Exploiting the potential of antisense: beyond phosphorothioate oligodeoxynucleotides

CA Stein

Phosphorothioate oligodeoxynucleotides, designed as nuclease-resistant antisense agents, appear to have a number of surprising biological effects that are unrelated to their intended antisense activity. These effects may be useful in themselves, but must be understood for the full potential of antisense technology to be realized.

Address: Department of Medicine, Columbia University, College of Physicians and Surgeons, 630 West 168 Street, New York, NY 10032, USA

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Antisense oligodeoxynucleotides can be used to specifically inhibit the intracellular expression of, theoretically, any given gene. It has been estimated that the sequence of an oligodeoxynucleotide of 15–17 residues in length may be unique in the entire human genome. Thus, a short DNA sequence could be used to form a specific mRNA–DNA hybrid, blocking the expression of that gene and that gene only. The attractions of such approaches have been obvious from the start, but their difficulties are only now becoming clear. Here I review some of the unexpected findings that make it difficult to interpret some antisense experiments, the potential uses of these unexpected findings, and the consequences for antisense design.

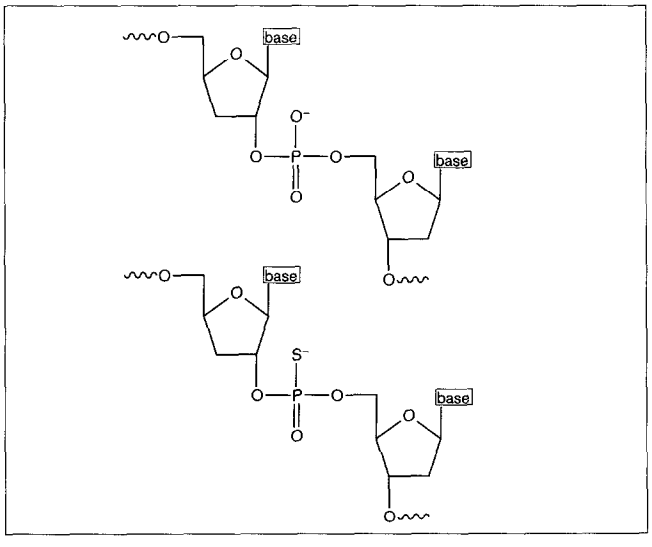
Successes and prospects of antisense

The power and elegance of the antisense approach to gene regulation has been demonstrated numerous times, both in tissue culture and *in vivo* [1]. Antisense oligodeoxynucleotides are now being used in clinical trials as therapeutic anti-neoplastic or anti-HIV agents. Target mRNAs have included a conserved region of the p53 mRNA, in an attempt to treat acute myelogenous leukemia [2], and the HIV-1 gag mRNA [3]. Although clinical efficacy has not yet been shown, one striking demonstration that antisense oligodeoxynucleotide technology can produce useful data was provided by Methia *et al.* [4]. Using a targeted antisense approach, these authors proved that the protooncogene c-*mpl* was the cell-surface receptor for a thrombopoietic factor. They used this information and affinity chromatography to isolate thrombopoietin, and then cloned and sequenced the gene that encodes it, and produced the recombinant protein. Thrombopoietin is now being used in clinical trials. As several tens of thousands of human genes will be sequenced over the next decade, it becomes essential to have a rapid, high through-put, relatively inexpensive method to determine their cellular function. Antisense oligodeoxynucleotide technology can, in theory, provide such a method.

Synthetic oligodeoxynucleotides

Normal DNA is not the ideal antisense agent, however, because it readily undergoes nuclease digestion, caused primarily by 3'–5' exonucleases, but also by endonucleases. To avoid this problem, Stec and collaborators [5] synthesized phosphorothioate oligodeoxynucleotides. These compounds have a sulfur for oxygen replacement at a non-bridging position at each phosphorus atom in the molecule (Fig. 1). They were originally synthesized by sulfurization of the phosphite linkage formed at the 5' terminus of the growing oligodeoxynucleotide by a solution of elemental sulfur in carbon disulfide/pyridine/1 % triethylamine. But this mixture tended to clog the automated synthesizer, and the sulfurization reagent in most common use today is a solution of tetraethylthiuram disulfide in acetonitrile. An alternative reagent, ^{3}H-1,2-benzodithiol-3-one 1,1-dioxide, also used with acetonitrile, has been developed by Beaucage and collaborators [6]. In both cases, sulfurization proceeds rapidly and is highly efficient, although reverse-phase high-performance liquid chromatography purification of the product is commonly performed, and is a virtual necessity for removing cytotoxic reagents used in the synthesis reaction.

Figure 1



Structures of the normal phosphodiester linkage in DNA (top) and the nuclease-resistant phosphorothioate linkage (bottom) used in antisense oligodeoxynucleotides.

Properties of phosphorothioate oligodeoxynucleotides

Phosphorothioate oligodeoxynucleotides share many of the properties of phosphodiester polymers. Although they are nuclease-resistant (though not nuclease-proof), they retain the aqueous solubility of the parent phosphodiester. The modified oligodeoxynucleotides hybridize strongly to their target mRNA, although not as strongly as does the parent molecule. For example, each G–C and A–T base pair of a phosphorothioate oligodeoxynucleotide contributes ~2.5 °C and 1.5 °C, respectively, to the duplex melting temperature (T_m), compared to 4 °C and 2 °C, respectively, for phosphodiesters. This results in a ΔH of melting for a phosphorothioate DNA–mRNA duplex that is substantially lower than that of a phosphodiester molecule of the same sequence [7]. Most importantly, phosphorothioate oligodeoxynucleotides, like their phosphodiester counterparts, can elicit the activity of RNAse H. This enzyme, found predominantly in the cell nucleus, cleaves the mRNA strand of an mRNA–DNA duplex. The oligodeoxynucleotide can thus be thought of as a catalyst for mRNA cleavage.

Here we come to the first puzzle of how antisense compounds work. RNAse H activity has been proven to be a major contributor to antisense activity in *Xenopus* oocytes, and this is also believed to be true in mammalian cells. If this is so, it should be possible to isolate mRNA cleavage fragments of the appropriate length from cells treated with an antisense oligodeoxynucleotide. It has generally been difficult to find such fragments. Recently, RNAse H cleavage fragments of p53 and *bcr–abl* mRNA have been detected in streptolysin-O permeabilized KY01 chronic myelogenous leukemia cells after treatment with appropriate antisense (but not control) oligodeoxynucleotides [8]. Unfortunately, it is unclear to what extent these data can be extrapolated to other cellular systems.

In using phosphorothioate oligodeoxynucleotides, one must consider the chirality at the phosphorus atom created by the sulfur for oxygen substitution. LaPlanche *et al.* [9] studied diastereomerically pure, duplex DNA with either a single *R*p or *S*p phosphorothioate linkage in the sequence $d(GG_SAATTCC)_2$. The value of T_m for the *R*p–*R*p duplex was diminished by 2.4 °C relative to the parent phosphodiester, whereas the T_m of the *S*p–*S*p duplex was almost identical to it. This is probably because, for the *R*p form, the P–S bond axis lies in the major groove of the duplex, whereas for the *S*p diastereomer, the sulfur atom lies on the face of the sugar-phosphate backbone, oriented 'out' of the major groove. Unfortunately, the *S*p diastereomer is much more sensitive to the nucleases found in mammalian cells than is the *R*p form.

The sulfurizing reagents commonly used in the automated phosphorothioate synthesis produce an approximately random mixture of *R*p and *S*p stereoisomers. In any given 28-base oligodeoxynucleotide, for example, 2^{27} stereoisomers may be present. To circumvent this problem, Stec *et al.* [10] designed a stereocontrolled synthesis of P-chiral phosphorothioates using a novel oxathiaphospholane synthon. Using this technology, and with subsequent modifications, it is possible to obtain milligram quantities of phosphorothioate oligodeoxynucleotides with >90 % (and, depending on the sequence, in some cases >95 %) stereopurity. The biological consequences of the improvement in the stereopurity of phosphorothioate oligodeoxynucleotides are currently being evaluated in a number of test systems.

The confounding effects of heparin-binding proteins

There is no question that phosphorothioate oligodeoxynucleotides can, under certain conditions, act as antisense reagents. One dramatic example of this was provided by Dean and Mackay [11]. They synthesized a 20-base phosphorothioate oligodeoxynucleotide targeted to the translation initiation codon of the mRNA encoding the murine protein kinase C-α isoform in liver, and administered it systemically to SK-1 hairless mice. At a concentration of 400 nM, the oligomer reversibly and sequence specifically decreased the intra-hepatocyte concentration of PKC-α mRNA and protein by 70–80 %, whereas the levels of the δ, ε and ζ isoforms were unchanged.

But phosphorothioate oligodeoxynucleotides, despite their physicochemical similarity to normal DNA, have an unexpected property that seriously complicates interpretation of experimental data obtained from their use. They bind avidly to proteins, particularly (and, as far as we know, exclusively) to those proteins that bind the negatively charged glycosaminoglycan heparin [12]. In general, the dissociation constant (K_d) of a phosphorothioate oligodeoxynucleotide–protein complex is approximately two to three orders of magnitude lower than that of the complex formed by the parent phosphodiester molecule. This may be a reflection of the fact that, at least for the complex formed between a phosphorothioate oligodeoxynucleotide and basic fibroblast growth factor (bFGF), the rate of dissociation of the phosphorothioate molecule from the protein appears to be extremely slow.

Examples of heparin-binding proteins now known to bind directly to phosphorothioate oligodeoxynucleotides include members of the FGF family, including bFGF, acidic FGF and FGF-4 (K-fgf/hst) [12], and other heparin-binding growth factors, including platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF). PDGF and VEGF have been implicated in regulating the rate of growth of certain tumor cells and in the process of angiogenesis. Phosphorothioate oligodeoxynucleotides do not seem to bind to epidermal growth factor (EGF), which binds only weakly, if at all, to heparin. It is interesting to note that there seems to be no difference between the affinities of all-*R*p, all-*S*p or stereorandom

forms of phosphorothioate oligodeoxynucleotides for bFGF, or for several other proteins [13]. The reasons for this are not yet entirely clear.

Heparin-binding cell-surface receptors

It has become apparent more recently that at least some phosphorothioate oligodeoxynucleotides can also bind to several cell-surface receptors, including the epidermal growth factor receptor (EGFR) and the VEGF receptor (flk-1) (P. Rockwell, N. Goldstein, L.M. Zhang & C.A.S., unpublished data), which both bind to heparin. Non-sequence-specific phosphorothioate oligodeoxynucleotides can stimulate EGFR phosphorylation in the absence of EGF in the KB epidermoid carcinoma line, but can antagonize ligand-mediated activation of EGFR in the presence of the growth factor. The modified oligodeoxynucleotides partially block the binding of certain anti-receptor monoclonal antibodies to flk-1, and block ligand-induced phosphorylation of this receptor. *In vivo*, a 28-base phosphorothioate homopolymer of cytidine (SdC_{28}) almost entirely blocked the growth of implanted GBM-18 (glioblastoma multiforme) cells in athymic nude mice. The effect of SdC_{28} was similar to that of a monoclonal antibody targeted to flk-1 (P. Rockwell, N. Goldstein, L.M. Zhang & C.A.S., unpublished data).

The mechanism of this inhibition has nothing to do with the classical antisense strategy, that is, the interaction of the oligodeoxynucleotide with mRNA, and we therefore term these effects 'non-sequence-specific'. This does not mean, however, that different phosphorothioate oligodeoxynucleotides all have the same level of inhibitory activity. Undoubtedly there will be some level of sequence selectivity in these effects, as discussed below.

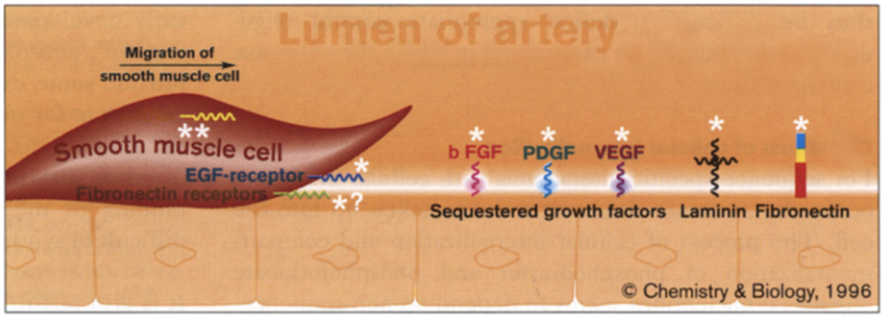
Phosphorothioate oligodeoxynucleotides block cellular adhesion by multiple mechanisms

Phosphorothioate oligodeoxynucleotides have a variety of effects on cellular adhesion, which have recently been shown to be non-sequence specific [14]. For example, there are many reports that these molecules can block heterotypic cellular adhesion to plastic substrata. Interestingly, this non-sequence-specific block to adhesion can be almost completely overcome by pretreatment of the plastic with either of the extracellular matrix proteins laminin or fibronectin, both of which bind to heparin. When the concentration of the phosphorothioate oligodeoxynucleotides is high enough, however, they can also block cellular adhesion to laminin. These compounds bind to the A (α_1) subunit (Mr = 400 kDa) of laminin at or near the heparin-binding site; this blocks the ability of laminin to bind to its ligand sulfatide (galactosyl ceramide sulfate), a compound secreted by cells and on which they can spread and grow. Phosphorothioate oligodeoxynucleotides also bind to fibronectin, and some data suggest that they bind to the fibronectin receptor as well. Although these effects were not the ones expected, it may be possible to exploit them for experimental and clinical purposes.

Effects on restenosis

Considering all the various non-sequence-specific effects of the phosphorothioate oligodeoxynucleotides, it seems likely that these compounds could also prove useful as agents that block coronary restenosis after balloon angioplast, since inhibition of the effects of growth factors of the FGF family should block smooth muscle proliferation (Fig. 2). Indeed, these compounds have already been evaluated as sequence-specific inhibitors of restenosis in a rat model [15]. The phosphorothioate oligodeoxynucleotides used in this study were targeted to the mRNA of the c-*myb* protooncogene, which has been implicated in the proliferation of smooth muscle cells. The results initially appeared positive, but their interpretation is complicated by the fact that the antisense c-*myb* phosphorothioate oligodeoxynucleotide contains a sequence motif consisting of four contiguous guanosine bases. Burgess *et al.* [16] have provided evidence that virtually all of the activity of the anti-c-*myb* oligomer against restenosis resides in this

Figure 2



Multiple sites at which phosphorothioate oligonucleotides might block restenosis. Potential binding sites for phosphorothioate oligonucleotides are indicated with asterisks; sequence-specific binding sites are shown as double asterisks. In one study [15], phosphorothioate oligonucleotides were evaluated as sequence-specific inhibitors of the c-*myb* oncogene and found to inhibit smooth muscle cell proliferation; however, it now seems more likely that non-sequence specific effects are more important [16]. Such non-specific effects include: inhibition of the activity of heparin-binding growth factors such as bFGF, PDGF and VEGF (shown sequestered in the extracellular matrix of the artery wall); inhibition of growth factor receptors such as the EGF receptor; and inhibition of cellular adhesion, for example by binding to the extracellular matrix proteins laminin or fibronectin, or possibly to the fibronectin receptor.

motif alone, suggesting that the inhibition of proliferation is not a true antisense effect.

We have recently shown [17] that the non-sequence-specific phosphorothioate SdC_{28} is a potent inhibitor of smooth muscle cell proliferation in response to EGF and bFGF, and that it is an effective inhibitor of restenosis when applied to the adventitial surface of a rat carotid artery in a pluronic gel after balloon angioplasty. On the basis of evidence of this type, it seems obvious that the biological activity of phosphorothioate oligodeoxynucleotides is based on their pleiotropic effects; at least in some cases, these are a composite of sequence-specific and non-sequence-specific effects.

Influence of G-quartets

The binding of phosphorothioate oligodeoxynucleotides to proteins, and in particular to heparin-binding proteins, seems to be dependent on the length of the oligomer, and to some extent on the sequence as well. For example, phosphorothioate oligodeoxynucleotides that contain the G-quartet motif (four contiguous guanosine residues) are more potent non-sequence-specific inhibitors, probably because they bind more strongly to proteins (e.g., to recombinant soluble CD4, bFGF or the laminin α_1 chain). Some phosphorothioate oligodeoxynucleotides (e.g., $SdT_2G_4T_2$ [18]) actually form parallel-stranded quadruple helices by Hoogsteen base-pairing, which may account for the fact that this compound binds more strongly to recombinant soluble gp120 than other phosphorothioate oligodeoxynucleotides [19]. Our understanding of the role of the G-quartet motif in 'non-sequence specific' effects is incomplete, however. For example, at least some phosphorothioate oligodeoxynucleotides containing the G-quartet motif have increased avidity for protein despite the demonstrable absence of the higher order structure caused by the formation of Hoogsteen base pairs ([12], C.A.S. and coworkers, unpublished data). Furthermore, Maltese *et al.* [20] have recently shown that the extent of the 'non-sequence-specific' effects observed with phosphorothioate oligodeoxynucleotides containing a G-quartet motif is dependent on the nature of the flanking sequences. It can thus be difficult to define appropriate control oligodeoxynucleotides for an experiment in which the antisense construct contains a G-quartet motif.

Questions of cellular internalization

For sequence-specific interactions with cellular mRNA to occur, the antisense construct must be taken up into the cell. The process of cellular internalization and compartmentalization of phosphodiester and phosphorothioate oligodeoxynucleotides seems to depend on non-sequence-specific interactions, however. The first step in internalization takes place at the cell surface, where charged oligodeoxynucleotides adsorb to binding sites on the cell membrane. In the cell lines examined thus far, >90 % of these sites are heparin-binding sites, and may include, as described above, Mac-1, EGFR and flk-1. Oligodeoxynucleotides that are uncharged, such as methylphosphonates, and peptide nucleic acids, in which the nitrogenous bases are covalently linked to a polypeptide scaffold, cannot bind to heparin-binding proteins, do not adsorb well to the cell surface, and tend not to be internalized in cells to a great extent. Since even non-charged oligodeoxynucleotides cannot passively diffuse through cell membranes, they must be internalized by fluid-phase endocytosis (also known as pinocytosis). This process tends to be inefficient, particularly for non-adherent cells. Thus, it may be difficult to construct antisense agents that are efficiently taken up by cells and yet fail to bind to heparin-binding proteins. There is some recent evidence, however, for the existence in some cells of a membrane channel that transports oligodeoxynucleotides [21].

Future directions

Where does this leave us? The advantages of phosphorothioate oligodeoxynucleotides are clear: they are soluble and nuclease resistant, form stable duplexes with mRNA, elicit RNAse H activity, adsorb well to cell membranes, probably by binding to heparin-binding sites, and are internalized efficiently (although their ability to penetrate endosomes or lysosomes in the absence of cationic lipids seems to be limited). On the other hand, phosphorothioates longer than 15 bases can have effects that are P-chirality-independent, non-sequence-specific, and which may confound the interpretation of experimental results. At least in some cases, these effects can be seen at a concentration of no greater than 2 μM. Furthermore, it may occasionally be impossible to establish appropriate control sequences when phosphorothioate oligodeoxynucleotides containing certain sequence motifs (e.g., G-quartets) are used. Thus, phosphorothioates have their limitations, although this by no means rules out the possibility that they may be extremely active anti-proliferative, clinical therapeutic agents.

There is no question that there is a great deal of room in this field for creative chemistry. The recent syntheses and early development of C5-propyne phosphorothioate [22] and of phosphoroamidate oligodeoxynucleotides [23] provide some examples. The phosphoroamidates contain an oxygen for nitrogen substitution at a bridging position on phosphorus. They seem to be potent antisense reagents with little or none of the non-sequence-specific binding of the phosphorothioates. They are, however, difficult to synthesize in high yield.

It is also possible that combinations of various classes of backbones, incorporated into what have been called chimeric oligodeoxynucleotides, will eventually render the ultimate antisense construct more efficacious, with fewer non-sequence-specific effects. It is possible that

the chimera will have to be optimized for variables such as targeted cell type and oligomer sequence, and may well have to make use of chemistries that are not yet available. Although antisense technology is now entering its second decade, our understanding of this complicated, controversial field is, to all intents and purposes, still in its infancy.

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