003) **112**(4):487-494.

6. VASQUEZ KM, GLAZER PM: Triplex-forming oligonucleotides: principles and applications. *Q. Rev. Biophys.* (2002) **35**(1):89-107.

7. CROOKE ST, BENNETT CF: Progress in antisense oligonucleotide therapeutics. *Ann. Rev. Pharmacol. Toxicol.* (1996) **36**:107-129.
8. ZAMECNIK PC, STEPHENSON ML: Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. *Proc. Natl. Acad. Sci. USA* (1978) **75**(1):280-284.
9. CROOKE ST: Vitravene-another piece in the mosaic. *Antisense Nucleic Acid Drug Dev.* (1998) **8**(4):vii-viii.
10. LEE M, HAN SO, KO KS *et al.*: Repression of GAD autoantigen expression in pancreas beta-Cells by delivery of antisense plasmid/PEG-γ-PLL complex. *Mol. Ther.* (2001) **4**(4):339-346.
11. LONG MB, JONES JP, 3rd, SULLENGER BA *et al.*: Ribozyme-mediated revision of RNA and DNA. *J. Clin. Invest.* (2003) **112**(3):312-318.
12. BUCHARDT O, EGHOLM M, BERG RH *et al.*: Peptide nucleic acids and their potential applications in biotechnology. *Trends Biotechnol.* (1993) **11**(9):384-386.
13. WALL NR, SHI Y: SMALL RNA: Can RNA interference be exploited for therapy? *Lancet* (2003) **362**(9393):1401-1403.
14. MAHATO RI, TAKAKURA Y, HASHIDA M: Development of targeted delivery systems for nucleic acid drugs. *J. Drug Target.* (1997) **4**(6):337-357.

• **Detailed information on how physicochemical characteristics influence the biodistribution profiles of ODNs and genes.**

15. WICKSTROM E: Oligodeoxynucleotide stability in subcellular extracts and culture media. *J. Biochem. Biophys. Methods* (1986) **13**(2):97-102.
16. MICKLEFIELD J: Backbone modification of nucleic acids: synthesis, structure and therapeutic applications. *Curr. Med. Chem.* (2001) **8**(10):1157-1179.

•• **Excellent review on how backbone modification of ODNs can enhance their stability and efficacy.**

17. KURRECK J: Antisense technologies. Improvement through novel chemical modifications. *Eur. J. Biochem.* (2003) **270**(8):1628-1644.

• **Comprehensive review on the chemical modification of antisense agents.**

18. AKHTAR S, JULIANO RL: Cellular uptake and intracellular fate of antisense oligonucleotides. *Trends Cell Biol.* (1992) **2**(5):139-144.
19. HU Q, BALLY MB, MADDEN TD: Subcellular trafficking of antisense oligonucleotides and down regulation of bcl-2 gene expression in human melanoma cells using a fusogenic liposome delivery system. *Nucleic Acids Res.* (2002) **30**(16):3632-3641.
20. BULMUS V, WOODWARD M, LIN L *et al.*: A new pH-responsive and glutathione-reactive, endosomal membrane-disruptive polymeric carrier for intracellular delivery of biomolecular drugs. *J. Control. Release* (2003) **93**(2):105-120.
21. ALAHARI SK, DEAN NM, FISHER MH *et al.*: Inhibition of expression of the multi-drug resistance-associated P-glycoprotein of by phosphorothioate and 5′ cholesterol-conjugated phosphorothioate antisense oligonucleotides. *Mol. Pharmacol.* (1996) **50**(4):808-819.
22. JENSEN KD, KOPECKOVA P, KOPECEK J: Antisense oligonucleotides delivered to the lysosome escape and actively inhibit the hepatitis B virus. *Bioconjug. Chem.* (2002) **13**(5):975-984.
23. NORI A, JENSEN KD, TIJERINA M *et al.*: Tat-conjugated synthetic macromolecules facilitate cytoplasmic drug delivery to human ovarian carcinoma cells. *Bioconjug. Chem.* (2003) **14**(1):44-50.
24. MOULTON HM, HASE MC, SMITH KM *et al.*: HIV Tat peptide enhances cellular delivery of antisense morpholino oligomers. *Antisense Nucleic Acid Drug Dev.* (2003) **13**(1):31-43.
25. DAGLE JM, WEEKS DL: Oligonucleotide-based strategies to reduce gene expression. *Differentiation* (2001) **69**(2-3):75-82.
26. FURDON PJ, DOMINSKI Z, KOLE R: RNase H cleavage of RNA hybridized to oligonucleotides containing methylphosphonate, phosphorothioate and phosphodiester bonds. *Nucleic Acids Res.* (1989) **17**(22):9193-9204.
27. DASH P, LOTAN I, KNAPP M *et al.*: Selective elimination of mRNAs *in vivo*: complementary oligodeoxynucleotides promote RNA degradation by an RNase H-like activity. *Proc. Natl. Acad. Sci. USA* (1987) **84**(22):7896-7900.
28. WALDER RY, WALDER JA: Role of RNase H in hybrid-arrested translation by antisense oligonucleotides. *Proc. Natl. Acad. Sci. USA* (1988) **85**(14):5011-5015.
29. DAGLE JM, WEEKS DL, WALDER JA: Pathways of degradation and mechanism of action of antisense oligonucleotides in *Xenopus laevis* embryos. *Antisense Res. Dev.* (1991) **1**(1):11-20.
30. MONIA BP, LESNIK EA, GONZALEZ C *et al.*: Evaluation of 2′-modified oligonucleotides containing 2′-deoxy gaps as antisense inhibitors of gene expression. *J. Biol. Chem.* (1993) **268**(19):14514-14522.
31. HEASMAN J: Morpholino oligos: making sense of antisense? *Dev. Biol.* (2002) **243**(2): 209-214.
32. Fomivirsen approved for CMV retinitis: first antisense drug. *AIDS Treat. News* (1998) **302**:7.
33. ECKSTEIN F: Phosphorothioate oligodeoxynucleotides: what is their origin and what is unique about them? *Antisense Nucleic Acid Drug Dev.* (2000) **10**(2):117-121.
34. CAMPBELL JM, BACON TA, WICKSTROM E: Oligodeoxynucleoside phosphorothioate stability in subcellular extracts, culture media, sera and cerebrospinal fluid. *J. Biochem. Biophys. Methods* (1990) **20**(3):259-267.
35. THIERRY AR, VIVES E, RICHARD JP *et al.*: Cellular uptake and intracellular fate of antisense oligonucleotides. *Curr. Opin. Mol. Ther.* (2003) **5**(2):133-138.
36. CROOKE ST: Progress in antisense technology: the end of the beginning. *Methods Enzymol.* (2000) **313**:3-45.
37. TONKINSON JL, STEIN CA: Antisense oligodeoxynucleotides as clinical therapeutic agents. *Cancer Invest.* (1996) **14**(1):54-65.
38. KRIEG AM, STEIN CA: Phosphorothioate oligodeoxynucleotides: antisense or antiprotein? *Antisense Res. Dev. (*1995) **5**(4):241.
39. GALBRAITH WM, HOBSON WC, GICLAS PC *et al.*: Complement activation and hemodynamic changes following intravenous administration of phosphorothioate oligonucleotides in the monkey. *Antisense Res. Dev.* (1994) **4**(3):201-206.
40. SARMIENTO UM, PEREZ JR, BECKER JM *et al.*: *In vivo* toxicological effects of rel A antisense phosphorothioates in CD-1 mice. *Antisense Res. Dev.* (1994) **4**(2):99-107.
41. AGRAWAL S, KANDIMALLA ER: Antisense therapeutics: is it as simple as complementary base recognition? *Mol. Med. Today (*2000) **6**(2):72-81.
42. ZAMARATSKI E, PRADEEPKUMAR PI, CHATTOPADHYAYA J: A critical survey

of the structure-function of the antisense oligo/RNA heteroduplex as substrate for RNase H. *J. Biochem. Biophys. Methods* (2001) **48**(3):189-208.

43. BAKER BF, LOT SS, CONDON TP *et al.*: 2′-O-(2-Methoxy)ethyl-modified anti-intercellular adhesion molecule 1 (ICAM-1) oligonucleotides selectively increase the ICAM-1 mRNA level and inhibit formation of the ICAM-1 translation initiation complex in human umbilical vein endothelial cells. *J. Biol. Chem.* (1997) **272**(18):11994-12000.
44. RUSCKOWSKI M, QU T, ROSKEY A *et al.*: Biodistribution and metabolism of a mixed backbone oligonucleotide (GEM 231) following single and multiple dose administration in mice. *Antisense Nucleic Acid Drug Dev.* (2000) **10**(5):333-345.
45. AGRAWAL S, JIANG Z, ZHAO Q *et al.*: Mixed-backbone oligonucleotides as second generation antisense oligonucleotides: *in vitro* and *in vivo* studies. *Proc. Natl. Acad. Sci. USA* (1997) **94**(6):2620-2625.
46. VERMA S, ECKSTEIN F: Modified oligonucleotides: synthesis and strategy for users. *Ann. Rev. Biochem.* (1998) **67**: 99-134.
47. ENGELS JW, UHLMANN E: Chemistry of oligonucleotides; in Couvreur P, Malvy C (eds):*Pharmaceutical Aspects of Oligonucleotides*. London, Taylor & Francis, (2000), pp 35-68.
48. HEIDENREICH O, GRYAZNOV S, NERENBERG M: RNase H-independent antisense activity of oligonucleotide N3 '- > P5 ' phosphoramidates. *Nucleic Acids Res.* (1997) **25**(4):776-780.
49. SKORSKI T, PERROTTI D, NIEBOROWSKA-SKORSKA M *et al.*: Antileukaemia effect of c-myc N3'- > P5' phosphoramidate antisense oligonucleotides *in vivo*. *Proc Natl Acad SciUSA* (1997) **94**(8):3966-3971.
50. GRYAZNOV SM, LLOYD DH, CHEN JK *et al.*: Oligonucleotide N3'- > P5' phosphoramidates. *Proc. Nat.l Acad. Sci. USA* (1995) **92**(13):5798-5802.
51. SUMMERTON J, WELLER D: Morpholino antisense oligomers: design, preparation, and properties. *Antisense Nucleic Acid Drug Dev. (*1997) 7(3):187-195.
52. SUMMERTON J: Morpholino antisense oligomers: the case for an RNase H-independent structural type. *Biochim. Biophys. Acta.* (1999) **1489**(1):141-158.

•• **Excellent review on morpholino antisense ODNs.**

53. HUDZIAK RM, BAROFSKY E, BAROFSKY DF *et al.*: Resistance of morpholino phosphorodiamidate oligomers to enzymatic degradation. *Antisense Nucleic Acid Drug Dev.* (1996) **6**(4):267-272.
54. DOMINSKI Z, KOLE R: Restoration of correct splicing in thalassemic premRNA by antisense oligonucleotides. *Proc. Natl. Acad. Sci. USA* (1993) **90**(18):8673-8677.
55. SUWANMANEE T, SIERAKOWSKA H, LACERRA G *et al.*: Restoration of human beta-globin gene expression in murine and human IVS2-654 thalassemic erythroid cells by free uptake of antisense oligonucleotides. *Mol. Pharmacol.* (2002) **62**(3):545-553.
56. HEASMAN J: Morpholino oligos: making sense of antisense? *Dev. Biol. (*2002) **243**(2):209-214.
57. SUMMERTON J, STEIN D, HUANG SB *et al.*: Morpholino and phosphorothioate antisense oligomers compared in cell-free and in-cell systems. *Antisense Nucleic Acid Drug Dev.* (1997) 7(2):63-70.
58. TAYLOR MF, PAULAUSKIS JD, WELLER DD *et al.*: *In vitro* efficacy of morpholino-modified antisense oligomers directed against tumour necrosis factor-alpha mRNA. *J. Biol. Chem.* (1996) **271**(29):17445-17452.
59. GILES RV, SPILLER DG, CLARK RE *et al.*: Antisense morpholino oligonucleotide analog induces missplicing of C-myc mRNA. *Antisense Nucleic Acid Drug Dev.* (1999) **9**(2):213-220.
60. STEIN DA, SKILLING DE, IVERSEN PL *et al.*: Inhibition of Vesivirus infections in mammalian tissue culture with antisense morpholino oligomers. *Antisense Nucleic Acid Drug Dev.* (2001) **11**(5):317-325.
61. ARORA V, IVERSEN PL: Redirection of drug metabolism using antisense technology. *Curr. Opin. Mol. Ther.* (2001) **3**(3):249-257.
62. ARORA V, CATE ML, GHOSH C *et al.*: Phosphorodiamidate morpholino antisense oligomers inhibit expression of human cytochrome P450 3A4 and alter selected drug metabolism. *Drug Metab. Dispos.* (2002) **30**(7):757-762.
63. VIKRAM A, DEREK CK, MURALIMOHAN TR, DWIGHT DW, PATRICK LI: Bioavailability and Efficiency of Antisense Morpholino Oligomers Targeted to c-*myc* and Cytochrome P450 3A2 Following Oral Administration in Rats. *J.Pharm. Sci.* (2002) **91**(4):1009-1018.
64. MCCAFFREY AP, MEUSE L, KARIMI M *et al.*: A potent and specific morpholino antisense inhibitor of hepatitis C translation in mice. *Hepatology* (2003) **38**(2):503-508.
65. IVERSEN PL, ARORA V, ACKER AJ *et al.*: Efficacy of antisense morpholino oligomer targeted to c-myc in prostate cancer xenograft murine model and a Phase I safety study in humans. *Clin. Cancer Res.* (2003) **9**(7):2510-2519.
66. SCHMAJUK G, SIERAKOWSKA H, KOLE R: Antisense oligonucleotides with different backbones. Modification of splicing pathways and efficacy of uptake. *J. Biol. Chem.* (1999) **274**(31):21783-21789.
67. SAZANI P, KANG SH, MAIER MA *et al.*: Nuclear antisense effects of neutral, anionic and cationic oligonucleotide analogs. *Nucleic Acids Res.* (2001) **29**(19):3965-3974.
68. SAZANI P, GEMIGNANI F, KANG SH *et al.*: Systemically delivered antisense oligomers upregulate gene expression in mouse tissues. *Nat Biotechnol* (2002) **20**(12):1228-1233.
69. KRIEG AM: Immune effects and mechanisms of action of CpG motifs. *Vaccine* (2000) **19**(6):618-622.
70. SCHEULE RK: The role of CpG motifs in immunostimulation and gene therapy. *Adv Drug Deliv Rev* (2000) **44**(2-3):119-134.

•• **Excellent review on CpG motifs.**

71. KRIEG AM, YI AK, MATSON S *et al.*: CpG motifs in bacterial DNA trigger direct B cell activation. *Nature* (1995) **374**(6522):546-549.
72. PAYETTE PJ, DAVIS HL, KRIEG AM: Role of CpG motifs in immunostimulation and gene expression; in Mahato RIaK, S.W. (ed):*Pharmaceutical Perspectives of Nucleic Acid-Based Therapeutics.* London, Taylor & Francis, (2002), pp 467-486.

•• **Excellent review on CpG motifs.**

73. KANDIMALLA ER, BHAGAT L, ZHU FG *et al.*: A dinucleotide motif in oligonucleotides shows potent immunomodulatory activity and over-rides species-specific recognition observed with CpG motif. *Proc Natl. Acad. Sci. USA* (2003) **100**(24):14303-14308.
74. DOVE A: Antisense and sensibility. *Nat. Biotechnol.* (2002) **20**(2):121-124.
75. LONSDORF AS, KUEKREK H, STERN BV *et al.*: Intratumour CpG-oligodeoxynucleotide injection induces

protective antitumour T cell immunity. *J. Immunol.* (2003) **171**(8):3941-3946.

76. DALPKE A, ZIMMERMANN S, HEEG K: Immunopharmacology of CpG DNA. *Biol. Chem.* (2002) **383**(10):1491-1500.
77. AGRAWAL S, KANDIMALLA ER: Medicinal chemistry and therapeutic potential of CpG DNA. *Trends Mol. Med.* (2002) **8**(3):114-121.
78. KANDIMALLA ER, BHAGAT L, YU D *et al.*: Conjugation of ligands at the 5′-end of CpG DNA affects immunostimulatory activity. *Bioconjug. Chem.* (2002) **13**(5):966-974.
79. YU D, ZHU FG, BHAGAT L *et al.*: Potent CpG oligonucleotides containing phosphodiester linkages: *in vitro* and *in vivo* immunostimulatory properties. *Biochem. Biophys. Res. Commun.* (2002) **297**(1):83-90.
80. PRASEUTH D, GUIEYSSE AL, HELENE C: Triple helix formation and the antigene strategy for sequence-specific control of gene expression. *Biochim. Biophys. Acta* (1999) **1489**(1):181-206.
81. MAHER LJ 3rd: Prospects for the therapeutic use of antigene oligonucleotides. *Cancer Invest.* (1996) **14**(1):66-82.
82. POTAMAN VN: Applications of triple-stranded nucleic acid structures to DNA purification, detection and analysis. *Expert Rev. Mol. Diagn.* (2003) **3**(4):481-496.
83. GEWIRTZ AM, SOKOL DL, RATAJCZAK MZ: Nucleic acid therapeutics: state of the art and future prospects. *Blood* (1998) **92**(3):712-736.

•• **Excellent review on nucleic acid-based therapeutics.**

84. OLIVAS WM, MAHER LJ 3rd: Competitive triplex/quadruplex equilibria involving guanine-rich oligonucleotides. *Biochemistry* (1995) **34**(1):278-284.
85. FARUQI AF, KRAWCZYK SH, MATTEUCCI MD *et al.*: Potassium-resistant triple helix formation and improved intracellular gene targeting by oligodeoxyribonucleotides containing 7-deazaxanthine. *Nucleic Acids Res.* (1997) **25**(3):633-640.
86. BASYE J, TRENT JO, GAO D *et al.*: Triplex formation by morpholino oligodeoxyribonucleotides in the HER-2/neu promoter requires the pyrimidine motif. *Nucleic Acids Res.* (2001) **29**(23):4873-4880.
87. LACROIX L, ARIMONDO PB, TAKASUGI M *et al.*: Pyrimidine morpholino oligonucleotides form a stable triple helix in the absence of magnesium ions. *Biochem. Biophys. Res. Commun.* (2000) **270**(2):363-369.
88. FERDOUS A, WATANABE H, AKAIKE T *et al.*: Poly(L-lysine)-graft-dextran copolymer: amazing effects on triplex stabilization under physiological pH and ionic conditions (*in vitro*). *Nucleic Acids Res.* (1998) **26**(17):3949-3954.
89. MARUYAMA A, WATANABE H, FERDOUS A *et al.*: Characterization of interpolyelectrolyte complexes between double-stranded DNA and polylysine comb-type copolymers having hydrophilic side chains. *Bioconjug. Chem.* (1998) **9**(2):292-299.
90. FERDOUS A, AKAIKE T, MARUYAMA A: Mechanism of intermolecular purine-purine-pyrimidine triple helix stabilization by comb-type polylysine graft copolymer at physiologic potassium concentration. *Bioconjug. Chem.* (2000) **11**(4):520-526.
91. CARBONE GM, MCGUFFIE E, NAPOLI S *et al.*: DNA binding and antigene activity of a daunomycin-conjugated triplex-forming oligonucleotide targeting the P2 promoter of the human c-myc gene. *Nucleic Acids Res.* (2004) **32**(8):2396-2410.
92. FARIA M, WOOD CD, PERROUAULT L *et al.*: Targeted inhibition of transcription elongation in cells mediated by triplex-forming oligonucleotides. *Proc. Natl. Acad. Sci. USA* (2000) **97**(8):3862-3867.
93. VASQUEZ KM, NARAYANAN L, GLAZER PM: Specific mutations induced by triplex-forming oligonucleotides in mice. *Science* (2000) **290**(5491):530-533.
94. VUORIO E, DE CROMBRUGGHE B: The family of collagen genes. *Ann. Rev. Biochem.* (1990) **59**: 837-872.
95. VAN Der REST M, GARRONE R: Collagen family of proteins. *Faseb J.* (1991) **5**(13):2814-2823.
96. BRENNER DA, RIPPE RA, VELOZ L: Analysis of the collagen alpha **1**(I) promoter. *Nucleic Acids Res.* (1989) **17**(15):6055-6064.
97. JOSEPH J, KANDALA JC, VEERAPANANE D *et al.*: Antiparallel polypurine phosphorothioate oligonucleotides form stable triplexes with the rat alpha1(I) collagen gene promoter and inhibit transcription in cultured rat fibroblasts. *Nucleic Acids Res.* (1997) **25**(11):2182-2188.
98. DHALLA AK, KANDALA JC, WEBER KT *et al.*: Identification of negative and positive regulatory elements in the rat alpha **1**(I) collagen gene promoter. *Int. J. Biochem. Cell Biol.* (1997) **29**(1):143-151.
99. WEBER KT, SWAMYNATHAN SK, GUNTAKA RV *et al.*: Angiotensin II and extracellular matrix homeostasis. *Int. J. Biochem. Cell Biol.* (1999) **31**(3-4):395-403.
100. MUSSO M, VAN DYKE MW: Polyamine effects on purine-purine-pyrimidine triple helix formation by phosphodiester and phosphorothioate oligodeoxyribonucleotides. *Nucleic Acids Res.* (1995) **23**(12):2320-2327.
101. NAKANISHI M, WEBER KT, GUNTAKA RV: Triple helix formation with the promoter of human alpha1(I) procollagen gene by an antiparallel triplex-forming oligodeoxyribonucleotide. *Nucleic Acids Res.* (1998) **26**(22):5218-5222.

•• **Excellent research article on TFOs.**

102. FIRE A, XU S, MONTGOMERY MK *et al.*: Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* (1998) **391**(6669):806-811.
103. COUZIN J: Breakthrough of the year. Small RNAs make big splash. *Science* (2002) **298**(5602):2296-2297.
104. PLASTERK RH: RNA silencing: the genome's immune system. *Science* (2002) **296**(5571):1263-1265.
105. MILHAVET O, GARY DS, MATTSON MP: RNA interference in biology and medicine. *Pharmacol. Rev.* (2003) **55**(4):629-648.

•• **Excellent review on siRNA.**

106. LIEBERMAN J, SONG E, LEE SK *et al.*: Interfering with disease: opportunities and roadblocks to harnessing RNA interference. *Trends Mol. Med.* (2003) **9**(9):397-403.
107. ZENG Y, CULLEN BR: RNA interference in human cells is restricted to the cytoplasm. *RNA* (2002) **8**(7):855-860.
108. ZAMORE PD, TUSCHL T, SHARP PA *et al.*: RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* (2000) **101**(1):25-33.
109. NYKANEN A, HALEY B, ZAMORE PD: ATP requirements and small interfering RNA structure in the RNA interference pathway. *Cell* (2001) **107**(3):309-321.

110. HAMMOND SM, BERNSTEIN E, BEACH D *et al.*: An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *Nature* (2000) **404**(6775):293-296.

111. DYKXHOORN DM, NOVINA CD, SHARP PA: Killing the messenger: short RNAs that silence gene expression. *Nat. Rev. Mol. Cell Biol.* (2003) **4**(6):457-467.

•• **Excellent review on siRNA.**

112. SCHWARZ DS, HUTVAGNER G, DU T *et al.*: Asymmetry in the assembly of the RNAi enzyme complex. *Cell* (2003) **115**(2):199-208.

•• **Excellent research work demonstrating that the two strands of an siRNA duplex are not equally eligible for assembly into RISC.**

113. SCHIFFELERS RM, WOODLE MC, SCARIA P: Pharmaceutical prospects for RNA interference. *Pharm. Res.* (2004) **21**(1):1-7.

114. SCHERER LJ, ROSSI JJ: Approaches for the sequence-specific knockdown of mRNA. *Nat. Biotechnol.* (2003) **21**(12):1457-1465.

•• **This paper discusses the application of antisense ODNs, ribozymes, DNAzymes and RNAi.**

115. HOLEN T, AMARZGUIOUI M, WIIGER MT *et al.*: Positional effects of short interfering RNAs targeting the human coagulation trigger Tissue Factor. *Nucleic Acids Res.* (2002) **30**(8):1757-1766.

116. HENSCHEL A, BUCHHOLZ F, HABERMANN B: DEQOR: A web-based tool for the design and quality control of siRNAs. *Nucleic Acids Res.* (2004) **32**(Web Server issue):W113-W120.

117. YANG D, BUCHHOLZ F, HUANG Z *et al.*: Short RNA duplexes produced by hydrolysis with Escherichia coli RNase III mediate effective RNA interference in mammalian cells. *Proc. Natl. Acad. Sci. USA* (2002) **99**(15):9942-9947.

118. KAWASAKI H, SUYAMA E, IYO M *et al.*: siRNAs generated by recombinant human Dicer induce specific and significant but target site-independent gene silencing in human cells. *Nucleic Acids Res.* (2003) **31**(3):981-987.

119. AN DS, XIE Y, MAO SH *et al.*: Efficient lentiviral vectors for short hairpin RNA delivery into human cells. *Hum. Gene Ther.* (2003) **14**(12):1207-1212.

120. XIA H, MAO Q, PAULSON HL *et al.*: siRNA-mediated gene silencing *in vitro* and *in vivo*. *Nat. Biotechnol.* (2002) **20**(10):1006-1010.

121. BRUMMELKAMP TR, BERNARDS R, AGAMI R: A system for stable expression of short interfering RNAs in mammalian cells. *Science* (2002) **296**(5567):550-553.

122. SUI G, SOOHOO C, AFFAR El B *et al.*: A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. *Proc. Natl. Acad. Sci. USA* (2002) **99**(8):5515-5520.

123. KAYKAS A, MOON RT: A plasmid-based system for expressing small interfering RNA libraries in mammalian cells. *BMC Cell Biol.* (2004) **5**(1):16.

124. ZHENG L, LIU J, BATALOV S *et al.*: An approach to genomewide screens of expressed small interfering RNAs in mammalian cells. *Proc. Natl. Acad. Sci. USA* (2004) **101**(1):135-140.

125. TOMAR RS, MATTA H, CHAUDHARY PM: Use of adeno-associated viral vector for delivery of small interfering RNA. *Oncogene* (2003) **22**(36):5712-5715.

126. ZHAO LJ, JIAN H, ZHU H: Specific gene inhibition by adenovirus-mediated expression of small interfering RNA. *Gene* (2003) **316**: 137-141.

127. DEVROE E, SILVER PA: Retrovirus-delivered siRNA. *BMC Biotechnol.* (2002) **2**(1):15.

128. MATTA H, HOZAYEV B, TOMAR R *et al.*: Use of lentiviral vectors for delivery of small interfering RNA. *Cancer Biol. Ther.* (2003) **2**(2):206-210.

129. TISCORNIA G, SINGER O, IKAWA M *et al.*: A general method for gene knockdown in mice by using lentiviral vectors expressing small interfering RNA. *Proc. Natl. Acad. Sci. USA* (2003) **100**(4):1844-1848.

130. ABBAS-TERKI T, BLANCO-BOSE W, DEGLON N *et al.*: Lentiviral-mediated RNA interference. *Hum. Gene Ther.* (2002) **13**(18):2197-2201.

131. UI-TEI K, NAITO Y, TAKAHASHI F *et al.*: Guidelines for the selection of highly effective siRNA sequences for mammalian and chick RNA interference. *Nucleic Acids Res.* (2004) **32**(3):936-948.

132. NAITO Y, YAMADA T, UI-TEI K *et al.*: siDirect: highly effective, target-specific siRNA design software for mammalian RNA interference. *Nucleic Acids Res.* (2004) **32**(Web Server issue):W124-W129.

133. YUAN B, LATEK R, HOSSBACH M *et al.*: siRNA Selection Server: an automated siRNA oligonucleotide prediction server. *Nucleic Acids Res.* (2004) **32**(Web Server issue):W130-W134.

134. FRIEDRICH I, SHIR A, KLEIN S *et al.*: RNA molecules as anticancer agents. *Semin. Cancer Biol.* (2004) **14**(4):223-230.

135. MAHATO RI, TAKEMURA S, AKAMATSU K *et al.*: Physicochemical and disposition characteristics of antisense oligonucleotides complexed with glycosylated poly(L-lysine). *Biochem. Pharmacol.* (1997) **53**(6):887-895.

136. BRUMMELKAMP TR, BERNARDS R, AGAMI R: Stable suppression of tumorigenicity by virus-mediated RNA interference. *Cancer Cell* (2002) **2**(3):243-247.

137. KARIKO K, BHUYAN P, CAPODICI J *et al.*: Small interfering RNAs mediate sequence-independent gene suppression and induce immune activation by signalling through toll-like receptor 3. *J. Immunol.* (2004) **172**(11):6545-6549.

138. ELBASHIR SM, MARTINEZ J, PATKANIOWSKA A *et al.*: Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. *Embo J.* (2001) **20**(23):6877-6888.

139. JACKSON AL, BARTZ SR, SCHELTER J *et al.*: Expression profiling reveals off-target gene regulation by RNAi. *Nat. Biotechnol.* (2003) **21**(6):635-637.

140. SCACHERI PC, ROZENBLATT-ROSEN O, CAPLEN NJ *et al.*: Short interfering RNAs can induce unexpected and divergent changes in the levels of untargeted proteins in mammalian cells. *Proc. Natl. Acad. Sci. USA* (2004) **101**(7):1892-1897.

141. SNOVE O Jr, HOLEN T: Many commonly used siRNAs risk off-target activity. *Biochem. Biophys. Res. Commun.* (2004) **319**(1):256-263.

142. SAWAI K, MAHATO RI, OKA Y *et al.*: Disposition of oligonucleotides in isolated perfused rat kidney: involvement of scavenger receptors in their renal uptake. *J. Pharmacol. Exp. Ther.* (1996) **279**(1):284-290.

•• **Excellent research paper on renal disposition of ODNs.**

143. YOSHIDA M, MAHATO RI, KAWABATA K *et al.*: Disposition characteristics of plasmid DNA in the single-pass rat liver perfusion system. *Pharm. Res.* (1996) **13**(4):599-603.

144. TAKAKURA Y, MAHATO RI, YOSHIDA M *et al.*: Uptake characteristics of

oligonucleotides in the isolated rat liver perfusion system. *Antisense Nucleic Acid Drug Dev.* (1996) **6**(3):177-183.

145. TAKAKURA Y, MAHATO RI, HASHIDA M: Control of pharmacokinetic profiles of drug-macromolecule conjugates. *Adv. Drug Del. Rev.* (1996) **19**:377.

146. TAKAKURA Y, HASHIDA M: Macromolecular carrier systems for targeted drug delivery: pharmacokinetic considerations on biodistribution. *Pharm. Res.* (1996) **13**(6):820-831.

147. INAGAKI M, TOGAWA K, CARR BI *et al.*: Antisense oligonucleotides: inhibition of liver cell proliferation and *in vivo* disposition. *Transplant. Proc.* (1992) **24**(6):2971-2972.

148. BIJSTERBOSCH MK, MANOHARAN M, RUMP ET *et al.*: *In vivo* fate of phosphorothioate antisense oligodeoxynucleotides: predominant uptake by scavenger receptors on endothelial liver cells. *Nucleic Acids Res.* (1997) **25**(16):3290-3296.

149. RAPPAPORT J, HANSS B, KOPP JB *et al.*: Transport of phosphorothioate oligonucleotides in kidney: implications for molecular therapy. *Kidney Int.* (1995) **47**(5):1462-1469.

150. GEARY RS, LEEDS JM, FITCHETT J *et al.*: Pharmacokinetics and metabolism in mice of a phosphorothioate oligonucleotide antisense inhibitor of C-raf-1 kinase expression. *Drug Metab. Dispos.* (1997) **25**(11):1272-1281.

151. GEARY RS, YU RZ, LEADS JM *et al.*: Pharmacokinetic properties in animals; in Crooke ST (ed):*Antisense Drug Technology.* New York, Marcel Dekker Inc. (2001) pp 119-154.

152. DELONG RK, NOLTING A, FISHER M *et al.*: Comparative pharmacokinetics, tissue distribution, and tumour accumulation of phosphorothioate, phosphorodithioate, and methylphosphonate oligonucleotides in nude mice. *Antisense Nucleic Acid Drug Dev.* (1997) 7(2):71-77.

153. YAKUBOV LA, DEEVA EA, ZARYTOVA VF *et al.*: Mechanism of oligonucleotide uptake by cells: involvement of specific receptors? *Proc. Natl. Acad. Sci. USA* (1989) **86**(17):6454-6458.

154. LOKE SL, STEIN CA, ZHANG XH *et al.*: Characterization of oligonucleotide transport into living cells. *Proc. Natl. Acad. Sci. USA* (1989) **86**(10):3474-3478.

155. WU-PONG S, BARD J, HUFFMAN J *et al.*: Oligonucleotide biological activity: relationship to the cell cycle and nuclear transport. *Biol. Cell* (1997) **89**(4):257-261.

156. SHOJI Y, AKHTAR S, PERIASAMY A *et al.*: Mechanism of cellular uptake of modified oligodeoxynucleotides containing methylphosphonate linkages. *Nucleic Acids Res.* (1991) **19**(20):5543-5550.

157. AKHTAR S, BASU S, WICKSTROM E *et al.*: Interactions of antisense DNA oligonucleotide analogs with phospholipid membranes (liposomes). *Nucleic Acids Res.* (1991) **19**(20):5551-5559.

158. STUART DD, ALLEN TM: A new liposomal formulation for antisense oligodeoxynucleotides with small size, high incorporation efficiency and good stability. *Biochim. Biophys. Acta* (2000) **1463**(2):219-229.

159. BENNETT CF, MIREJOVSKY D, CROOKE RM *et al.*: Structural requirements for cationic lipid mediated phosphorothioate oligonucleotides delivery to cells in culture. *J. Drug Target.* (1998) **5**(3):149-162.

160. ZELPHATI O, SZOKA FC Jr: Mechanism of oligonucleotide release from cationic liposomes. *Proc. Natl. Acad. Sci. USA* (1996) **93**(21):11493-11498.

•• **Excellent research paper on mechanism of ODNs release from liposomes.**

161. LUKACS GL, HAGGIE P, SEKSEK O *et al.*: Size-dependent DNA mobility in cytoplasm and nucleus. *J. Biol. Chem.* (2000) **275**(3):1625-1629.

162. BIESSEN EA, Vietsch H, Rump ET *et al.*: Targeted delivery of oligodeoxynucleotides to parenchymal liver cells *in vivo*. *Biochem. J.* (1999) **340** (Pt 3):783-792.

163. GINOBBI P, GEISER TA, OMBRES D *et al.*: Folic acid-polylysine carrier improves efficacy of c-myc antisense oligodeoxynucleotides on human melanoma (M14) cells. *Anti-Cancer Res.* (1997) **17**(1A):29-35.

164. WANG S, LEE RJ, CAUCHON G *et al.*: Delivery of antisense oligodeoxyribonucleotides against the human epidermal growth factor receptor into cultured KB cells with liposomes conjugated to folate via polyethylene glycol. *Proc. Natl. Acad. Sci. USA* (1995) **92**(8):3318-3322.

165. ROJANASAKUL Y, WEISSMAN DN, SHI X *et al.*: Antisense inhibition of silica-induced tumour necrosis factor in alveolar macrophages. *J. Biol. Chem.* (1997) **272**(7):3910-3914.

166. LIANG WW, SHI X, DESHPANDE D *et al.*: Oligonucleotide targeting to alveolar macrophages by mannose receptor-mediated endocytosis. *Biochim. Biophys. Acta* (1996) **1279**(2):227-234.

167. SCHLEPPER-SCHAFER J, HULSMANN D, DJOVKAR A *et al.*: Endocytosis via galactose receptors *in vivo*. Ligand size directs uptake by hepatocytes and/or liver macrophages. *Exp. Cell Res.* (1986) **165**(2):494-506.

168. KAWABATA K, TAKAKURA Y, HASHIDA M: The fate of plasmid DNA after intravenous injection in mice: involvement of scavenger receptors in its hepatic uptake. *Pharm. Res.* (1995) **12**(6):825-830.

169. RAJUR SB, ROTH CM, MORGAN JR *et al.*: Covalent protein-oligonucleotide conjugates for efficient delivery of antisense molecules. *Bioconjug. Chem.* (1997) **8**(6):935-940.

170. MAIER MA, YANNOPOULOS CG, MOHAMED N *et al.*: Synthesis of antisense oligonucleotides conjugated to a multivalent carbohydrate cluster for cellular targeting. *Bioconjug. Chem.* (2003) **14**(1):18-29.

171. CROOKE ST, GRAHAM MJ, ZUCKERMAN JE *et al.*: Pharmacokinetic properties of several novel oligonucleotide analogs in mice. *J. Pharmacol. Exp. Ther.* (1996) **277**(2):923-937.

172. BIJSTERBOSCH MK, MANOHARAN M, DORLAND R *et al.*: *bis*-Cholesteryl-conjugated phosphorothioate oligodeoxynucleotides are highly selectively taken up by the liver. *J. Pharmacol. Exp. Ther.* (2002) **302**(2):619-626.

173. MANOHARAN M: Oligonucleotide conjugates in antisense technology; in Crooke ST (ed):*Antisense Drug Technology.* New York, Marcel Dekker, (2001), pp 391-469.

•• **Excellent review on ODN conjugates.**

174. BIJSTERBOSCH MK, MANOHARAN M, DORLAND R *et al.*: Delivery of cholesteryl-conjugated phosphorothioate oligodeoxynucleotides to Kupffer cells by lactosylated low-density lipoprotein. *Biochem. Pharmacol.* (2001) **62**(5):627-633.

175. DESJARDINS J, MATA J, BROWN T *et al.*: Cholesteryl-conjugated phosphorothioate oligodeoxynucleotides

modulate CYP2B1 expression *in vivo*. *J. Drug Target.* (1995) **2**(6):477-485.

176. EPA WR, RONG P, BARTLETT PF *et al.*: Enhanced down regulation of the p75 nerve growth factor receptor by cholesteryl and *bis*-cholesteryl antisense oligonucleotides. *Antisense Nucleic Acid Drug Dev.* (1998) **8**(6):489-498.
177. LEDOAN T, ETORE F, TENU JP *et al.*: Cell binding, uptake and cytosolic partition of HIV antigag phosphodiester oligonucleotides 3′-linked to cholesterol derivatives in macrophages. *Bioorg Med. Chem* (1999) 7(11):2263-2269.
178. GRYAZNOV SM, LLOYD DH: Modulation of oligonucleotide duplex and triplex stability via hydrophobic interactions. *Nucleic Acids Res.* (1993) **21**(25):5909-5915.
179. LI S, DESHMUKH HM, HUANG L: Folate-mediated targeting of antisense oligodeoxynucleotides to ovarian cancer cells. *Pharm. Res.* (1998) **15**(10):1540-1545.
180. BONORA GM, IVANOVA E, ZARYTOVA V *et al.*: Synthesis and characterization of high-molecular mass polyethylene glycol-conjugated oligonucleotides. *Bioconjug. Chem.* (1997) **8**(6):793-797.
181. HARADA-SHIBA M, YAMAUCHI K, HARADA A *et al.*: Polyion complex micelles as vectors in gene therapy-pharmacokinetics and *in vivo* gene transfer. *Gene Ther.* (2002) **9**(6):407-414.
182. VINOGRADOV SV, BRONICH TK, KABANOV AV: Self-assembly of polyamine-poly(ethylene glycol) copolymers with phosphorothioate oligonucleotides. *Bioconjug. Chem.* (1998) **9**(6):805-812.
183. BRUS C, PETERSEN H, AIGNER A *et al.*: Physicochemical and biological characterization of polyethylenimine-graft-poly(ethylene glycol) block copolymers as a delivery system for oligonucleotides and ribozymes. *Bioconjug. Chem.* (2004) **15**(4):677-684.
184. JEONG JH, KIM SW, PARK TG: A new antisense oligonucleotide delivery system based on self-assembled ODN-PEG hybrid conjugate micelles. *J. Control. Release* (2003) **93**(2):183-191.

• **Research paper demonstrating that complex micelles, pegylated ODNs and PEI can effectively supress tumour growth.**

185. JEONG JH, KIM SW, PARK TG: Novel intracellular delivery system of antisense oligonucleotide by self-assembled hybrid micelles composed of DNA/PEG conjugate and cationic fusogenic peptide. *Bioconjug. Chem.* (2003) **14**(2):473-479.
186. HANGELAND JJ, LEVIS JT, LEE YC *et al.*: Cell type specific and ligand specific enhancement of cellular uptake of oligodeoxynucleoside methylphosphonates covalently linked with a neoglycopeptide, YEE(ah-GalNAc)3. *Bioconjug. Chem.* (1995) **6**(6):695-701.
187. DUFF RJ, DEAMOND SF, ROBY C *et al.*: Intrabody tissue-specific delivery of antisense conjugates in animals: ligand-linker-antisense oligomer conjugates. *Methods Enzymol.* (2000) **313**:297-321.
188. BIESSEN EA, VIETSCH H, RUMP ET *et al.*: Targeted delivery of antisense oligonucleotides to parenchymal liver cells *in vivo*. *Methods Enzymol.* (2000) **314**:324-342.
189. COREY D: 48000-fold accelaration of hybridization by chemically modified oligonucleotides. *J. Am. Chem. Soc.* (1995) **117**(36):9373-9374.
190. NEUMAN BW, STEIN DA, KROEKER AD *et al.*: Antisense morpholino-oligomers directed against the 5′ end of the genome inhibit coronavirus proliferation and growth. *J. Virol.* (2004) **78**(11):5891-5899.
191. MURATOVSKA A, ECCLES MR: Conjugate for efficient delivery of short interfering RNA (siRNA) into mammalian cells. *FEBS Lett.* (2004) **558**(1-3):63-68.
192. BERTRAND JR, POTTIER M, VEKRIS A *et al.*: Comparison of antisense oligonucleotides and siRNAs in cell culture and *in vivo*. *Biochem. Biophys. Res. Commun.* (2002) **296**(4):1000-1004.
193. JULIANO RL, ALAHARI S, YOO H *et al.*: Antisense pharmacodynamics: critical issues in the transport and delivery of antisense oligonucleotides. *Pharm. Res.* (1999) **16**(4):494-502.

Websites

201. http://design.RNAi.jp
202. http://jura.wi.mit.edu/bioc/siRNA
203. http://cluster-1.mpi-cbg.de/Deqor/deqor.html

Affiliation

Dr Ram I Mahato PhD[†1,2], Dr Kun Cheng[1] & Dr Ramareddy V Guntaka[3]
[†]Author for correspondence
[†1]University of Tennessee Health Science Center, Department of Pharmaceutical Sciences, 26 South Dunlap Street, Feurt Bldg RM 406, Memphis, TN 38163, USA
Tel: +1 901 448 6929; Fax: +1 901 448 6092; E-mail: rmahato@utmem.edu
[2]University of Tennessee Health Science Center, Biomedical Engineering, Memphis, TN 38163, USA
[3]University of Tennessee Health Science Center, Molecular Sciences, 101 Molecular Science Building, 858 Madison Avenue, Memphis, TN38163, USA
Tel: +1 901 448 8230; Fax: +1 901 448 8462
E-mail: rguntaka@utmem.edu