In pursuit of antisense

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The first generation of antisense oligodeoxynucleotides (ODNs) are now undergoing clinical trials, but their effects may reflect biological activities unrelated to their ability to bind RNA. Nevertheless, preclinical animal studies now suggest that phosphorothioate ODNs may be more permeable in certain animal tissues than in cell culture, raising hopes that antisense mechanisms can be exploited pharmacologically.

THE development of antisense oligodeoxynucleotides (ODNs) as therapeutic agents relies on the ability of ODNs to bind to a disease-causing RNA, by Watson–Crick base pairing, and thereby inactivate it. Phosphorothioate-modified ODNs have progressed the furthest in clinical development for a number of indications (Table 1). We discuss the limitations of these first-generation compounds and the more potent molecules that are being progressed through cell culture and preclinical models.

First-generation ODNs

Much has been learned from the use of phosphorothioate ODNs that can be applied to the design of future molecules. A more rigorous set of criteria for controls in antisense studies has been used in the determination of whether antisense is the mechanism of action responsible for the observed effects[1,2]. From studies that have adhered to these guidelines, a generally accepted antisense model has evolved as follows: antisense molecules endocytose into cells, enter the cytoplasm by assisted (for example, cationic liposomes) or non-assisted means, translocate to the nucleus by diffusion, hybridize to their target RNA, and result in cleavage of the RNA portion of the hybrid by the cellular enzyme RNase H[3–6]. Virtually any region of the RNA can be successfully targeted by antisense phosphorothioate ODNs. The adage of "target the AUG initiation codon" had to be replaced after finding that many sites of a message need to be examined to locate an active ODN[3,4,7,8]: sites may not be accessible because of RNA secondary structure or may not impart enough binding affinity to support ODN hybridization.

Of particular note is that there is now the indication that phosphorothioate ODNs can penetrate at least some cells in animals. A number of cell culture studies previously led to the conclusion that antisense ODNs cannot permeate cells except in the presence of delivery methods (cationic liposomes[1,9,10] or electroporation[11,12]). In the best example of more permissive permeation in animals, daily treatment of tumour-bearing mice with an ODN (0.06–6 mg kg^{-1}) targeted to the c-*raf* kinase gene caused dose-dependent inhibition both of tumour growth and of c-*raf* kinase RNA levels in the tumours[8]. That this was attributable to an antisense mechanism of action was supported by a strong correlation between highly controlled cell culture results and antitumour activity. The potency of the antisense ODN decreased rapidly as the number of mismatches in the perfectly matched ODN were increased. The striking disparity between cell culture and *in vivo* observations surrounding ODN permeability highlights the need for a much better understanding about the permeable properties of phosphorothioate ODNs in animals. Sequence-dependent non-antisense effects could also cause tumour and oncogene suppression[1]. To prove that antisense inhibition operates in animals, future studies will need to examine inhibition of other targets that affect RNA and protein levels, but not tumour growth (in the case cited above, perhaps another *raf* family member that does not inhibit tumour cell growth). Also of interest will be whether tumour cells are particularly sensitive to ODN uptake. These results, if further supported, mark a notable advance in antisense technology.

The pharmacodynamic activities of phosphorothioate ODNs have been characterized in a number of animal species and in humans. Phosphorothioate ODNs are administered by systemic routes (not oral) and are quickly eliminated from plasma to give broad tissue distribution (mostly liver and kidney, but not brain)[13–15]. Phosphorothioate ODNs can cause immune stimulation and cytokine release depending on the sequence and modification of the ODN[16–18]. In monkeys, phosphorothioate ODNs may cause complement activation and hypotension[19], and their administration can increase clotting times[20]. None of these effects have been problematic in clinical trials but they have led to the need for long infusion times (to avoid hypotension) and may be dose-limiting for efficacy endpoints. Also, phosphorothioate ODNs can cause numerous non-antisense effects in cell culture studies because of their potential to bind proteins, such as growth factors and receptors[21] (for review, see refs 1, 22, 23). In addition, non-specific effects on cellular adhesion have been described[24–26]. So far the precise mechanisms of action of many of the compounds in clinical trials are unknown and may include both antisense and/or non-antisense mechanisms. Although this is a subtle distinction in terms of determining clinical efficacy, the design of improved agents relies on a rational mechanistic understanding.

TABLE 1 Oligodeoxynucleotide therapies currently under clinical development

Indication	Company	Product	Clinical stage
HIV	Aronex	AR177	Phase I
HIV	Chugai	GPs0193	Phase I
HIV	Hybridon	GEM91	Phase Ib/II
CMV	Isis	ISIS2922	Phase III
HPV	Isis	ISIS2105	Discontinued, Phase II
Inflammation	Isis	ISIS2302	Phase II
Lymphoma	Genta	G3139	Phase I/II
AML	Lynx	—	Phase I/II
CML	Lynx	—	Phase I/II
Cancer	Isis	ISIS3521	Phase I
Cancer	Isis	ISIS5132	Phase I
Restenosis	Lynx	—	Phase I

Source: ref. 50

Second- and third-generation compounds

Numerous modifications and design strategies have been applied to optimize the antisense and pharmacological properties of ODNs[27,28]. Central to this development is our increased

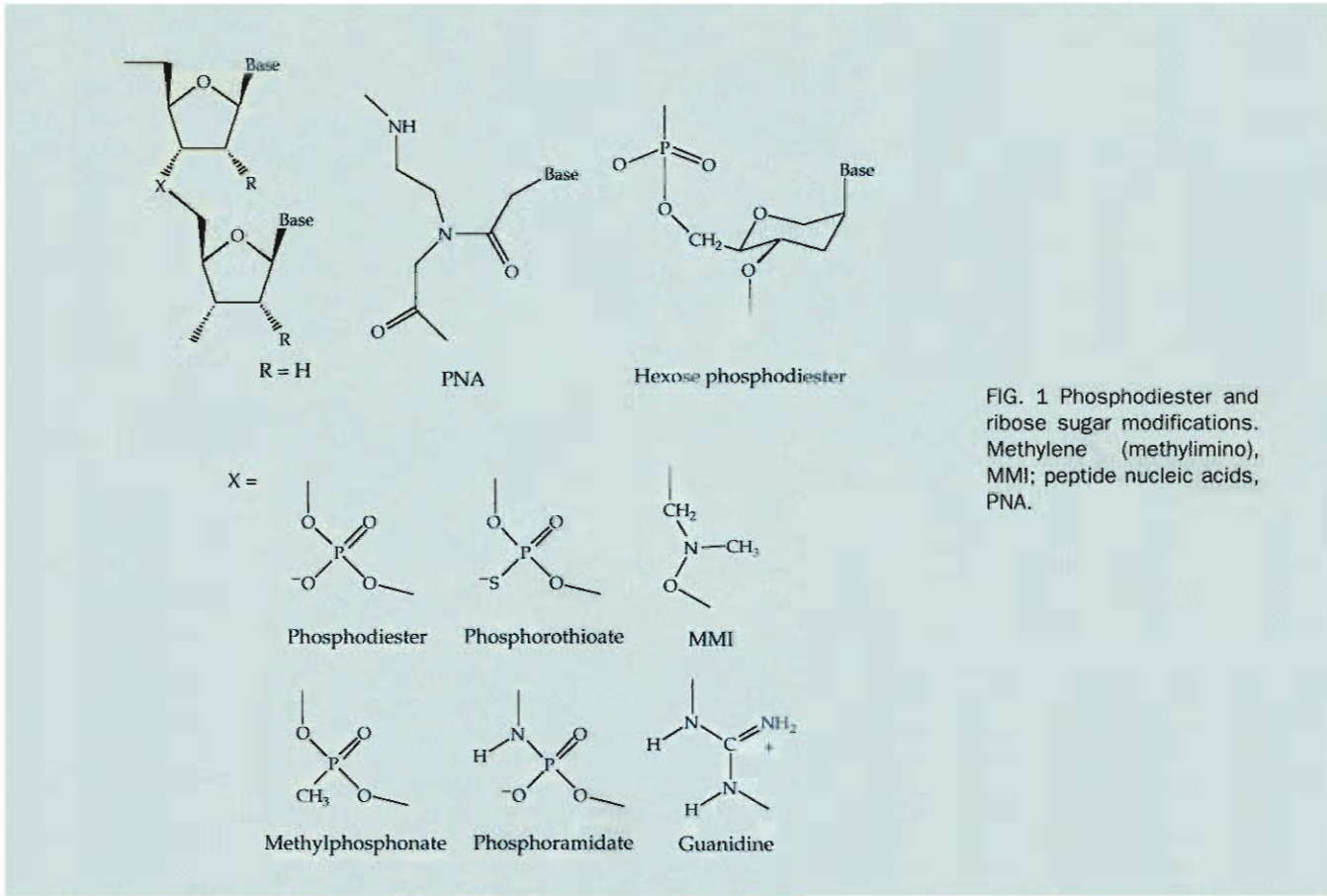

FIG. 1 Phosphodiester and ribose sugar modifications. Methylene (methylimino), MMI; peptide nucleic acids, PNA.

knowledge of antisense mechanisms. Studies now suggest that the most potent antisense effects can be obtained when RNase H is used instead of a non-cleaving steric blockade of RNA processing and translation[3–6]. This has prompted investigation of other RNA-degrading mechanisms, including ribozymes[29,30], RNase P (an endogenous RNA-protein enzyme involved in transfer RNA processing)[31] and RNase L (an endogenous enzyme which is activated by 2′–5′ $(A)_n$)[32].

A substantial effort has been made to develop structural modifications that preserve RNase H recruitment and enhance hybrid affinity. The frustration of the chemists has been that modification of the phosphate internucleotide linkage and ribose sugar moiety of ODNs usually results in the loss of RNase H recruitment. Only the affinity-reducing phosphorothioate and phosphorodithioate analogues of phosphate in combination with 2′-deoxyribose sugars (R = H, Fig. 1) have been shown to retain that activity[33].

Concerns about toxicity and the nonspecific protein binding of the phosphorothioate linkages has led to the development of 'chimaeric' molecules. These are sequences that contain a minimal core (~7 nucleotides) of RNase H-recruiting phosphorothioate DNA and flanking sequences that are modified to confer affinity, specificity and nuclease stability. One recent example of this approach incorporated 2′-methoxyethoxy substitutions with phosphodiester linkages (R = $CH_3O–CH_2–CH_2–O–$, Fig.1) in the flanking regions. This modification resulted in enhanced antitumour activity when incorporated into the c-*raf* kinase ODN, detailed above, and may have a superior side effect profile for haemodynamic changes[20].

Other flanking modifications that have been studied areneutral phosphate analogues. The methylene (methylimino) linkage (MMI, Fig. 1)[34] and methylphosphonates (Fig. 1)[35] have been used in the flanking regions. The hope is that the lower charge density from the neutral analogues will result in increased cellular permeation, decreased nonspecific binding to proteins, and/or slower clearance in animals. Currently there are no data that support the realization of these properties. In all cases, *in vitro* affinity, nuclease resistance and RNase H recruiting ability of the core phosphorothioate tract of the chimaeric molecule have been preserved.

The quest for higher-affinity ODNs has been driven by the belief that such ODNs would have enhanced potency in animals. Several provocative new modifications have recently emerged from this hunt. Peptide nucleic acids (PNA, Fig. 1) can bind both to single-stranded RNA and to double-stranded DNA with high affinity[36]. PNAs represent a dramatic departure from natural DNA because they do not contain nucleosides or nucleoside analogues. They can block translation of messenger RNA and initiation of transcription from duplex DNA in cell-free systems[37–39]. Cellular effects have been frustrating; despite

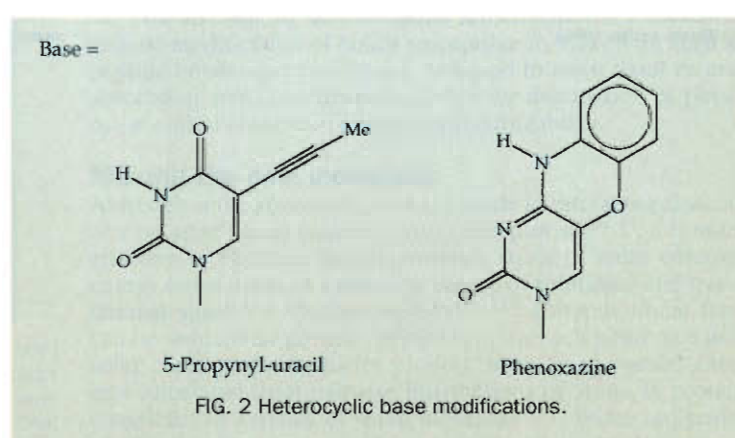

FIG. 2 Heterocyclic base modifications.

extensive effort, biological effects on cells resulting from RNA interaction have been modest[40]. The hexose nucleosides (Fig. 1) containing all four native heterocyclic bases have been linked with phosphodiesters and investigated *in vitro* but not in cell culture[41]. The 3´-phosphoramidate linkage (Fig. 1) has been completely substituted into ODNs containing all four bases[42]. Preliminary cell culture data suggest this analogue results in enhanced antisense potency[43]. The guanidine linkage has been incorporated into an oligothymine[44]. In all cases these modifications confer significantly enhanced *in vitro* affinity for a target RNA and nuclease stability to the ODN. Data on potency in cell culture are limited and *in vivo* results are not yet available.

Heterocyclic modifications (Fig. 2) can result in enhanced RNA affinity and enhanced antisense activity in cell culture. Antisense ODNs containing affinity-enhancing 5-propynyl-uracil and 5-propynylcytosine have been shown to be specific inhibitors of gene expression[45], including the cell-cycle regulator $p27^{kip1}$ (ref. 46), and Cdc2 kinase[47]. These ODNs areeffective in cell culture when introduced into cells using the serum-insensitive GS 2888 cytofectin[10,46,47]. The propynyl pyrimidines allow the use of very short (7 nucleotide-long) — and potentially cost-effective — antisense oligonucleotides where the secondary structure of a target RNA provides potency and specificity[48]. Neighbouring group stacking interactions and affinity can be enhanced by extended pyrimidine ring systems, such as the tricyclic cytosine analogue, phenoxazine[49]. Phenoxazine has conferred antisense potency, comparable to 5-propynylcytosine substitution, to some ODNs in cell culture (R.W. and M.M., unpublished results). The purine series has been less studied because of the complexities of synthesis.

Conclusions

The effort directed towards the chemistry needed to improve antisense agents has largely focused on improving ODN affinity for a target RNA. The attraction has been the detailed three-dimensional structural information available for natural DNA, which has served as a starting point for the rational design of oligonucleotide analogues. There have been many failures, the successes have been mentioned above. The other challenges facing antisense ODNs, such as improved pharmacokinetic properties, do not enjoy the benefit of a clear structural direction. These problems will, for the foreseeable future, be approached through the traditional medicinal chemistry method of synthesizing followed by testing. For such an approach, a reliable biological readout is critical. The recent developments of well controlled tissue culture assays, coupled with the potential for dependable *in vivo* assays, will allow for the definitive testing of emerging structural modifications of ODNs. □

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