

Nucleic Acid Therapeutics: Current Targets For Antisense Oligonucleotides And Ribozymes

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Nucleic acids are becoming increasingly important as therapeutic molecules mainly for the ease with which specificity for a vast range of targets of these drugs is achieved. Yet the dose rates of several mg/kg are not easy to provide. Reform of drug policy has been suggested earlier to facilitate newer therapeutic strategies to be put to use. With the current technology, each mg of the unmodified oligonucleotide would cost US\$ 20. That means someone with 50 kg of body weight requiring 50 mg would require to pay ca US\$1000/- per dose. That is where targeted delivery systems emerge as a possible hope for minimizing on the dose rates while new technology for synthesis for economical production of these drugs would remain welcome. The suspected toxicity and non-specificity associated with certain modifications need to be scrutinized. Studies on potential side effects and frequency of insertion of the administered oligonucleotides in recipient cell's genome must remain under vigilance. In antiviral and anticancer applications, it will be necessary to establish regimen that ensures complete cure to avoid resurgence on withdrawal of the drugs. Most of the current data show good but partial elimination of viral or cancer targets.

Introduction

With the increasing resolution of our understanding of the biological systems at molecular level, most of the biological expressions including onset of pathogenesis can more precisely be attributed to molecular dysfunctions. This opens up the possibility of defining molecular targets in case of disease, an information paving way to designing and developing novel drugs as well as evolving strategies for minimizing the dose rates required through targeted delivery (58). The entire range of activities in this area has necessitated recognition of 'Biomolecular Medicine' as the discipline within biotechnology addressing to disease related questions based on biomolecules involved in disease process. Targeting biomolecules involves construction of cognate molecules that specifically and, perhaps exclusively recognize these targets, effect necessary changes in their structural or functional property and mitigate the situation of the disease (10). The best of available software for visualizing structure either of the ligand or its cognate molecule is not accurate enough to precisely visualize the actual structure. Hence, development of cognate molecules based

on higher order structure of the target necessitates experimentally selecting for them e.g., producing antibodies against chosen targets or efficient binding versions of a protein by display on phage surface and repeated selection for those withstanding the most stringent binding conditions etc. Nevertheless, if the nucleic acid sequences e.g., of the gene or mRNA involved are defined, it becomes relatively easy to design a complementary sequence, the rules of intermolecular interactions being based on well-defined primary structure (1, 12, 13). One expects an interaction between complementary sequences present in the same milieu resulting in interference and inhibition of the normal functioning of the endogenous sequence by the administered complementary sequence. Since the target sequences are usually the ones coding for one of the functioning biomolecules (i.e., a sense sequence as in mRNA), the administered complementary sequence is commonly referred to as being antisense. The administered nucleic acids are expected to effect inhibition of an unwanted function in several ways e.g., they could titrate out the factors binding to the endogenous nucleic acid, or they could form duplexes in single stranded regions (like those undergoing replication, transcription and translation). Apart from simply forming duplexes the antisense sequence may also include catalytic motifs capable of causing cleavage of molecules annealing with them. This is particularly attractive as a strategy for targeted cleavage of RNA molecules with the help of catalytic RNA (6). The antisense approach has proved to be a convenient tool for dissecting out roles of certain molecules, like alpha-1 connexin, in embryo development (5). This article briefly outlines the antisense and ribozyme approach, considerations one makes, and issues facing this approach and enlists targets used for nucleic acid-mediated control over the past two years or so. The references cited are by no means comprehensive and I duly apologize to authors whose work may not have been reflected in this review.

Annealing with Complementary Sequences

Depending upon the nature of the administered nucleic acid, DNA-DNA, DNA-RNA and RNA-RNA complexes would be formed involving the target sequence. As to the mechanisms of action of antisense nucleic acids (see Table 1), formation of complexes with the target sequence may make the latter unsuitable as templates. Moreover, the DNA-RNA duplexes will act as substrate for cellular RNase H activity destroying the RNA component of the duplex (14, 17, 34, 69). The RNA-RNA duplexes are subjected to double stranded RNA specific ribonucleases (70) whereas several kinds of the administered ribozyme molecules cleave the target sequence subsequent to annealing (for a review see 65). Expression of hammerhead ribozyme targeted against TNF- α as intron-embedded, polyadenylated or cytoplasmically produced transcript, resulted in depletion of apoptotic factor in culture supernatants of the cytokine-producing cells (Dammai, 1998; our unpublished results).

Formation of Triple Helix

Application of triple helix forming oligonucleotides is limited to certain specialized sequences (3). Targets with oligopyrimidine-oligopurine sequence are known to form

triple helices with oligonucleotides having similar sequences. This hinders the normal functioning of the target nucleic acid molecule. Claude Helene of INSERM (Paris) has compared the suitability of variously modified oligonucleotides in forming triple helical structures, which requires oligopyrimidine-oligopurine target. They find that the stability can be increased by covalent attachment of intercalating agents while oligophosphoramidates seem to form more stable triple helices than oligophosphodiester (2). The target sequences are accessible to triplex-forming oligonucleotides within the chromatin structure of cell nuclei. This inhibits transcription of the corresponding genes.

Binding with Non-Nucleic Acid Targets

It is possible to select for oligonucleotide sequences assuming higher order structure capable of binding with non-nucleic acid targets. Oligonucleotide phosphoramidates and phosphorothioates with varied sequence and structural characteristics differ in their binding specificity towards cell surface proteins. Phosphorothioates containing a G-quartet and antisense to c-myc were found to display non-specific binding with many proteins (23). Binding of RNA with protein may be independent of sequence and secondary structure (51). This suggests for the need to establish higher order structure for RNA molecules in order to visualize intermolecular interactions involving RNA.

Determinants of Effectiveness

A problem that does cause concern is the rapid natural turnover of oligonucleotides in the cells and one may have to provide for stability or continued production from within the cells in order to overcome this problem. On the other hand half-life of the target RNA or the translation product is an important determinant of how effective antisense or ribozyme molecules will be (58).

Table 1. How do oligonucleotide therapeutics work ?

Nature of the oligonucleotide	Mechanism of action	Reference*
Sequence-targeted oligonucleotide	DNA-RNA duplex formation and clearance by cellular RNase H	14, 34, 68
	Triple helix formation with target sequence	3
	RNA-RNA duplex formation and clearance by double strand RNA-specific Rnase	69
Sequence-targeted ribozymes	Cleavage of target sequence	6, 46, 51, 63, 65
Oligonucleotides selected for non-nucleic acid targets	Binding with target molecule and occlusion or inhibition of its function	4, 24

Synthetic Oligonucleotides, Replicons and Expression Cassettes

The antisense nucleic acids can be administered as synthetic material into the recipient organisms. This also offers the possibility to make suitable chemical modifications enabling them to escape from cellular nucleases. Alternatively, antisense nucleic acids can be provided either as replicons or as expression cassettes. In case of catalytic RNA molecules, it becomes particularly necessary to attain high enough intracellular concentration because of their rather low catalytic turnover constant (K_{cat}). This may be achieved by constructing the ribozyme expression cassettes based on viral replicons. We have constructed CMV promoter-based expression cassettes for TNF- α targeting ribozyme and have demonstrated depletion in apoptotic factor accompanying expression of the ribozyme. This observation establishes the promise of in vivo application of the ribozyme for diminishing TNF- α concentration in pathological situations like rheumatoid arthritis.

Antisense Drug in the Market

Recently ISIS Pharmaceuticals have launched and have released for marketing Vitravene within months of securing approval of FDA for treatment of retinitis caused by CMV (16). This is first ever approved antisense drug and has got growing acceptance and was aptly unveiled at the annual meeting of the American Academy of Ophthalmology in October, 1998. It is projected that patients at risk of going blind from CMV retinitis will be protected against the malady by the new therapy.

Resolution of Single Base Differences

With regard to target specificity, it has been claimed that a hammerhead ribozyme targeted against fibrillin mRNA can discriminate single base mismatch between the target and the ribozyme (48).

Selecting a Suitable Target

The development of antisense reagents begins with selecting for a suitable target because most of the regions of mRNA are not open for duplex formation with oligonucleotides and the targeted bases may be involved in intramolecular interactions. An empirical way of looking at the openness of the target is to visualize its secondary structure employing computer programs like MFOLD and RNAFOLD. It may however, be necessary to experiment with an array of antisense oligonucleotides and experimentally select the ones binding optimally (20, 38). It is often exclaimed that the rules for ab initio prediction of patterns of interaction would emerge only after detailed characterization of a larger number of oligonucleotides and targets (40, 57). In addition to selection of target, cellular uptake and use of controls are other important considerations in developing antisense reagent. Targeting the coding sequences as well as the 5' UTR of a luciferase reporter gene ligated to the promoter of insulin-like growth factor 1 gene were effective for inhibiting gene expression(21). Accessibility of the target site to antisense oligonucleotides and to ribozymes in some situations seems to be different perhaps due to additional structural features of the ribozyme molecules.

The Need for High Throughput Production and Analysis

Development of modular automated nucleic acid research facility for rapid production and analysis of a wide variety of oligonucleotides and their analogues is considered necessary in view of the diverse applications in site as well as the requirements of each individual application(66). Newer targets are emerging as we learn more and more about genomic sequences (13). Table 2 is an illustrative list of some of the targets currently being tested for antisense therapy. Transcription factor 1 has been studied as a possible target for antisense oligonucleotide-mediated modulation (18). It has been proposed to use the antisense approach to aid functional genomics (8,15).

Dendrimers, Chimeric Oligonucleotides and other Modifications

The 2'-O-methoxyethyl RNA display improved RNA affinity. Use of dendrimers as poly-labeled probes has already been established (55). It will be worthwhile employing such molecules as multipronged or multivalent antisense oligonucleotides. Chimeric oligonucleotides with 2'-O-methoxyethyl RNA wings and a central DNA phosphorothioate effectively reduce the growth of tumors in animal models at low doses. Structural study of a self complementary dodecamer 2'-O-MOE r(CGCGAAUUCGCG) shows that the duplex adopts an 'A' conformation and all modified sugars display C3'-endo pucker. In most of the 2'-O-substituted oligonucleotides, the torsion angle around the C-C bond of the ethylene glycol linker has a gauche conformation. In the case of a G residue, the trans conformation around the ethylene bond leads to hydrogen bonding between oxygen (of

Table 2: A list of some molecules currently being targeted by oligonucleotide therapeutics

Target	Reference
Alpha-1 connexin	5
BCL2	9,68
Dopamine receptor	10
BRCA1	53, 64
CMV	16
c-myb, c-myc	56
C-raf	41, 42
Fibrillin 1 (Marfan's syndrome)	30, 48
FMDV polymerase	63
GABA(A) receptor gamma2 subunit	27-29
Ha-ras	2
HCV serine proteinase	26
Hepatitis C viral RNA	67, 70
Hepatitis B virus (5'-region of the preS gene of DHBV)	45
ICAM1	60-62
MDM2	7, 49
PCNA	52
Protein kinase A	64
Protein kinase C-alpha	33, 39, 56
Regulators of AP-1	18
Rex (protein)	4
Start site of the first exon of the IT15 gene	23
Trinucleotide repeat (for ribozyme mediated splicing)	46

methoxy group) and N2. Such fortuitous interactions contribute to the improved affinity of the 2'-O-substituted oligonucleotides with target RNA. Structural studies with the 2'-O-aminopropyl RNA shows that the positively charged 2'-O-substituents cannot effectively shield the negative charge of the phosphate groups on either the 5' or the 3' side. Yet the positive charge of the substituent interferes with the metal ion binding site of exonucleases. This accounts for the exceptional nuclease resistance of such oligonucleotides.

Mixed Backbone Oligonucleotides

It may be mentioned that, in the mixed backbone oligonucleotides, desirable properties of two oligonucleotide analogues are combined. Thus the phosphorothioate segments provide a site for RnaseH activity while other segments containing modified oligonucleotides provide a tool to modulate nuclease stability, affinity, ionic characteristics and protein binding (64, 73). Another modification for nuclease resistance and permeability has been the poly-2'-O-(2,4-dinitrophenyl)-oligoribonucleotides. They have been tested for application in antiviral and anticancer therapy (53).

A degree of caution must be exercised while adopting phosphorothioate oligonucleotides for diverse applications in view of the reduced RNA-binding affinity and potential for unspecific protein binding and the side effects that might result. More sugar, backbone and base modifications should be tested for their biophysical properties and nuclease resistance before adopting the oligonucleotides for administering in human subjects. Nevertheless, signal transduction pathway intermediates like PKC- α (39) and c-raf kinase (41, 42) have been used as targets for antisense therapy employing modified oligonucleotides. The 2'-O-methoxyethyl substituted ribonucleosides at appropriate positions allow greater than 50% reduction in phosphorothioate content and an increase in the biological potency.

Novel Cleavage Site Specificity in Ribozymes

In case of catalytic RNA molecules, a residue-by-residue modification reveals ways of enhancing the potential of the designed molecules. Thus hammerhead ribozymes bearing an inosine at position 15 of the catalytic core in place of the naturally occurring adenosine have an altered cleavage site specificity and cleave substrates containing an NCH (N is A, G, or C and H is not G) triplet in place of normal NUH sites that occur in nature. A problem facing development and testing of targeted ribozymes has been the non-availability of convenient assay system for cleavage reaction. However, development of real time assay for multiple turnover ribozyme reactions based on fluorescence polarization is likely to alleviate this difficulty (Sproat at *Antisense* 98).

Peptide Nucleic Acids

Nuclease resistance in antisense oligonucleotides as well as ribozymes can be achieved by chemical modifications of these molecules. One such modification is to use peptide backbone instead of phosphodiester. The entire backbone is replaced by N-(2-aminoethyl)glycine units. The resulting molecules are called peptide nucleic acid (PNAs)

which are very good structural mimics of DNA with reference to formation of double helical complexes with complementary oligonucleotides. PNAs can also bind to double-stranded DNA by strand displacement (31). Binding of PNA to dsDNA is largely restricted to purine-rich targets. PNAs show very high biochemical stability and exert antisense effects in prokaryotic and eukaryotic cells (43).

Antisense Oligonucleotides Specific for Isoforms of the Target

Antisense oligodeoxyribonucleotides directed against different isoforms of PKC were used to delineate which isoform mediated the intracellular effects of hyperglycemia in vascular tissue. It was inferred that the PKC isoform α was responsible for the glucose-induced increase in the endothelial cell permeability and enhances the expression of transforming growth factor β in vascular smooth muscle cells. Expression of surface adhesion molecule ICAM-1 could be inhibited by antisense oligonucleotides thereby preventing reperfusion induced neutrophil adhesion and cell damage. This promises application in transplantation medicine where reperfusion plays a major role (60-62).

Signal Transduction Intermediates as Targets

Down regulating PKC- α with the help of an antisense oligonucleotide resulted in induction of p53 and an insulin like growth factor-binding protein in glioblastoma cells (56). Antisense oligos against *c-myc* in porcine vascular smooth muscle cells cause increased apoptosis whereas there was no effect on apoptosis in porcine aortic endothelial cells over 24 hours.

Isoform-specific antisense molecules against members of the MAP kinase signaling pathways, including ras and raf kinase promise application in treatment of cancer (44). Some of these block tumor cell proliferation in vitro, and display considerable antitumor activity in animal models. They have already been adopted for clinical applications against a wide range of human neoplasms (2,9).

Modulating Central Nervous System Functions

Antisense oligonucleotides have also found application in blocking the brain dopamine receptor function (8). The classical drugs bind to receptors whereas the antisense drugs bind to receptor-coding mRNA and stop the production of the receptor. Dopamine receptors are coded by at least five genes, which can be divided into two families corresponding to the receptor types D1 and D2. The antipsychotic activity of neuroleptic drugs appears to be mediated by D2 receptor family. The D2 receptors consist of 3 gene products viz., D2, D3 and D4. Antisense oligonucleotides corresponding to each receptor subtype help to determine their neurophysiological function under given experimental conditions(10). Neuronal nitric oxide synthase (nNOS) is an important molecule in the CNS. Hammerhead ribozymes have been used to downregulate nNOS gene expression and can be further exploited to reveal neuronal gene function to tackle neurological disease.

Dysfunction of GABAergic inhibition via the GABA_A receptor is a probable prelude to pathogenesis of **epilepsy** (27). The receptor has a heterooligomeric pentamer structure and has cognate sites for compounds like benzodiazepine and barbiturates. In vivo, intrahippocampal administration of antisense oligonucleotides to GABA_A receptor $\gamma 2$ subunit affects seizure propensity (28, 29).

Targeting Cancer

Another application of the antisense oligonucleotides has been demonstrated in activating tumor suppressor genes like p53, by targeting oncoproteins like MDM2; the latter downregulates the expression of tumor suppressor gene. In such a study with 15 tumor types containing wild type p53, significant induction of nuclear p53 was observed following inhibition of MDM2 expression (7, 40). Increased p53 level is associated with an increase in transcriptional activity, growth inhibition, or apoptosis. It may be mentioned that different isoforms of MDM2 may be expressed by different cells (49). Targeting of BRCA1 gene with antisense oligonucleotides results in inhibition of growth and enhanced apoptosis in cultured cell lines (53, 64).

Inhibiting Tumor Angiogenesis

Caveolae and their components like prostacyclin synthase in human vascular endothelial cells are involved in the morphogenetic phase of **angiogenesis**. Antisense oligonucleotides targeting caveolin-1 mRNA decrease the level of caveolin and effectively inhibit angiogenesis.

Synergy with Other Therapies

Sequences complementary to mRNA of protein kinase A in combination with cytotoxic drugs or other biological agents have been employed as a potential antitumor therapeutic strategy (64). Protein kinase A plays a key role in the control of growth and differentiation of cells and has two distinct isoforms PKAI and PKAII in mammalian cells. While PKAII is expressed in normal cells and is involved in growth arrest and differentiation, PKAI is overexpressed in cancer cells in which a ligand-activated epidermal growth factor receptor is operating. Downregulation of R1a or PKAI by antisense oligonucleotides results in cell growth arrest and differentiation in a wide variety of cancer cell lines. However, phosphorothioate oligos often show toxicity in animal models and humans. Mixed backbone oligos containing either methyl phosphonate linkages or 2-O-methyl-ribonucleotides seem to have lower side effects and a much better pharmacokinetic profile (70). A non-inhibitory dose of the antisense oligonucleotide against R1a results in a synergistic growth inhibition and an induction of apoptosis in combination with taxanes, platinum-based compounds and topoisomerase II-selective drug. Other targets that have been successfully used for intervention with antisense therapy include PKC α (39, 56), C-raf kinase (41, 42) and Ha-ras (2). Antisense oligonucleotides targeting the EGF receptor inhibit growth of ovarian cancer cells. Alteration of β -adrenergic function and reversal of phorbol ester-induced reduction of isoproterenol follows application of antisense oligonucleotides against PKC- α (33, 39). Endogenously synthesized ribozymes directed against MDR1 alter the multidrug resistance phenotype in lung tumor cells

There is an apparent radiosensitization in cells downregulated for their type I insulin-like growth factor with the help of antisense oligonucleotides in B16.F1 murine melanoma cells. This points towards a possible application of the antisense oligos in radiation treatment of the otherwise radio-resistant tumors.

The Issues Facing Nucleic Acid Therapeutics

Despite remarkable progress made in different areas of application, the antisense approach to therapy must address the issues like:

- Elimination of side effects
- Large scale production of the required oligonucleotides
- Rational selection of target site
- Acceleration of annealing kinetics
- Improvement of carriers and vectors (e.g., for endogenous delivery of ribozymes)
- Uptake by the cells and transport within the cells
- Cell specific applications
- Residence time within the biological system
- Oral administrability
- Penetrating the blood brain barrier for targets in the CNS
- Unexpected consequences of an antisense oligonucleotide-based downregulation

Routes of Administration

Among the routes of administration of oligonucleotides, even intestinal absorption of antisense oligonucleotides when administered in combination with permeation enhancers has been demonstrated. Effect of various modifications of the oligos on their intestinal absorption are being studied. A combined approach of chemical modification and permeation enhancers yields bioavailability in the range of 15% to 20%. Topically applied oligos have been shown to undergo dermal absorption and distribution throughout the epidermis and into the dermis. The concentrations assayed using capillary electrophoresis suggest good bioavailability for pharmacological action. Inhalation of oligonucleotides results in substantial concentration build-up in pneumocytes, vascular endothelium and alveolar macrophages. About 50% of the material in lung remains intact 8 hours after exposure. Presence of intact oligonucleotides in plasma and other organs suggests for systemic distribution of inhaled oligos. Extracranially delivered peptide nucleic acids knock down rat neurotensin receptor (rNTR1) gene expression. Neurotensin affects CNS only when it is injected directly into the brain, causing hypothermia and antinociception. Intraperitoneally administered PNA complementary to rNTR-1 sequence reverts the effects of neurotensin injected into the brain within 24 hours. This perhaps suggests penetration of PNAs into the rat brain after their administration outside the brain.

The Problem of Drug Delivery

The delivery systems currently under investigation include liposomes, low density lipoproteins, a composite system consisting of a stearyl-poly-L-lysine, LDL and antisense oligonucleotide, biodegradable polyalkyl cyanoacrylate nanoparticles and linear and hyperbranched polymers. Water-soluble synthetic polymer-drug conjugates have also been developed and put to clinical trials for delivery of conventional antitumor agents. It is tempting to speculate application of the latter for antisense or gene delivery. The vector must be non-toxic, non-immunogenic and biodegradable. Also, it must be tightly bound with the material being carried in a reversible manner so as to incur minimal losses in

transit and optimal release at the target site. At the cellular level, the delivery must be made intracytoplasmically so that the oligonucleotide escapes from the endosomal or lysosomal compartments of the cell. Accumulation of drugs in solid tumor increases over 50-70 fold when N-(2-hydroxypropyl) methacrylamide copolymer-drug conjugates and dendrimer-drug conjugates as compared to intravenous administration of free compound. Other materials for antisense or gene delivery under investigation include low molecular weight chitosans (50) and polyamidoamines (PAAs).

The oligonucleotides reach a maximal level in the liver 8 hours after an intravenous application at a dose rate of 10mg/kg body weight. The concentration rapidly declines following this peak suggesting for an active efflux or metabolic clearance mechanism (21, 49, 72). Nevertheless, the oligonucleotides administered intravenously accumulate in proportion with the total dose administered (54) and there seems to be good tolerance for the intravitreally administered oligonucleotides (32). The observation that liposomal delivery systems accumulate within tumors and sites of inflammation has been extended to design antisense and gene delivery systems. Therapeutic index of phosphorothioate oligonucleotides is enhanced when they are encapsulated in liposomes. Unfortunately, it is difficult to efficiently encapsulate oligonucleotides in conventional liposomes. Some cationic lipid-based formulations produce stable lipid-oligonucleotide particles and yield encapsulation efficiencies of 90%. These formulations work independently of the oligonucleotide chemistry as long as negative charges are present. In the encapsulated form, the oligonucleotides are protected from nuclease degradation and interaction with plasma proteins. Thus formulated phosphorothioate oligos do not activate complement in serum. They accumulate in tumors and at sites of inflammation. Encapsulated oligonucleotides directed against ICAM-1 completely inhibit the inflammatory response in a murine model of delayed type hypersensitivity at an increased efficacy over the free drug. Certain observations suggest for involvement of receptors or binding molecules on cell surface in mediating uptake of oligonucleotides (24). Efficient delivery of phosphodiester antisense oligonucleotides complexed with ligand, poly-L-lysine and a human replication-defective adenovirus via the asialoglycoprotein receptor into the liver of Peking ducks has been demonstrated (45, Offensperger et al 1998). Dissociation of the phosphorothioate oligonucleotides from cationic lipids seems to be a pre-requisite for their uptake by nuclei (36). Encapsulation and release of the encapsulated materials in decylamine-carboxymethyl-cellulose may be influenced by certain modulators and also seem to be a function of molecular weight (37). Streptolysin permeabilisation and electroporation seem to significantly enhance the uptake of oligonucleotides compared to lipophilic conjugates (59).

Target Validation with Antisense Oligonucleotides

Effects of antisense oligonucleotides and ribozymes on the recipient can be undertaken to validate utility of a cellular molecule as appropriate drug target (Christoffersen, 1997). Downregulation of anti-apoptotic genes like BCL-2, Survivin and BCL-xL to eradicate the malignant cells and make them amenable to chemotherapy is being actively pursued as a possible co-therapeutic strategy. Decreased BCL-2 protein production, loss of cell viability and induction of apoptosis have been noticed using this approach in lymphoma as well as leukaemia cell lines that overexpress BCL-2 (9).

Antisense Oligonucleotides as Post-Surgical Help

Angioplasty is an important treatment for vascularization of blockade in coronary arteries. However in 25%-45% of successfully dilated coronary lesions restenose within six months requiring repeat treatment. Coronary restenosis results from enhanced proliferation of smooth muscle cells within the vessel wall causing myointimal hyperplasia (35). Antisense oligonucleotides against *c-myb* and *c-myc* have produced encouraging responses lessening the probability of restenosis (52).

Targeting Viral Nucleic Acids

Antisense oligonucleotide therapy has also found application in combating viral infections. Balb/c mice exposed to influenza virus A strain at dose of 100 LD₅₀s when administered with antisense oligos, showed significantly prolonged mean survival time. Partially thioated anti-HIV oligonucleotides were found to have comparable metabolic half-life and lower toxicity than completely thioated oligonucleotides. Anti-FMDV ribozymes have been used for efficient cleavage of FMDV RNA in the VP3, VP4 and viral polymerase- coding region (63). Effectiveness of antisense therapy for hepatitis B virus has been tested in Peking ducks as the experimental model (45).

Diverse Applications through Exon-Skipping, Cell Proliferation, and Blood Coagulation

Cultured dystrophin-deficient mouse muscle cells administered with 2' ribose modified oligoribonucleotides complementary to splice sites flanking exon 23 (bearing a point mutation in the mdx mouse model of DMD) resulted in exon skipping and restoration of the dystrophin reading frame. This strategy is being extended to primary cardiac myocytes from the mdx mouse and human cells.

Incubation of splenocytes from male Sprague-Dawley rats with anti-proopiomelanocortin oligonucleotides prior to stimulation by Con A significantly decreased [³H]-thymidine incorporation while similar treatment with anti-preproenkephalin oligonucleotide resulted in enhanced [³H]-thymidine incorporation showing the role of these endogenous molecules in modulating the proliferation of activated splenocytes in vitro.

A monocyte tissue factor acts as surface receptor for coagulation factors VII and VIIa initiating **blood coagulation**. Suppression of this factor by 2'-O-methyl RNA coupled to anti-CD14 monoclonal antibody has been achieved and its possible in vivo applications projected.

Antisense Therapy for Genetic Disorders

Dominant genetic disorders like the Huntington's disease and Marfan's syndrome may become amenable to antisense therapy. Initial studies using phosphorothioate oligodeoxynucleotides show inhibition of synthesis of huntingtin in a human teratocarcinoma cell line (23). Mutant fibrillin which exerts a dominant negative effect in patients with Marfan's syndrome can be downregulated with the help of antisense oligonucleotides for management of the disease state (30). A class of mutations in

characterized by expansion of trinucleotide repeats. This is associated with genetic disorders including Huntington's disease and myotonic dystrophy. Successful targeting and trans-splicing of such genes showing trinucleotide repeats with ribozymes has been achieved (46, 47).

Targeting Molecules of Protozoan Parasites

Targeting ribonucleotide reductase of protozoan parasites seems to hold promise as the enzyme convenes a rate-limiting step in DNA biosynthetic pathway (Ingram at *Antisense* 98).

Combinatorial Approaches and Molecular Mimics

Fitness to chosen target sequences as well as novel binding properties have been achieved employing combinatorial approaches (19, 40). When a pool of phosphorothioated RNA was bound to FGF in vitro, a family of sequences containing a 13-nucleotide stem-loop motif could be eluted. An aptamer with similar motif was shown to inhibit bFGF binding to cell surface heparin sulfate proteoglycans and to bFGF receptor thus also inhibiting the bFGF-induced cell growth (25). In another series of experiments, aptamer isolated for tight binding with a fusion protein Rex was shown to mimic the natural Rex-binding sequence element (4, 24). Apart from the therapeutic applications based on their endoribonuclease activity, ribozymes with ligase activity showing activation over 100-fold by oligonucleotides have been selected in vitro. These promise application in diagnosis and can be adopted for correcting mutant mRNAs (51). These observations illustrate the applicability of oligonucleotides against non-nucleic acid targets. Similarly, oligonucleotides inhibiting hepatitis C virus serine proteinase have been isolated (26, 67, 71).

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