Antisense DNA and RNA strategies: new approaches to therapy

This article is based on a talk given at the Science and Medicine conference at the Royal College of Physicians in November 1993.

ABSTRACT—This review describes the results both *in vitro* and *in vivo* of the application of small DNA molecules or their analogues to target sequences in messenger RNA (mRNA) or DNA. Biological effects on the replication of viruses and the expression of oncogenes are recorded. At the same time, RNA catalytic sequences ('ribozymes') have been used to target and cleave mRNA sequences. Before these treatments can be confidently applied to clinical situations, further work is needed on their stability, cellular uptake and selection of their target, and the mechanism of their action also needs to be understood and controlled. In this way, it may be possible to guarantee specific effects on gene or cell type. The review describes the current research and state of development.

In each coil of the DNA double helix, a base on one strand of DNA is bound to its complementary base on the other strand, namely: A (adenine) to T (thymine) and G (guanine) to C (cytosine). When a gene transcribes the information stored in its DNA, the helix opens and the 'sense' and its complementary 'antisense' strand separate. Each strand serves as a template for reconstructing its partner, thus producing two identical helices. To decode the genetic information in the DNA to produce the appropriate protein, the antisense strand now acts as a template for a chain of sense mRNA, which is subsequently translocated into a protein; similarly, the sense strand of DNA yields an antisense mRNA. The antisense mRNA can bind to its mirror image sense mRNA and prevent its translation into a protein.

Antisense therapeutics exploits the specificity involved in DNA or RNA base pairing. By targeting a specific gene or a sequence within it with an anti-gene, or its mRNA product with antisense, mRNA gene expression might be inhibited. Antisense technology may involve a double strand (mRNA/DNA) or a triple helix of DNA to modify gene expression and prevent or regulate the production of disease-specific proteins. The technique requires discrimination between perfect and partially mismatched sequences if it is to be of use in selecting out mutant cells from normal cells. The length of the oligomer would usually be in the 11–20 mer range to maintain such discrimination, and the base composition must be such as to reduce intra-strand secondary structure formation.

Dramatic results both *in vivo* and *in vitro* have been achieved with this technology and have occasionally resulted in the correction of physiological defects (Table 1). Furthermore, by targeting viral gene sequences or the genes involved in tumorigenesis ('oncogenes'), effects on viral replication, cell transformation and tumour growth have been achieved. (Some of these are shown in Table 2.)

Despite the burgeoning success stories, several unresolved problems remain (those which still require resolution are shown in Table 3); they will require a greater understanding before antisense strategies can be applied to the clinical situation.

Any clinical use of antisense compounds as new therapies will also require improvements in their stability in serum and cells and also their failure to interact with key components in the cells, better uptake into cells, and of course tight base pairing specificity with target RNA or DNA.

New compounds in antisense strategies

Most work has concentrated on oligodeoxynucleotides and their analogues, the phosphorothioates and methylphosphonates (Fig 1), which show greatly increased resistance to nucleases. As Table 4 shows, they may have limited effectiveness in some of the

Table 1. Successful examples of *in vivo* treatment with antisense DNA.

Suppression of growth of solid human colon carcinoma cells in athymic mice by systemic treatment (cholesterol pellet implant) with an oligomer antisense to the type 1 regulatory subunit of cAMP-dependent protein kinase.
Suppression of Philadelphia[1]-positive human leukaemia cells in severe combined immune deficiency mice by systemic treatment (iv injection, 1 mg/day) with a 26-mer antisense to the B2A2 breakpoint-junction of *bcr-abl*.
Inhibition of intimal arterial smooth muscle cell accumulation by an 18-mer complementary to *c-myb* RNA using local delivery (pluronic gel) in a rat carotid artery model.
Treatment of human leukaemia cells in a severe combined immune deficiency mouse model of chronic myeloid leukaemia by systemic treatment (Alzet™ pump implant, 100 µg/day) with a 24-mer antisense to *c-myb*.

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Table 2. Successes with *in vitro* treatment of viral replication and oncogene expression with antisense DNA.

Antivirals	Anticancer
Human immunodeficiency virus, type 1	*c-myc* *n-myc*
Influenza virus, types A and B	*l-myc* *ras*
Hepatitis virus, type B	p53 *c-myb*
Human papilloma virus, types 6 and 11	*bcl*-2 *bcr-abl*
Herpes simplex virus, types 1 and 2	MDR *c-erb* B-2
Cytomegalovirus	*jun* bFGF *wnt*-1 TGF-β *spi*-1

properties described in Table 3 and show relative differences in their effectiveness. It is essential to retain base-specific pairing whilst overcoming sensitivity to nucleases. Phosphorothioates are, for example, less sensitive to nucleases than are the phosphodiesters. The methylphosphonates are fairly inert, uncharged molecules and do not interact with cellular nucleic acid enzymes. Charged molecules, on the other hand, for example phosphorothioates, have cytotoxic side-effects and interact non-specifically with cell proteins. An analogue, peptide nucleic acid (PNA) chimaera, in which the entire deoxyribose-phosphate backbone has been exchanged with a polyamide (peptide) backbone, is a powerful DNA mimic (Fig 2) with potential for being developed into an antisense drug. It appears that a single strand of DNA is displaced and two DNA strands take its place. PNAs inhibit the elongatory action of RNA and DNA polymerases. Unlike other gene targeters, they are not restricted to attacking promoter or regulatory regions of DNA.

Homopurine/homopyrimidine nucleic acids can form three-stranded structures; that has made it possible to produce sequence-specific interactions with double-stranded DNA. The third strand interacts with the purine base in the major groove of the double

Table 3. Areas of investigation to improve antisense effects.

Synthesis and purification	Biodistribution
Structural characterisation	Biological stability
Solubility	Inhibitory efficacy
Cellular uptake	Safety and toxicity
Target binding	Drug formulation
Pharmacokinetics	Regulatory approval

Table 4. Comparisons of two antisense DNA molecules for effectiveness.

	Normal phosphodiester	Methylphosphonate analogues
Cell uptake	+	++
Biological stability	–	+++
Non-toxicity	±	++
Hybridisation efficacy	+++	+

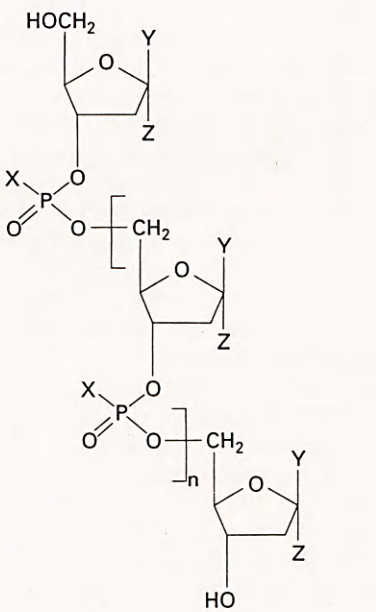
+ = effective
– = non-effective

helix. The target sequence requires contiguous purine bases on a DNA strand. New triplex formations are currently being developed with the aim of inhibiting gene expression at the transcriptional level. Figure 3 shows such a triplex DNA molecule which has proved to be stable under physiological conditions. It is likely that this will inhibit the movement of RNA polymerase or the action of transcription factors during transcription.

Ribozymes in antisense strategies

The discovery that certain molecules of RNA can cleave other RNA sequences has been one of the most

Fig 1. *DNA modifications with potential clinical application.*



	X	Y	Z
Phosphodiester	O	A,G,C,or T	H
Methylphosphonate	CH_2	A,G,C,or T	H

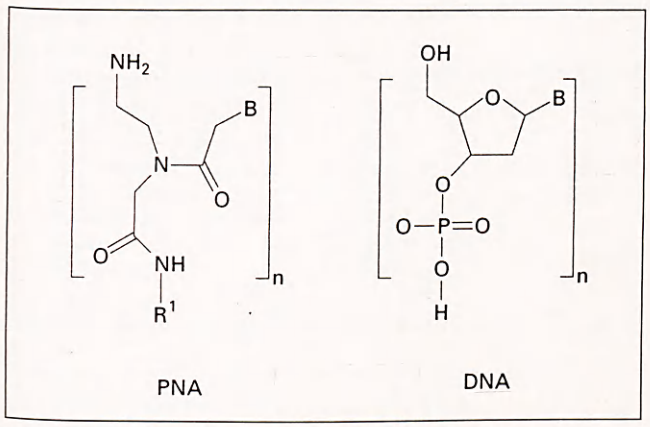


Fig 2. *Peptide nucleic acid (PNA) mimics DNA structure.*

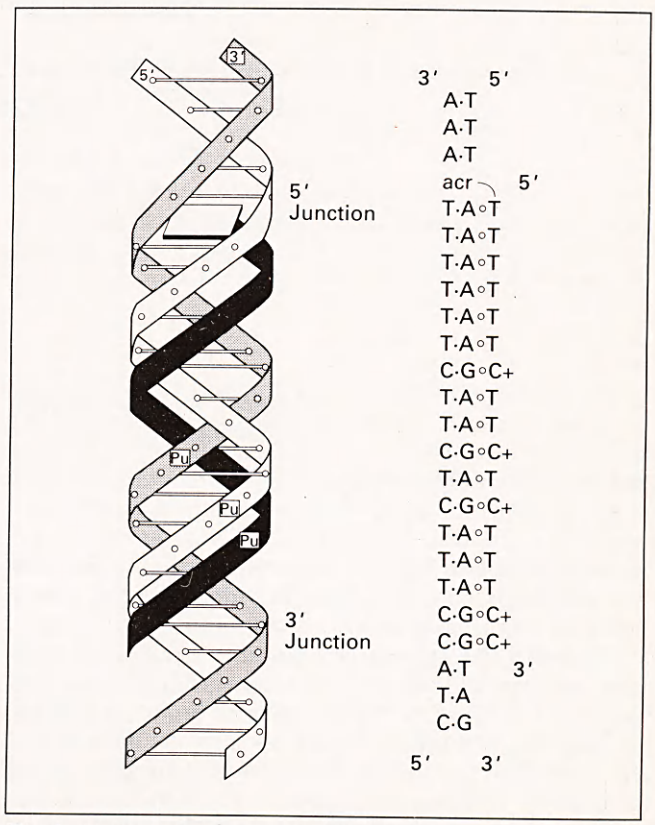


Fig 3. *Triple helix formation. The third strand interacts with purine bases in the major groove of the double helix.*

fascinating developments in recent molecular biology. RNA 'enzymes' (ribozymes) can be designed to cleave sequences unique to viral RNAs, and therefore have great potential in viral therapy. Cells expressing a ribozyme which cleaves HIV-1 RNA are more resistant to the virus. Ribozymes can be designed by taking an RNA sequence from a natural ribozyme, for example a plant satellite RNA virus, and adding flanking RNA or DNA arms. Figure 4 shows the 'hammerhead' structure of such a ribozyme designed to target an mRNA part of the *ras* oncogene message. This reaction leads to cleavage of the message into two fragments. *In vivo* action of a ribozyme against the *ras* oncogene has recently proved successful, and clinical trials against the HIV virus are planned shortly. Current research aims to maximise the catalytic activity and resistance of ribozymes to nucleases. Another approach involves the production of an RNA transcript from the opposite strand to that producing mRNA, thereby forming an RNA duplex; this reduces protein synthesis.

Strategies for cellular uptake

One of the greatest advances in antisense strategy would be achieved by increasing the import of the oligomers into cells and directing them to a site in the cytoplasm or nucleus where the pre-message or mRNA is to be found. Several routes of entry into cells have been disclosed (passive diffusion, receptor-mediated, fluid-phase and adsorptive-phase endocytosis), and seem to depend on the particular oligodeoxynucleotide analogue employed. Highly anionic phosphorothioated and phosphodiester oligomers bind to the cell surface and so get into cells. Methylphosphonates are non-ionic and are thought to enter by passive diffusion, although this is disputed. Our laboratory has recently isolated a surface binding protein for antisense DNA which appears to have the properties expected of a receptor. For example, the greater the amount of this protein, the greater the uptake of the antisense molecules into cells. This process of uptake is also energy dependent. After binding and entry, many molecules enter the endosome/lysosomal pathway and fail to reach their target; methods for circumventing endocytosis will be of great value.

Access to the nucleus or cytoplasm is clearly important for efficient antisense activity, and restricting antisense molecules in vesicles makes for inefficiency. Some of the experimental approaches for improving mechanisms of entry of DNA and RNA into cells and directing them to their targets are listed in Table 5. To improve delivery, cationic lipids have been used to disrupt membranes and allow the DNA to enter the cytosol. Similar mechanisms of entry with polylysine conjugated to various ligands bring about receptor-mediated endocytosis. Cationic polylysine tails on molecules may disrupt the endosomal membrane in a manner similar to cationic lipids, allowing vesicle-entrapped DNA molecules to escape into the cytosol.

Selecting the target

Isolating the sections of the target DNA or mRNA sequences which are available for strand interaction has proved difficult. Secondary structures within

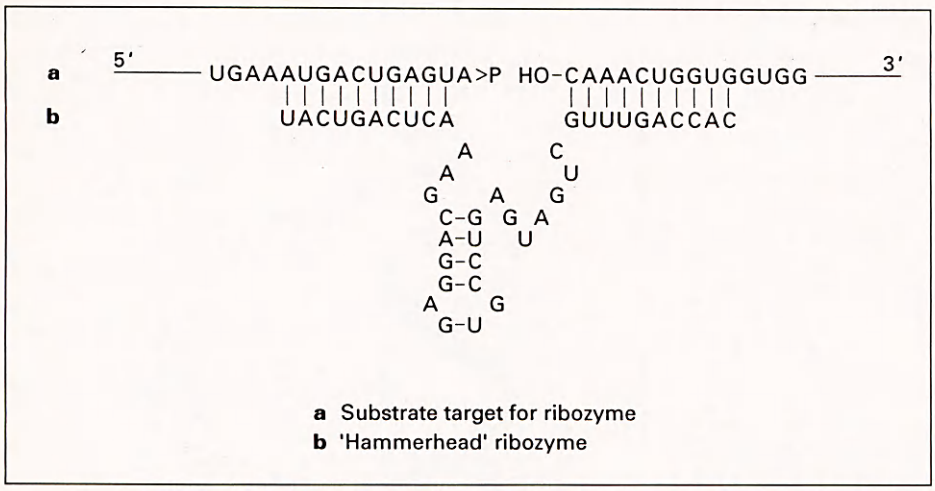


Fig 4. *Design of a ribozyme: (a) substrate target for ribozymes; (b) 'hammerhead' ribozyme.*

nucleic acids are hard to predict, although some successes have been claimed for the identification of single loop target sections.

Directing the antisense oligomer to such a target in the nucleus or cytoplasm is clearly important, but again the method of entry is critical. Some success for getting the oligomers into the nucleus can be achieved by microinjection or by adding polylysine tails to the oligomer. To form triplexes in the nucleus means identifying runs of purine bases in the target DNA; this too has been accomplished.

Recently, targeting ribozyme sequences to sites of viral RNA replication has been tackled by directing the ribozyme to the viral packaging sites. This problem is more easily tackled with viral replication than by attempting to track mRNA in the nucleus or cytoplasm. Directing two RNA molecules to the same site will require a knowledge of the physical pathways involved in RNA transit.

Methods of action of antisense DNA

Several mechanisms have been proposed to explain how protein synthesis might be inhibited. One is steric blocking of ribosome assembly and splicing, and capping, mechanisms in mRNA production. Other events in the maturation of mRNA or its translation into protein have also been implicated in some of the successful work. Aiming at the recognition sequences such as translation initiation or protein binding regulatory sites would appear attractive. (The mechanisms, including that of the ribozymes, are described in Table 6.)

The mechanism involving mRNA cleavage is mediated by cellular RNAase H, which attacks the RNA part of an RNA/DNA hybrid to give a fragment of mRNA. Such inhibition will depend on the cellular activity of RNAase H which is known to vary. It should also be noted that methylphosphonate oligomers are not substrates for RNAase H.

Table 5. Improving uptake.

Liposomes.
Receptor-mediated endocytosis (adenovirus/antisense complex).
Retroviruses.
Modification of oligomer, eg addition of lipophilic moieties at the 2^1 position of the ribose ring.

Towards clinical applications

Application of antisense compounds by *ex vivo*, regional or systemic administration are now strong possibilities. Additions to stem cells as a treatment for haematological tumours can involve antisense treatments to remove abnormal cells before transferring the surviving cells back to the patient. *Ex vivo* treatment of this nature avoids the toxic problems which ensue following the administration of drugs directly to patients. Direct application of the compounds to brain or lung, or injection into portal veins for treatment of liver tumours, would ensure high levels of antisense being site-directed to the relevant tumour. The essential requirement for stability against tissue nucleases with such treatment is shown in Table 7. The 3' end of the molecules is the end more susceptible to attack, and the resultant effects following protection are recorded in Table 7 both *in vivo* and *in vitro*.

Table 6. Mechanisms of action (*in vitro* and *in vivo*?).

Oligomers	Activation of RNAase H binding to mRNA (translation inhibition).
Ribozymes	Cleavage of substrate, antisense (?).
Triplexes	Inhibition of polymerases and RNA target as inhibitors of translation.

Table 7. Stability (oligomers and ribozymes).

Protection at 3' end (thioated, methylphosphonate).
Confers stability without toxic effect up to:
72 hours *in vitro;*
seven days *in vivo* following regional application to cerebrospinal fluid.

Table 8. Pharmacokinetics of antisense DNA molecules in mice.

Rapid ($t_{\frac{1}{2}}$, 10–20 minutes) plasma clearance.
Slow ($t_{\frac{1}{2}}$, 30–40 hours) urinary clearance.

Specific targeting of genes involved in tumour progression has been achieved with antisense technology, including those genes that initiate the progress of the multistage process of tumorigenesis. For example, the *bcr-abl* fusion gene which produces a specific mRNA and protein in chronic myeloid leukaemias can be targeted, it is claimed, without affecting the activity of normal *abl* or *bcr* genes. Genes which result in apoptosis (programmed cell death) or enhancement of chemotherapy and radiation sensitivity have also been targeted. One strategy to inhibit tumour cell proliferation that is often discussed is the induction of terminal differentiation. Targeting genes which block differentiation, for example *c-myc* or *c-myb* homeobox transcription factors, might allow tumour cells to differentiate and lose their proliferative capacity, but this has not yet been convincingly demonstrated.

Conclusion

Antisense oligonucleotide technology is being applied both in animal studies and in clinical trials. Studies on pharmacokinetics, adsorption, distribution, metabolism and excretion of the compounds are needed before they can be used clinically. Table 8 shows the results of a pharmacokinetic study in mice where both slow and rapid clearance mechanisms operate. The choice of antisense compounds will require many properties to be understood, including their mode of action. In some instances, RNA cleavage may be of benefit; in others, the neutral backbone of the methylphosphonates which utilises steric hindrance mechanisms may be favoured. Chimaeric antisense DNA molecules combining several potential mechanisms for activity within the structure may be even more appropriate. There is much to learn, but the experimental pace is quickening; some of the experimental areas likely to result in improvements for drug development are listed in Table 9.

Table 9. Improvements.

Combined antisense applications to different sequences on target.
Triplex-combined treatment to different strands.
Ribozymes: increased production (?).
Targeting RNA substrate for specificity.
In vivo studies (rate-limiting steps, ionic conditions, protein binding).

Further reading

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