JOURNAL OF
MEDICINAL
CHEMISTRY

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Volume 36, Number 14 July 9, 1993

Perspective

Current Concepts in Antisense Drug Design

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Received April 14, 1993

Introduction

Antisense oligodeoxynucleotides (ODNs) have been proposed as a major class of new pharmaceuticals. In general, antisense refers to the use of small, synthetic oligonucleotides, resembling single-stranded DNA, to inhibit gene expression.[1-3] Gene expression is inhibited through hybridization to coding (sense) sequences in a specific messenger RNA (mRNA) target by Watson-Crick base pairing in which adenosine and thymidine or guanosine and cytidine interact through hydrogen bonding (Figure 1). These simple base-pairing rules govern the interaction between the antisense ODNs and the cellular RNA, allowing the design of ODNs to target any gene of a known sequence. A major advantage of this strategy is in the potential specificity of action. In principle, an ODN can be designed to target any single gene within the entire human genome, potentially creating specific therapeutics for any disease in which the causative gene is known. As a result, there have been numerous studies of antisense ODN activity for potential antiviral[4-6] and anticancer[7,8] applications. Although the field has progressed over the past decade, recent papers indicate that the observed activity of ODNs in tissue culture may be predominantly through non-antisense mechanisms.[5,6,9-11] Selected studies will be used to demonstrate that there are significant hurdles which are limiting progress in the development of ODNs both as research tools and therapeutics. Understanding these hurdles to antisense efficacy provides a framework for the future development of potent, antisense ODNs.

Antisense RNA

Antisense was first described as a naturally occurring phenomenon in which cells transcribe an antisense RNA complementary to a cellular mRNA.[1,2,12] This antisense RNA was found to be a repressor of gene expression, hybridizing to a target mRNA, inhibiting its translation, and decreasing the cellular levels of the protein. Subsequent investigations revealed that small fragments of genes could be used to construct antisense "genes" which could be transfected into cells in culture.[13] These smaller antisense constructs specifically inhibited the target gene's expression, and hence activity of the protein product, and provided molecular biologists with a powerful new tool to study the function of many genes.[1,2,13] Antisense RNAs inhibit gene expression through the activity of a cellular enzyme which modifies double-stranded RNAs.[14] This enzyme recognizes the RNA:RNA duplex, disrupts the base pairing, and changes many of the adenosine residues to inosine.[14,15] Gene expression is inhibited since the modified mRNA is no longer competent for translation.

Table I: Properties of Selected Phosphodiester Backbone Analogues

backbone analogue	activation of RNase H	resistance to nucleases[a]	chiral center
Phosphorus Analogues			
phosphodiester	yes	–	no
phosphorothioate	yes	+	yes
phosphorodithioate	yes	++	no
methylphosphonate	no	++	yes
phosphoramidate	no	+	yes
alkyl phosphotriester	no	+	yes
Non-Phosphorus Analogues			
sulfamate	no	++	no
3′-thioformacetal	no	++	no
methylene(methylimino) (MMI)	no	++	no
3′-*N*-carbamate	no	++	no
morpholino carbamate	no	++	no
peptide nucleic acids (PNAs)	no	++	no

[a] –, rapidly degraded by nucleases; +, resistant to nucleases; ++, no nuclease degradation.

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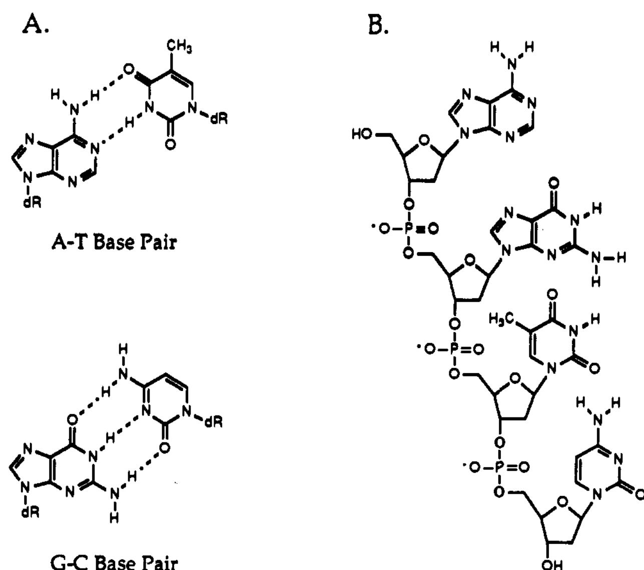

Figure 1. (A) Base-pairing interactions for adenosine with thymidine (A-T) and guanosine with cytidine (G-C), where dR is the 2′-deoxyribose sugar. (B) Structure of an oligodeoxynucleotide (5′-AGTC-3′).

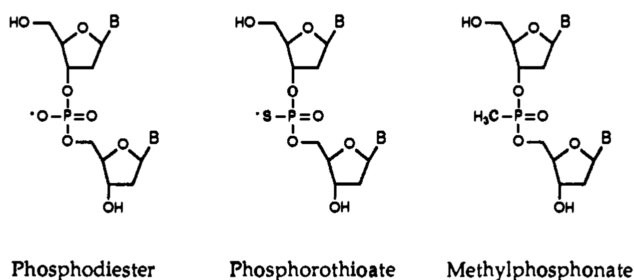

Figure 2. Structures of phosphodiester, phosphorothioate, and methylphosphonate internucleotide linkages.

These unwinding and modification activities occur ubiquitously in primary mammalian tissues,[16] but it is possible that there are other mechanisms through which antisense RNA can inhibit gene expression. Antisense RNA strategies have been used to elucidate the functions of many genes in culture and in transgenic animals and plants.[1,2,12,13]

Antisense ODNs

In contrast to antisense RNA, no naturally occurring systems are known which utilize antisense DNA to inhibit gene expression. Theoretically, a small ODN of 15 nucleotides (nt) in length has the specificity necessary to inhibit the expression of a single target gene through complementary hybridization with a cellular mRNA.[2] Inhibition of gene expression through the use of an exogenously added ODN was first reported in 1978 when Zamecnik and Stephenson described inhibition of Rous sarcoma viral replication after addition of a phosphodiester ODN to tissue culture media, postulating that the inhibition was through an antisense mechanism.[17] Since these early experiments many researchers have observed that ODNs containing phosphodiester, phosphorothioate, or methylphosphonate linkages (Figure 2) also have inhibitory activity against viral replication or expression of cellular genes in a number of different tissue culture systems.[3-8] The hallmark of most of these assays is that the exogenously applied ODN decreases the mRNA or protein levels of the target gene or causes changes in the growth characteristics or shapes of the cells.[1-8] As a demonstration of the specificity of the antisense effect, other ODNs, which are not complementary to the target mRNA, have been included as controls. An antisense mechanism is implicated when the antisense ODNs inhibit better than the control ODNs. Frequently, the control ODNs do show activity, which is often attributed to a nonspecific and non-antisense mechanism.[5,6] To avoid some of the obvious sources of nonspecific inhibition, most researchers now use a scrambled control ODN, which contains the same base composition as the antisense ODN but in a different order. In early studies, researchers often used a sense control ODN, which is a sequence complementary to the antisense ODN, but not necessarily related chemically to the antisense ODN. For example, an antisense ODN rich in guanosine and adenosine would have a sense ODN rich in cytidine and thymidine. Upon nuclease digestion, these ODNs would yield very different levels of mononucleotide (deoxynucleoside 5′-monophosphate) degradation products, which, in turn, can have substantially different effects on cell morphology. It has been established that micromolar concentrations of dAMP or dGMP can have pronounced cytotoxic or cytostatic effects on cells, particularly those derived from hematopoietic tissues.[18-21] The differential degradation products complicate the analysis of antisense inhibition; therefore, many researchers now favor the nuclease resistant phosphorothioate or methylphosphonate ODNs.

The three-dimensional molecular structure of ODNs may be responsible for some of the observed non-antisense inhibitory activity. These structures are highly dependent upon the sequences within the ODN and scrambled or sense control oligos, which do not contain these exact sequences, serve as poor controls, and could falsely support an antisense mechanism. Recently, a sequence of four consecutive guanosines (G-4 tract) within a larger phosphorothioate ODN was found to have antiproliferative effects, which are not due to an antisense mechanism.[22] An antisense ODN containing the G-4 tract inhibited cell growth. The sense and scrambled control ODNs did not contain a G-4 tract and did not inhibit cellular proliferation. These data would normally support an antisense mechanism, but other scrambled control ODNs which contained the G-4 tract also inhibited cellular proliferation. These recent results demonstrate that ODNs have novel non-antisense activities which can inhibit cell growth, adding further complexity to the analysis of ODN inhibition of gene expression.

Like proteins, cellular RNAs are thought to be highly ordered in secondary and tertiary structures.[23] If the antisense target site is buried within an RNA structure it may not be accessible for binding to the antisense ODN.[24,25] In many experiments, the failure of antisense ODNs to inhibit as anticipated is thought to imply that such a structure is present at the RNA target site. There is little data on *in vivo* mRNA secondary structure, and a systematic search of the literature failed to establish a correlation between predicted RNA secondary structure and antisense activity.[26] Because RNA secondary structure is difficult to predict, drug development may require that accessible regions of the RNA be determined empirically by synthesizing ODNs to multiple target sites. This process may select for accessible single-stranded regions in the target RNA, but alternatively, could select for ODNs which inhibit via non-antisense mechanisms or through impurities in the ODN preparation. Support of

antisense mechanisms would require additional ODN controls and analysis of non-targeted RNA and protein levels. Analysis of ODNs from different synthetic preparations also ensures that there is not batch-to-batch variation in ODN activity which may implicate impurities responsible for the observed activity.

Hurdles to Antisense Drug Development

The proposed antisense activity of ODNs is rather remarkable, given the theoretical barriers. First, the ODNs must be able to cross the cellular membrane to reach the cytoplasm or nucleus. Once inside the cell the ODN must be resistant to degradation. Finally, the ODN must be able to bind specifically and with high-affinity to the RNA target in order to inhibit expression of the disease causing gene. The observed activity of ODNs in tissue culture was earlier thought to imply that these theoretical barriers were not a concern. However, analysis of the data from recent articles indicates that these theoretical barriers are indeed real.[10,11] Initial experiments indicate that under carefully controlled conditions many of these barriers are in fact hurdles which may be dissected and overcome.

Permeation

Cells have receptors which bind to and internalize DNA as part of their normal receptor-mediated endocytosis mechanisms,[27–29] and ODNs have been shown to accumulate rapidly with cells in tissue culture.[30–34] Assays for ODN uptake generally measure the accumulation of radiolabeled ODNs within cells.[32,33] The cells are incubated in media containing a radiolabeled ODN, after which they are extensively washed and the extent of association determined by scintillation counting of the intact cells. These methods allow for easy quantitation of the amount of ODN associated with the cell but do not discriminate between ODNs localized in vacuoles or in the cytoplasm or nucleus. Others have gone on to lyse the cells, analyzing cellular fractions to determine the apparent nuclear or cytoplasmic localization.[33] Such techniques generally rely on the presence of ODNs in the nuclear fraction to indicate true intracellular localization. These techniques are prone to artifacts and, in the absence of extensive controls, are not a precise analytical tool for determining the intracellular localization of ODNs. In fact, during fractionation, endosomal and cytoplasmic components leak into nuclear fractions (and vice versa), which, in these studies, is exacerbated by the strong tendency for ODNs to concentrate in the nucleus in a very rapid manner.[35–37] Due to these technical problems associated with isolating the different intracellular compartments of cells, these fractionation techniques may greatly overestimate the amount of ODN in the nucleus and cytoplasm.

Fluorescence microscopy has been used to ascertain the intracellular location of ODNs tagged with fluorescent labels. Leonetti *et al.*[35] and Chin *et al.*[36] have examined the intracellular distribution of ODNs following microinjection into cells. Fluorescently labeled ODNs are found to rapidly concentrate into the nucleus following microinjection into the cytoplasm of cells. Concentration into the nucleus is observed within seconds for phosphodiester, phosphorothioate, methylphosphonate, and 2′-*O*-allyl ODNs.[35–37] When fluorescently labeled ODNs are placed in tissue culture media, the fluorescence accumulates in vacuoles within the cell, forming a punctate perinuclear pattern which are presumably endosomes and lysosomes.[10,30,31,34–37] The release of ODNs from vacuoles must be a very inefficient process since there is no visible fluorescence in the nucleus. Coadministration with cationic lipids enhances the uptake of fluorescently labeled ODNs as judged by nuclear localization of fluorescence. Several research groups have recently reported that, in the presence of cationic lipids, phosphorothioate ODNs have strong antisense activity, while in the absence of the cationic lipid, no activity was observed.[10,11,38–41] The correlation between the observations with fluorescence microscopy and biological activity strengthens the argument that phosphodiester and phosphorothioate ODNs do not cross the cellular membranes to an appreciable extent.

Methylphosphonate ODNs are uncharged and have been reported to enter cells via passive diffusion.[42] Shoji *et al.* explored the cellular distribution of fluorescently labeled methylphosphonate ODNs.[31] However, these ODNs did contain one phosphodiester linkage which could alter the permeation characteristics of the molecules. The observed pattern of uptake was again perinuclear, and rhodamine-labeled ODNs colocalized with fluorescein-labeled dextran, a marker for endosomal and lysosomal localization. This pattern of fluorescence is similar to that observed for phosphorothioate and phosphodiester ODNs. The methylphosphonate ODNs did appear to enter the cells through an absorptive endocytosis (pinocytosis) process.[31] This pathway is distinct from that reported for phosphodiester and phosphorothioate ODNs but ultimately appears to end with the same consequences: methylphosphonate ODNs trapped in vacuoles. Thus, these first lead ODN analogues do not efficiently permeate into the cell and second generation molecules will be desired for enhanced antisense activity.

Stability

The naturally occurring phosphodiester linkages in ODNs are susceptible to degradation by endogenously occurring cellular nucleases, while phosphorothioate and methylphosphonate linkages are highly resistant to nuclease degradation.[2,3,41] Phosphodiester ODNs are known to be rapidly degraded in serum containing tissue culture media by 3′-exonuclease activity.[43,44] Protection from degradation can be achieved by use of a "3′-end cap" strategy in which nuclease-resistant linkages are substituted for phosphodiester linkages at the 3′ end of the ODN.[43,44] Phosphoramidates, phosphorothioates, and methylphosphonate linkages all function adequately in this manner.[43–45]

Cellular nucleases have significant levels of endonuclease activity as well, and Hooke *et al.* demonstrated that neither 3′-end caps nor 5′-end caps, nor both, were sufficient to protect ODNs from degradation in HeLa cell extracts.[45] The presence of a single phosphodiester within a larger phosphorothioate ODN rendered it susceptible to the same level of rapid degradation. In an *in vivo* assay, Woolf *et al.* demonstrated that, after microinjection into *Xenopus* embryos, phosphodiester ODNs have an intracellular half-life of less than 30 min.[46] In microinjection studies in mammalian cells, Leonetti *et al.* observed a loss in fluorescence intensity from the nucleus with fluorescently labeled phosphodiester ODNs, resulting in the complete loss of fluorescence by 3 h postinjection.[35] The authors concluded that the loss of fluorescence is a result of degradation of the ODN, as fluorescently labeled phos-

phorothioate ODNs showed a strong nuclear fluorescence for over 24 h. Fisher *et al.* have also examined the intracellular disposition of fluorescently labeled ODNs and agree that phosphodiester ODNs are rapidly degraded with a 75% loss in observed fluorescence intensity within 1 h of microinjection.[37] 2′-*O*-methyl ODNs are also degraded rapidly, while 2′-*O*-allyl and phosphorothioate ODNs have half-lives of over 12 h inside cells.[37] These data indicate that phosphodiester ODNs are poor candidates as antisense agents due to their short intracellular half-life. Phosphorothioate and methylphosphonate ODNs do have sufficiently long intracellular half-lives to act as antisense agents if they could permeate sufficiently into cells.

ODN Affinity and Specificity

Few studies have examined the effect of ODN affinity in determining the potency and specificity of antisense inhibition of gene expression. Affinity is determined by both the length, increasing as the ODN gets longer, and the base composition of an ODN, increasing with the number of G-C base pairs. Specificity for target sequences also increases as the length of an ODN increases, but the affinity for closely related sequences also increases and, beyond a certain length, specificity begins to decrease.[47] Sequences of A and T bind more weakly than sequences of G and C, and are thought to increase specificity by spreading recognition and binding over a larger number of bases, decreasing the affinity to sites with mismatch base pairs and increasing the discrimination between target and nontarget sequences. This analysis assumes a termination event, such as RNase H cleavage, which is fast relative to the equilibrium binding of the ODN.[47] In practice, the necessary affinity and specificity of ODNs will depend upon the cellular mechanisms of antisense inhibition, and further studies are clearly necessary to better establish direct correlations between activity and ODN base composition and length in mammalian cells.

Mechanism of Antisense Inhibition

Once inside the cell, the ODN binds to its target mRNA, in either the cytoplasm, nucleus, or both. Following hybridization to the mRNA, ODNs can inhibit gene expression by at least two distinct mechanisms.[1-8] In one mechanism, cellular RNase H cleaves the RNA portion of an RNA:DNA duplex.[48-50] Once cleaved, the mRNA is no longer competent for translation and may be rapidly degraded. ODNs which can inhibit gene expression via RNase H activation may have an advantage in that each message is permanently inactivated upon cleavage and each ODN can potentially inhibit multiple copies of each target mRNA. RNase H cleavage may decrease the specificity of antisense ODNs since transient hybridization to other mRNAs may be sufficient to activate RNase H cleavage.[47,51]

In a second antisense mechanism, high-affinity binding of an ODN to a target mRNA inhibits gene expression through simple steric blocking.[52] In this model, the ODN:RNA duplex forms and physically blocks the RNA from interacting with cellular components required for subsequent translation of the RNA into a protein. In the nucleus, steric blocking could possibly inhibit pre-mRNA splicing, polyadenylation, or mRNA transport from the nucleus to the cytoplasm.[1,2] While in the cytoplasm, steric blocking could also inhibit the protein synthesis processes such as translational initiation or ribosome elongation.[2,52,53] Steric blocking should not be effective at all regions of an RNA since the ODN must physically inhibit protein interactions with the RNA, eliminating most of the intron portions of RNAs as target sites. Thus, a steric blocking mechanism would decrease the opportunities for binding to related RNA sequences, compared with an RNase H based mechanism. Additionally, inhibition of gene expression by steric blocking is dependent upon the lifetime of the ODN:RNA complex and binding of an ODN to sites with base pair mismatches should result in complexes with short half-lives, which should not substantially disrupt the expression of these nontargeted genes.[47] Phosphodiester and phosphorothioate ODNs are competent for RNase H activated cleavage of RNA while methylphosphonate and 2′-*O*-allyl ODNs are not. Future studies comparing these different types of analogues are necessary to elucidate the role of each mechanism at various target sites within an RNA, allowing optimization of antisense strategies for high potency and specificity.

Antisense Inhibition of Viral Activity

Since the first experiments of Zamecnik and Stephenson, many viruses have been shown to be inhibited by antisense ODNs, as reviewed recently by Cohen,[4] Stein *et al.*,[5] and Bischofberger and Wagner.[6] The list is lengthy and includes many important human pathogens such as HIV, HSV 1 and 2, HPV, and influenza. Viruses are attractive therapeutic targets since their genetic sequences are unique with respect to the human host, and antisense ODNs of sufficient length should hybridize only to the targeted viral RNAs. Most of the important viral genes have been sequenced and are well-characterized for highly conserved sequences and essential functions. However, the results from ODN inhibition of viruses are rather complicated, and most of the studies indicate a large, non-antisense component to the inhibition of viral replication.[4-6]

Human Immunodeficiency Virus

Numerous phosphorothioate ODNs have been tested for the inhibition of HIV replication in cell culture systems. In an early antisense study, Matsukura *et al.* targeted a 27 nt phosphorothioate ODN against the HIV *rev* gene for inhibition of virus activity in chronically infected H9 cells.[54] Addition of the ODN significantly reduced viral titer, as assessed by a p24 immunoassay, and also decreased the levels of full-length viral RNA. In a more recent study, Vickers *et al.* looked at a similar series of ODNs for HIV inhibition in CEM cells instead of H9 cells.[39] This study included ODNs of 18, 26, and 28 nt as both phosphodiesters and phosphorothioates directed against the TAR element and a series of 20 nt phosphorothioate ODNs directed against the *tat* gene, comparing them with the 27 nt phosphorothioate *rev* ODN that Matsukura *et al.* had studied. In a chronic infection assay both the antisense TAR and the scrambled control ODN showed substantial, but identical, activity in reducing viral titer. The 27 nt antisense *rev* ODN showed only slightly better activity and a similar dose response, indicating a non-antisense mechanism of inhibition by these phosphorothioate ODNs. In an acute infection assay for the inhibition of reverse transcriptase activity, the 27 nt antisense *rev* ODN had an IC_{50} of 0.2 μM while a 26 nt scrambled control had an IC_{50} of 0.15 μM. It is interesting to note that only the phosphorothioate ODNs had anti-HIV activity; none of

these sequences showed any activity as phosphodiester ODNs. These data suggest that ODNs containing phosphorothioate linkages have properties which inhibit viral activity through a nonspecific and non-antisense mechanism. Similar antiviral activity has been demonstrated for a phosphorothioate poly-dC 28 nt ODN (S-dC-28) which exhibits anti-HIV activity at 1 μM, inhibiting *de novo* DNA synthesis, but not *gag* protein production, in chronically infected cells.[55] Stein *et al.* also showed that the phosphorothioate dC-10 with a 5′-cholesterol (chol-S-dC-10) could inhibit HIV infection in CEM cells with an IC_{50} of 0.8 μM.[56] The authors postulated that both chol-S-dC-10 and S-dC-28 protect cells from infection by inhibiting viral uptake, interfering with the interaction of gp120 and CD4 in CEM cells.[56,57] The 5′-cholesterol conjugate may increase the potency of S-dC-10 by enhancing cellular association, but clearly inhibits by a non-antisense mechanism, indicating that phosphorothioate ODN interactions at the cell surface can inhibit viral uptake. Furthermore, S-dC-28 was able to inhibit syncytia formation in MOLT-3 cells with an ID_{50} of 0.6 μM, similar to the anti-HIV activities reported for the polyanions, dextran sulfate[58] or heparin.[59]

Lisziewicz *et al.* showed that five different 27 nt phosphorothioate antisense ODNs inhibited HIV virus production in chronically infected MOLT-3 cells.[60] Early in the infection cycle, all five antisense ODNs, as well as mismatch, random, and S-dC-28 showed substantial activity against HIV infection. However, in a long-term infection model the mismatch, random, and an antisense ODN targeted to the 5′-splice-site-acceptor (SA) region of the *tat* gene all showed a loss of activity by day 25. The authors speculated that the loss in potency of the antisense SA ODN was due to mutations in the viral RNA sequences, although it is not clear why this loss of activity is different from the loss of activity with the mismatch and random control ODNs. By day 32 the antisense ODNs targeted to *rev*, *gag*, and the *rev* response element (RRE) all still showed strong inhibitory activity. The antisense *rev* ODN was again the same sequence first identified by Matsukura *et al.* for its anti-HIV activity. This *rev* ODN was compared with S-dC-28 and both were found to have identical inhibitory activity for 39 days. At day 39 the culture treated with S-dC-28 was judged to show evidence of cytotoxicity, leading to speculation that the anti-HIV activity was a non-antisense cytotoxic affect. These results are intriguing but fail to explain the apparent lag in specific antisense activity with the random and mismatch controls, which mimic the loss of activity observed with the SA antisense ODN. A specific antisense mechanism should occur rapidly and the identical long-term activity of S-dC-28 and the antisense *rev* ODN may indicate that a similar non-antisense mechanism of anti-HIV activity was observed for all ODNs.

Morvan *et al.* compared eight different antisense ODNs for their ability to inhibit HIV infection.[9] Each ODN consisted of the identical 12 nt sequence, also targeted to the HIV *tat* gene 5′-splice-site-acceptor region. The ODNs were modified in backbone linkage, sugar, or both, including 2′-deoxyphosphodiester or phosphorothioate ODNs (both the natural β-anomer and the α-anomer, in which the base is inverted), a 2′-deoxy-methylphosphonate (β-anomer), a ribose ODN (α-anomer), and 2′-*O*-methyl- or 2′-*O*-allylribose ODNs (β-anomers). This study compared affinity and nuclease resistance of the various modifications with activity against HIV infection in MT4 cells. Every ODN protected cells against HIV infection, with IC_{50} ranging from >100 μM for the phosphodiester ODN to 0.25 μM for a phosphorothioate ODN. For each antisense ODN the scrambled control, containing the same modifications, showed nearly identical activity and the authors concluded that this activity was not due to an antisense mechanism. The relative activity of the ODNs correlated strongly with resistance to nuclease digestion, but did not correlate well with affinity of the ODNs for the target RNAs, which may indicate that hybridization with the target RNA is not occurring in these assays.

Herpes Simplex Virus

Phosphorothioate ODNs have also been shown to be inhibitors of HSV 1 and HSV 2 infection in cell culture. A 21 nt phosphorothioate antisense ODN targeted to the UL13 mRNA in HSV 1 was found to inhibit viral yield by over 90% at 4 μM.[45] This same sequence showed no activity if used as a phosphodiester ODN or if it contained three phosphorothioates at the 5′-end, 3′-end, or both, to protect it from exonuclease degradation. However, inhibition of both HSV 1 and HSV 2 replication also shows a significant non-antisense component when phosphorothioate ODNs are added exogenously in cell culture.[61–63] S-dC-28 was again found to be a potent inhibitor of both HSV 1 and HSV 2 with a 90% reduction in viral yield at 1 μM, while at 3 μM the inhibition was about 95% for HSV 1 and >99% for HSV 2.[61] The degree of inhibition was slightly dependent upon the base composition, with 28 nt ODNs inhibiting as follows: dC-28 = T-28 > dA-28. The mechanism of inhibition was thought to be related to viral absorption, a virus-induced increase in ODN uptake, or inhibition of virally encoded DNA polymerases.[63] In this study HSV 2 was thought to dramatically increase the uptake of ODNs into cells.[62] Viral entry into the cell may facilitate the passage of ODN from vacuoles into the cytoplasm, which in turn inhibits viral replication, perhaps by interacting selectively with the HSV polymerase.[62] Phosphorothioate ODNs show very low levels of toxicity in uninfected cells[64] and may provide lead compounds for drug development as selective inhibitors of viral infection, even in the absence of a specific antisense mechanism.

Methylphosphonate ODNs have also been evaluated for antisense activity against HSV 1 and HSV 2. A series of four 12 nt ODNs containing 1 phosphodiester and 10 methylphosphonate linkages were targeted to overlapping sequences at the exon–intron boundary at the 5′-splice-site-acceptor of the HSV 1 immediate early 4 (IE4) RNA.[65] The four ODNs were tested individually at 100 μM for the inhibition of infectious virus production in Vero cells and showed a range of inhibition from 9% to 98%. The 12 nt ODN which showed the highest activity in tissue culture was evaluated for anti-HSV activity in a mouse ear pinna model.[66] In animals treated with a single intradermal injection of 500 μM at the time of infection, there was no discernable decrease in viral titer. Animals were then treated with 500 μM ODN at the time of infection followed by daily topical applications of 500 μM ODN in a polyethylene glycol solution. At day 4 postinfection, viral titer was reduced by 82–98% with similar inhibition seen at day 6. However, similar treatment with 100 μM ODN did not decrease the viral titer, indicating a very small therapeutic range. The extremely high concentrations necessary for biological effects would indicate that the

use of methylphosphonate ODNs may be limited to systems where local delivery is acceptable.

Human Papilloma Virus

Antisense ODNs were used to target both the human papilloma E6 and E7 proteins in cells infected with HPV16.[67] Phosphorothioate ODNs were the most potent inhibitors of virus-induced, cellular proliferation as judged by [^{3}H]thymidine incorporation. ODNs which contained phosphodiesters plus a 3′-terminal phosphorothioate linkage to protect against 3′-exonuclease degradation were much less effective and neither full phosphodiester nor methylphosphonate ODNs had any measurable activity. While the phosphorothioate ODNs inhibited proliferation of both CaSki and HeLa cells at a range of 1–5 μM, they did not demonstrate significant decreases in either E6 or E7 protein levels. E7 is known to undergo serine phosphorylation and was found to be phosphorylated to a lesser extent than in the untreated, control cells. The cells incubated with the phosphorothioate ODNs displayed an overall decrease in protein synthesis when compared with untreated cells. The authors speculated that inhibition of cellular proliferation was only partially brought about by inhibition of HPV specific proteins and that nonspecific inhibition of gene expression was being observed, which may include antisense targeting of cellular genes or non-antisense mechanisms. It is interesting that HPV does not encode its own polymerase, and this particular assay is not dependent upon active production of virus.[68] The non-antisense mechanism of inhibition cannot involve inhibition of viral uptake or selective inhibition of viral DNA polymerases and must involve non-specific inhibition of endogenous, cellular pathways of proliferation.[69]

The effects of antisense ODNs on HPV infection are difficult to evaluate due to the lack of suitable tissue culture and animal model systems for the virus. Cowsert *et al.* established a cellular model system in which the HPV-11 E2 protein transactivates the chloramphenicol acetyltransferase (CAT) gene.[70] A 20 nt phosphorothioate ODN was incubated with C127 cells overnight prior to calcium phosphate transfection of the HPV-11 E2 plasmid and the E2-CAT reporter plasmid. After transfection the cells were incubated for 48 h and transactivation of the CAT gene by the E2 protein was assessed by CAT enzymatic activity. The antisense ODN was found to inhibit E2 transactivation of CAT activity with an IC_{50} of about 5 μM, while a noncomplementary ODN did not inhibit E2 transactivation. It seems likely that, in this system, the calcium phosphate transfection procedure introduced high concentrations of the ODN into the cells. Once inside the cells, the antisense ODN inhibited E2-induced CAT activity in a manner consistent with an antisense mechanism.

Conclusions on Antiviral Activity

The antiviral activity demonstrated in these and other assays indicates that phosphorothioate ODNs show efficacy, even if not necessarily by an antisense mechanism. The precise mechanisms of viral inhibition are not known, although several possibilities have been reported. The ODNs may enter the cell and act as viral polymerase inhibitors. Selectivity for the virus could then be achieved if the K_i for the viral DNA polymerase is significantly lower than the K_i for the cellular DNA polymerases *in vivo*.[62] The ODNs could inhibit viral absorption on the cell surface, acting principally through interactions of the phosphorothioate backbone with cell surface or viral proteins.[55–57,62,63] Abasic phosphorothioates, in which the bases are not present on the deoxyribose sugars, have inhibitory properties against HIV infection which cannot possibly be attributed to any antisense effect on cellular genes and probably inhibit viral absorption.[71,72] Dextran sulfate and other polyvalent anions inhibit both HIV[58,59] and HSV[73] infection by blocking absorption of the virus to the cell surface. A second mechanism of nonspecific inhibition requires that the ODNs enter into intracellular vesicles through classic endocytic mechanisms, where they concentrate and disrupt the proper intracellular vesicle trafficking essential for viral release into the cytoplasm. Polyanion disruption of endosomal trafficking has been described previously.[74,75] This observed non-antisense mechanism may warrant further validation as the activity may be sufficient for therapeutic antiviral strategies. Future development of potent antisense ODNs will require modifications which decrease the concentrations necessary for antiviral activity by enhancing specific antisense effects.

Antisense Inhibition of Oncogenes

Antisense ODNs have been reported to inhibit a variety of oncogenes including *c-ras*, *c-myc*, *c-myb* and *bcr/abl* (see Reviews by Tidd[7] and Callabretta[8]). Most studies have focused on the use of phosphorothioate or methylphosphonate derivatives or have used heat-inactivated or serum-free media to reduce the extracellular degradation of phosphodiester ODNs. Inhibition of gene expression is generally seen at high concentrations of ODNs, again creating a scenario where nonspecific effects due to the presence of a polyanion could mimic the specific effects sought.[69] This may be particularly pertinent in assays where inhibition is judged by the response, or lack thereof, to cellular mitogens following treatment with an ODN. Since the expression and activities of many oncogenes are regulated in response to external stimuli,[76] non-antisense inhibition of mitogen stimulation may be misinterpreted as antisense activity. In the future, more potent antisense agents could help elucidate the functions of many of these oncogenes, by reducing the non-antisense effects of ODNs on cellular functions.

c-*myb*

c-*myb* is a nuclear oncogene which is thought to play an important role in regulating hematopoietic cell proliferation and perhaps differentiation.[77] c-*myb* is expressed in immature hematopoietic tissues and certain hematopoietic tumors.[78] Decreased expression of c-*myb* correlates with cellular maturation and differentiation, while constituitive (continuous) expression of c-*myb* causes inhibition of differentiation in erythroleukemia cells from mice.[78] An 18 nt antisense phosphodiester ODN to c-*myb* was reported to inhibit the proliferation of bone marrow mononuclear cells as reflected in their inability to respond to normal mitogens.[79] Other cells were not inhibited by the antisense ODN and a sense ODN did not inhibit mitogen response. The c-*myb* antisense ODN was reported to inhibit the proliferation of both normal human T-lymphocytes and HL-60 cells at a concentration of 10 μM, arresting cells at the G_1/S transition in the cell cycle.[80,81] Inhibition of the G_1/S transition in the cell cycle by antisense ODNs has also been observed for the oncogene c-*myc*[82] and the cell cycle regulatory gene *cdc*2.[83] This

result is unexpected since the expression of the c-*myb*, c-*myc*, and *cdc*2 genes are known to occur at different and distinct stages of the mitogenic process of T lymphocytes.[8] It has been speculated that this apparent paradox likely reflects ignorance of the control points in the biochemical pathway leading to DNA synthesis.[8] It is also possible that the inhibition of proliferation is a reflection of a non-antisense mechanism of ODNs on the cell cycle, perhaps inhibition of a common signal transduction pathway. The nonspecific effects are reminiscent of reports of polyanionic polymers which have antiproliferative effects on transformed cell lines in culture.[69]

Antisense c-*myb* ODNs have been shown to inhibit the proliferation of primary, human *bcr/abl* positive cells from both acute myelogenous (AML) and chronic myelogenous (CML) leukemias.[84] The antisense phosphodiester ODN was found to inhibit leukemic colony formation in 18 of 23 AML patient cell lines and 4 out of 5 CML patient cell lines with a ID_{50} of 20 μg/mL.[84] This is the first report that c-*myb* plays a role in the proliferation of BCR-ABL-positive cells. Antisense c-*myb* phosphorothioate ODNs have also been reported to inhibit the growth of the *bcr/abl*-positive cell line, K562, in a SCID mouse model system.[85] After injection into SCID mice, K562 cells will rapidly proliferate, ultimately causing death. Treatment with a 24 nt antisense phosphorothioate c-*myb* ODN increased the mean survival time from 7 ± 2 days to 24 ± 11 days when the mice were treated with 100 μg ODN/mouse/day for 14 days. Control ODNs did not increase the mean survival time. The mice were not cured, as all died after cessation of treatment. The antisense ODN was targeted to the human c-*myb* gene and was not expected to have any effect on the endogenous mouse c-*myb* gene, which has five mismatches in this target sequence. This c-*myb* antisense ODN demonstrates a substantial *in vivo* antiproliferative activity, but the mechanism of inhibition has yet to be determined.

The transient expression of c-*myb* has also been implicated in the proliferation of smooth muscle cells and is a potential target for antisense strategies for diseases such as restenosis following coronary and angioplasty.[88] Serum-starved smooth muscle cells (SMCs) become quiescent and will resume proliferation only if mitogenically stimulated by the addition of serum to the culture media. Addition of an 18 nt phosphorothioate ODN to SMCs, prior to the addition of serum, caused about a 2-fold decrease in cellular proliferation in response to serum, at a concentration of 25 μM. The addition of the antisense ODN had a long-lasting antiproliferative effect, which was observed for over 72 h, while sense and mismatch ODNs had no effect on cellular proliferation. However, two additional control ODNs, sense and antisense to thrombomodulin, had a greater antiproliferative effect than the antisense c-*myb* ODN, again indicating that a non-antisense mechanism may be responsible for the antiproliferative effect.

The c-*myb* antisense ODN inhibits restenosis in a rat aortic model system.[87] Following injury to the aortic endothelium, SMC-mediated restenosis is strongly inhibited by treatment with the antisense ODN, but not with the sense or mismatch ODNs. This result is particularly striking since the ODNs were applied to the exterior of the aorta following injury to the vascular endothelium by a balloon catheter. This external application requires that the ODNs diffuse through the exterior of the aorta to reach the smooth muscle cell layer on the inside of the vessel wall. The control of the proliferation of SMCs following injury is complex, and it has been reported previously that simple polyanions such as low-molecular weight heparin also inhibit smooth muscle cell proliferation in animal model systems, again suggesting that the antiproliferative effect may be due to a non-antisense mechanism.[88] The proliferation of SMCs can also be inhibited by the addition of antisense ODNs targeted to c-*myc*,[89,90] proliferating cell nuclear antigen (PCNA),[91] or basic fibroblast growth factor (basic FCF).[92] The growing number of target genes which are reported to inhibit SMC proliferation may well be a reflection of the intrinsic activity of ODNs rather than the role of the target genes in the proliferation of SMCs.

c-*ras*

c-*ras* has been extensively studied to determine the specificity of antisense methylphosphonate ODNs.[93-95] Chang *et al.* studied 11 nt methylphosphonate ODNs targeted to a region around the c-*ras* codon 12, which is often mutated in tumors.[96] ODNs were added to cultures containing both RS504 cells (mutated c-*ras*, A to T) and RS453 cells (wild type c-*ras*), and the inhibition was analyzed by a pulse-radiolabel immunoprecipitation assay. In this system the mutant *ras* p21 protein migrates slightly slower on an SDS PAGE gel, allowing analysis of the two proteins in a single culture. The 11 nt ODN targeted to the wild type c-*ras* gene partially inhibited the wild type c-*ras* expression at 75 μM and fully inhibited at 150 μM, with no inhibition of the mutant c-*ras* expression. Conversely, an 11 nt ODN targeted to the mutant c-*ras* gene partially inhibited the mutant c-*ras* expression at 150 μM, with no inhibition of the wild type c-*ras* expression. When a photoactivatable psoralen derivative was conjugated to the 5′-end of the ODNs, the inhibitory dose dropped about 10-fold, while the specificity of the ODNs for their respective targets remained. This is the first report that methylphosphonates (without psoralen) can inhibit translational elongation *in vivo*. In cell-free systems, methylphosphonate ODNs inhibit translation poorly when they are targeted to regions downstream of the translational initiation region, even at concentrations in excess of 100 μM.[53] In these studies, the c-*ras* genes are not physically in the same cell, and there is a possibility that the 11 nt ODNs could be selectively cytotoxic to one cell type but not the other. In this coculture assay, nonspecific cytostatic effects on only one cell type would not be detected and no data was presented on inhibition c-*ras* p21 in the individual cell lines or of other control ODNs. Although limited specificity with the ODNs was observed, the 75–100 μM concentration used in this study is far too high for these ODNs to be considered candidates for drug development.

Monia *et al.* also studied the ability of ODNs to discriminate between the wild type c-*ras* and a codon 12 mutant of c-*ras* (G to T).[40] This model system assayed the expression of a *ras*–luciferase fusion protein under control of the inducible MMTV promoter. HeLa cells, which had been transfected with the plasmids, were treated with the ODN in the presence of the cationic lipid, DOTMA, to facilitate uptake. Following ODN treatment, *ras*–luciferase expression was stimulated by the addition of dexamethasone, and luciferase activity was assayed 15 h later. A series of phosphorothioate ODNs between 5 and 25 nt in length, targeted to the c-*ras* codon 12 mutation,

were assayed for their ability to inhibit both mutant and wild type expression. ODNs of 5, 7, 9, 11, or 13 nt in length were unable to inhibit either the mutant or wild type expression at 100 nM. The ODNs of 15 and 16 nt in length both inhibited the mutant c-*ras* with no apparent inhibition of the wild type c-*ras*. The 15 nt ODN inhibited the mutant by only about 20% at 250 nM, while the 16 nt ODN had an IC_{50} of 100 nM. Antisense ODNs of 17, 19, 21, 23, and 25 nt in length all inhibited both the mutant and the wild type expression at doses of 100 nM or less. The concentrations of ODNs used in this study were lower than those used in most other studies, again indicating that treatment with DOTMA can substantially increase intracellular concentrations and activities of ODNs. This demonstration of the ability of an ODN to show discrimination between a mutant versus a wild type gene with only one base pair difference validates the potential selectivity of antisense drugs.

Conclusions on Antisense Biology

Although antisense ODNs have been reported to have activity in literally hundreds of published articles,[97] there are still many inconsistencies with respect to the mechanisms and specificity of antisense ODNs. Our approach has been to dissect the variables in antisense assays by developing a rapid, quantitative microinjection-based assay system to separate antisense activity from issues of ODN uptake and nuclease degradation.[11] In this two-gene assay system, a plasmid containing the T antigen gene (a nontransforming mutant) and a plasmid containing the gene for β-galactosidase (β-gal) are both microinjected into the nuclei of cells along with an ODN. At 4.5 h postinjection, the cells are fixed and protein expression is assessed by immunofluorescence microscopy. Antisense activity is assessed as the complete inhibition of the T antigen expression without observable inhibition of β-gal expression. Thus, β-gal expression serves as an internal control for the specificity of antisense inhibition. The ODNs were targeted to a region 150 nt downstream of the translational initiation codon, predicted to be in an accessible single-stranded region of the mRNA.[98]

A series of modified ODNs of the sequence 5′-ATTTTTCTTCATTTTTTCTTC-3′ (where C is 5-methyl-2′-deoxycytidine) were assayed by microinjection to examine the relative importance of nuclease resistance, binding affinity, and RNase H activation on antisense activity. The 20 nt ODNs containing a phosphodiester backbone or containing 2′-*O*-allyl sugars showed no inhibition of T antigen expression at intracellular concentrations up to 20 μM. The 20 nt ODN containing a phosphorothioate backbone showed no inhibition of T antigen at 5 μM and nonspecific inhibition of both T antigen and β-gal at 25 μM. ODNs were then synthesized substituting 5-propynyl-2′-deoxyuridine for thymidine and 5-propynyl-2′-deoxycytidine for 5-methyl-2′-deoxycytidine to enhance the binding affinity.[99] The 5-propynyl-substituted ODN with a phosphodiester backbone showed specific inhibition of T antigen expression with an intracellular IC_{50} of 2.5 μM. More dramatically, the 5-propynyl-substituted phosphorothioate ODN specifically inhibited T antigen with an IC_{50} of 0.05 μM. The 5-propynyl-substituted 2′-*O*-allyl ODN did not inhibit T antigen expression at concentrations up to 20 μM, despite having the highest T_m (89.5 °C) of all ODNs tested.

As a further test for the specificity of inhibition, a series of 15 nt 5-propynyl phosphorothioate ODNs, containing base-pair mismatches, were compared for activity. The fully complementary 15 nt ODN showed an IC_{50} of 0.1 μM. Control ODNs, with one or two mismatches, each had a decreased T_m against the target RNA, and showed a 5-fold or 10-fold decrease in potency, respectively. 15 nt ODNs with 4 or 10 mismatches showed no activity. As a further control, 15 nt ODNs were also targeted to the β-gal gene. A 15 nt phosphorothioate ODN showed no inhibition of β-gal at 20 μM in the absence of the 5-propynyl modification. The phosphorothioate ODN with 5-propynyl modifications inhibited β-gal expression with an IC_{50} of 0.25 μM with no apparent inhibition of T antigen expression. These experiments were carried out with the same results in multiple cell types including CV1 (green monkey kidney), Rat1 (rat fibroblast), ccd45sk (primary human fibroblast), SKOV-3 (human ovarian carcinoma), HeLa (human epithelium carcinoma), and BC3H1 (rat brain tumor, smooth muscle like).

Several conclusions on antisense activity and mechanism can be derived from this limited SAR study of the 5-propyne modification. The enhanced activity of these ODNs indicates that the minimum level of affinity necessary for antisense activity is relatively high. The unmodified ODN had a low affinity for the target RNA since it was extremely rich in thymidine, indicating that the 5-propynyl substitutions are desirable but perhaps not absolutely necessary. A 20 nt ODN targeted to the T antigen 5′-untranslated region did show specific inhibition without the 5-propynyl modifications, although only over a narrow dose–response range. A size titration experiment with modified ODNs also indicates that as size and T_m decrease, so does activity. The lack of activity for the 2′-*O*-allyl ODNs indicates that RNase H cleavage of the mRNA is probably the mechanism of action and that a steric blocking mechanism may not work at target sites in the translational coding regions. The decreased activity for the 5-propynyl-modified phosphodiester ODN is probably due to nuclease digestion of the ODN inside the cell. In additional experiments, cells were injected with 20 μM ODN to determine the persistence of the antisense effect. The 20 nt modified phosphodiester ODN showed activity for up to 6 h postinjection while the phosphorothioate ODN showed activity for more than 48 h, again indicating that the phosphodiester ODNs have short intracellular half-lives. To assess ODN uptake in cells, the 15 nt phosphorothioate ODN (intracellular $IC_{50} = 0.1$ μM) was added to tissue culture at 50 μM for 24 h prior to microinjection with the T antigen and β-gal plasmids. The ODN failed to inhibit T antigen expression, indicating that the intracellular concentration of ODN was well below 0.1 μM at the time of the injection. When the same experiment was repeated in the presence of lipofectin (a cationic lipid), T antigen expression was inhibited with an extracellular IC_{50} of 0.005 μM. The 20-fold increase in potency from the extracellular addition experiment indicates that cationic lipids concentrate ODNs into cells. These observations are consistent with other studies using cationic lipids as permeation enhancers[10,38–41] and again strongly suggest that phosphorothioate ODNs do not effectively cross the cellular membrane.

Design of Potent Antisense Therapeutics: Overcoming the Hurdles

The microinjection experiments of Wagner *et al.*[11] suggest that the three obvious hurdles to antisense activity are very real. The strong antisense activity with the

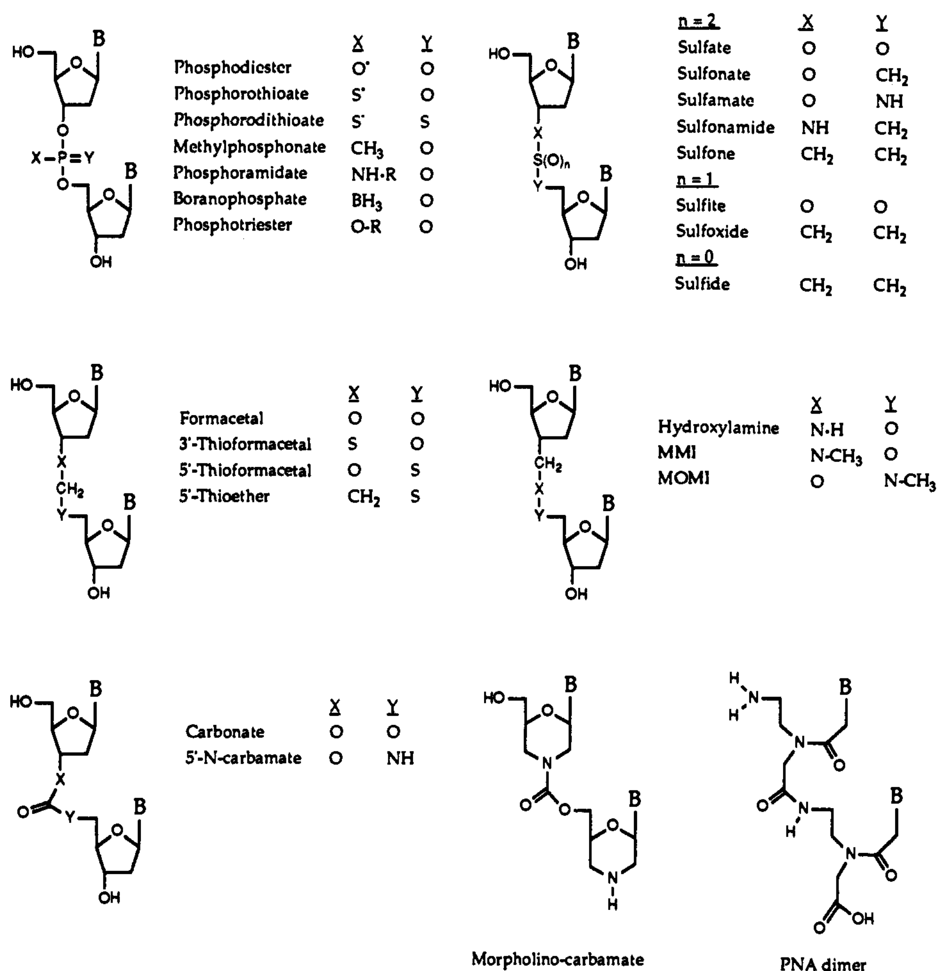

Figure 3. Backbone analogues for replacement of the natural phosphodiester linkage. MMI is a methylene(methylimino) linkage and MOMI is a methyleneoxy(methylimino) linkage.

5-propynylpyrimidines indicates that rational modifications can be designed to enhance the antisense activity of ODNs. Chemical strategies to enhance specific properties of ODNs can take advantage of the large body of knowledge of nucleic acids structure and thermodynamics to enhance specific properties of antisense ODNs. The fundamental Watson–Crick hydrogen-bonding scheme is central to the formation of the double helix and is unlikely to change substantially, but all other structural features of the phosphodiester backbone, heterocyclic bases, and sugars can be modified or replaced. Much of this chemistry has been reviewed thoroughly in detail elsewhere,[100,101] but in most respects, the medicinal chemistry of antisense ODNs is in its infancy. ODNs are large molecules and analogues are often difficult to synthesize in sufficient quantities for *in vitro* or *in vivo* analysis. In addition, methods for the *in vitro* analysis of ODN modifications are not always agreed upon, making it difficult to compare analogues from different investigators. For example, T_m as a measure of affinity is dependent upon both the salt conditions and the sequence of the ODN, and is usually measured with complementary DNA which is not necessarily predictive for duplex formation with RNA.[101] For these reasons, this review will serve to show the scope of the current strategies for ODN analogues (see Figure 3) since direct quantitative comparisons may not be accurate.

Modifications of the Phosphodiester Backbone

Modification of the phosphodiester backbone has been shown to impart stability and may allow for enhanced affinity and increased cellular permeation of ODNs. In addition, many different chemical strategies have been employed to replace the entire phosphodiester backbone with novel linkages. The chemistry required to produce these linkages is often quite challenging and many groups prefer to produce and characterize dimers containing the novel linkage, rather than synthesize fully substituted polymers. In this form they may be introduced during automated ODN synthesis, creating a polymer with alternating phosphodiester and novel linkages. The rapid advancements in chemistry have allowed some groups to produce small ODNs fully substituted with a novel linkage, allowing biophysical characterization.

Phosphorothioate- and methylphosphonate-modified ODNs have been attractive to biological researchers due to their availability through automated ODN synthesis. Both ODNs are stable to degradation by nucleases, but in general hybridize to target sequences with a lesser affinity than a phosphodiester ODN.[102] The ODNs containing either of these modifications are a mixture of 2^n diastereomers (where n is the number of linkages), and it is possible that an ODN containing all *R*P or all *S*P isomers would hybridize with better affinity. Lesnikowski *et al.* showed that for a 7 nt oligothymidine with a methylphosphonate backbone, the all-*R*P ODN had a significantly higher T_m than the ODN which was a mixture of diastereomers, while the all-*S*P ODN had a T_m too low to be determined.[103] These data indicate that a strategy for chirally pure methylphosphonates or phosphorothioates could lead to a superior antisense ODN. Unfortunately,

chiral synthesis of these backbone analogues will require considerable technology development and does not seem likely in the near future.[104] It may not be necessary to produce chirally pure ODNs for therapeutic purposes, but chirality still may introduce unwanted issues. For example, batch-to-batch variation in the mixtures of isomers could affect activity and assuring consistent ratios of isomers will be difficult. To avoid these issues, and in attempts to improve the properties of antisense ODNs, a diverse series of backbone analogues have been synthesized, many of which are achiral (Figure 3).

A phosphorodithioate version of the phosphorothioate has been synthesized and characterized.[105] In the dithioate linkage, both of the nonbridging oxygens have been substituted with sulfur. This linkage is highly nuclease resistant and achiral, and the ODNs bind with slightly less affinity than a phosphorothioate.[106-108] Preliminary results indicate reduction in HIV viral titer in tissue culture.[108] Other interesting, but less well-characterized, backbones derived from the phosphodiester include phosphoramidates,[109,110] alkyl phosphotriesters,[111] and boranophosphates.[112] New, achiral phosphate derivatives include the 3′-*O*-5′-*S*-phosphorothioate,[113] the 3′-*S*-5′-*O*-phosphorothioate,[114] the 3′-CH_2-5′-*O*-phosphonate,[115] and the 3′-*NH*-5′-*O*-phosphoramidate.[116] These analogues are likely to be nuclease resistant, but little is known of their hybridization properties.

Sulfur, at the central position of the backbone, is an isosteric replacement for phosphorus, offering a variety of options for modified isosteric linkages (Figure 3). A central sulfate or sulfite linkage would be susceptible to cleavage by internal or external nucleophiles and are probably not useful in ODNs. Additional modifications allow the central sulfur to be stably linked to the sugars via methylenes to produce sulfonate,[117-119] sulfone,[120-122] sulfoxide,[120] or sulfide[121] linkages. Changing a linker atom to nitrogen creates a sulfamate[125] and the combination of methylene and nitrogen creates a sulfonamide linkage.[118] These linkages are very resistant to nucleases, but the affinity of such ODNs has not yet been reported. These derivatives are achiral and, unlike the phosphorodithioate analogues, electronically neutral at physiological pH. There is no report of the use of these analogues in tissue culture experiments.

Another strategy to avoid chiral linkages replaces the central phosphorus with carbon (Figure 3). Matteucci *et al.* introduced a formacetal linkage in which the 5′- and 3′-oxygens are linked via a central methylene and a 5′-thioformacetal in which the 5′-oxygen of the formacetal is replaced with sulfur.[123] The formacetal linkage is electronically neutral and isosteric to a phosphodiester in that it contains free rotation about all the bonds of the linkage. ODNs partially substituted with formacetal linkages hybridize to RNA with only slightly less affinity than a control phosphodiester ODN.[124,125] The 5′-thioformacetal-containing ODNs do not hybridize as well to either RNA or DNA. In an effort to improve the binding properties, Jones *et al.* have introduced a 3′-thioformacetal variant in which the 3′-oxygen of the formacetal is replaced by sulfur.[126] ODNs containing this linkage, alternating with phosphodiesters, bind to RNA better than the corresponding phosphodiester ODN. The 3′-thioformacetal linkage is completely stable to nucleases and is also electronically neutral. Fully substituted polymers have not yet been reported or tested for antisense activity.

Another variation on the carbon-central, nonionic backbone is the 5′-*S*-thioether linkage, which has been made as a dimer.[127]

Carbonate and carbamate linkages have been introduced as novel replacements for the phosphodiester backbone (Figure 3). The carbonate dimers are unstable to mild basic conditions and have been synthesized as dimers but have not been incorporated into ODNs.[128,129] The 5′-*N*-carbamate linkage is chemically stable and was synthesized as a hexamer of cytidine (C-6) or thymidine (T-6).[130-132] The C-6 carbamate ODN was found to bind a complementary DNA or RNA with high affinity,[131] but the T-6 carbamate ODN bound with relatively low affinity.[132] Stirchak *et al.* introduced the morpholino carbamate derivative, in which the deoxyribose sugar is replaced with a 6-member morpholino "sugar" which is in turn connected via a carbamate linkage (Figure 3).[133] Fully substituted polymers have been synthesized and are found to bind with slightly less affinity than phosphodiester ODNs.[133-135] Other nitrogen-containing derivatives include the hydroxylamine linkage,[136,137] methylene(methylimino) (MMI) and methyleneoxy(methylimino) (MOMI) linkages[138] (Figure 3). All these modification have been introduced into ODNs and all show slight increases in T_m with complementary RNA.

Peptide nucleic acids (PNAs) were introduced by Nielsen *et al.* as a replacement of the entire ribose–phosphodiester backbone (Figure 3).[139] An advantage of this strategy is that the monomers can be polymerized using standard peptide coupling chemistry, which is highly efficient. PNAs which contain a terminal lysine moiety have been shown to have unusually high T_ms and strong activity for the inhibition of reverse transcriptase, RNA polymerase II, and elongating ribosomes *in vitro*.[140,141] PNAs have been shown to bind to RNA in a 2 PNA: 1 RNA complex for T-rich PNAs, indicating that the high T_ms may be a result of triple helix formation and complicate the interpretation of the biophysical data.[142] The high affinity may also be a reflection of the positively charged lysine on the end. PNAs containing T and C have been synthesized and are found to bind in either the NH to 5′ or NH to 3′ orientation with almost equal affinity; presumably this lack of preference for the polarity of binding is a reflection of the lack of any chiral centers in these molecules.[141,142] In an *in vivo* microinjection experiment, a 15 mer PNA, containing, T, C, and A, was shown to inhibit expression of a temperature-sensitive T antigen mutant.[140]

Sugar Modifications

Sugar modifications have also been used to enhance stability and affinity. The α-anomer of a 2′-deoxyribose sugar has the base inverted with respect to the natural β-anomer. ODNs containing α-anomer sugars are resistant to nuclease degradation and bind parallel to the RNA target.[143] A 12 nt α-anomer ODN was reported to inhibit *de novo* HIV infection, but without sequence specificity and by a non-antisense mechanism.[144] The natural 2′-deoxyribose sugar is the D isomer. An L-2′-deoxyribose analogue of cytidine (L-2′-dC) has been synthesized and incorporated into ODNs.[145] The presence of the L-2′-dC at the 3′-end of the ODN enhanced resistance to exonuclease digestion but lowered the T_m with a complementary DNA. Modification of the 2′-OH of the ribose sugar to form 2′-*O*-methyl or 2′-*O*-allyl sugars is found to enhance

resistance to degradation, compared with normal RNA, without compromising affinity.[146] Shibahara *et al.* showed anti-HIV activity for a 25 nt ODN which contained 2′-*O*-methyl sugars and phosphorothioate backbone, reporting an IC_{50} of 1 μM.[147] The same 2′-*O*-methyl ODN with a phosphodiester backbone showed no activity. The 2′-*O*-allyl ODNs are more resistant to degradation than the 2′-*O*-methyl ODNs[148,149] and have been reported to bind specifically to small nuclear RNAs targeted in the nucleus following microinjection.[150] As reported previously, 2′-*O*-allyl ODNs targeted to the coding region of T antigen were unable to inhibit gene expression in a microinjection experiment, despite very high affinity.[11] The 2′-fluororibose sugars also enhance affinity, but do not increase stability unless used in conjunction with phosphorothioate backbones.[101,146] 2′-Deoxy-carbocyclic sugars, in which the 4′-oxygen is replaced with a methylene, have been incorporated into ODNs as thymidine and 5-methylcytidine derivatives;[151,152] however, both of these carbocyclic sugars decrease the affinity of the ODNs. Hexose sugars have also been substituted for the deoxyribose sugar and have been found to enhance stability but compromise affinity.[153] None of these sugar analogues are known to activate the RNase H cleavage of the target RNA in an ODN:RNA hybrid, but they may prove useful when incorporated into ODNs targeted to regions of the RNA where a steric blocking mechanism is desirable.

Heterocyclic Base Modifications

Modifications of the heterocyclic bases offer an opportunity to enhance the affinity without compromising RNase H cleavage of the target RNA. Modifying the heterocycles is a challenge since base pairing must be maintained through a combination of proper hydrogen-bonding and base-stacking interactions. Hydrophobic modifications at the 5-position of pyrimidines can enhance the T_m of ODNs. The substitution of 2′-deoxyuridine (dU) for thymidine (T) decreases the affinity of ODNs targeted to DNA or RNA.[101,154] ODNs containing 5-fluoro-2′-deoxyuridine or 5-bromo-2′-deoxyuridine show a modest enhancement in T_m against DNA ODNs but do not increase T_m against RNA targets, as compared with ODNs containing thymidine.[155,156] The substitution of 5-methyl-2′-deoxycytidine (5-Me-dC) and 5-bromo-2′-deoxycytidine for 2′-deoxycytidine also enhances the T_m of ODNs targeted to either DNA or RNA targets.[154] Froehler *et al.* have shown that a 5-propynyl modification to pyrimidines greatly enhances ODN duplex stability as measured by T_m.[99] Both 5-propynyl-2′-deoxyuridine and 5-propynyl-2′-deoxycytidine enhance the T_m of ODNs bound to RNA, as compared with ODNs containing T and 5-Me-dC, independent of context, for both phosphodiester and phosphorothioate backbones. As mentioned previously, these modifications have also been shown to strongly enhance the biological activity of antisense ODNs, indicating that the minimum affinity necessary for an *in vivo* antisense affect is relatively high.[11]

Purine derivatives have also been modified in attempts to enhance affinity. 2-Amino-2′-deoxyadenosine (diaminopurine) introduces a third hydrogen bond into an A:T base pair and stabilizes duplex formation.[157,158] Seela *et al.* synthesized both 7-deazaguanosine (dzaG) and 7-deazaadenosine (dzaA) derivatives.[159,160] In ODNs with alternating dzaA and thymidine, the substitution of dzaA enhanced stability of the duplex.[159] No information is available on the affinity of dzaG in a duplex.[160] *N*2-(Imidazolylpropyl)-2′-deoxyguanosine has been found to increase the T_m against DNA targets, but has little effect on the T_m against RNA targets.[101] Future experiments could determine if purine modifications which enhance affinity also can increase the biological activity of antisense ODNs.

Modifications to Enhance Permeation

Another strategy to increase the biological potency of ODNs has been to create chimeric molecules consisting of an antisense ODN attached to a macromolecule which is known to be internalized by cells.[100] These ODN conjugates utilize cellular uptake pathways to increase the intracellular concentration of ODNs. Receptor-mediated uptake pathways may provide a route of ODN internalization which is distinct from that observed with non-conjugated ODNs.[161] Early experiments showed that conjugating an ODN to transferrin, a protein ligand for a cellular receptor, dramatically increased cellular association of the ODN.[162] Increased cellular association and activity have also been reported for an ODN–asialoglycoprotein conjugate (bound via polylysine) targeted to the hepatitis B virus.[163] In a similar uptake study, a fluoresceinated ODN was biotinylated and then bound to streptavidin which had 12 mannose residues attached.[164] This conjugate was then internalized preferentially in liver cells via the cellular mannose receptors. The ODN conjugate showed greater cellular association compared with the nonconjugated controls, but fluorescence microscopy clearly indicated a punctate, endosomal localization. Poly(L-lysine) has been shown to enhance the transfection efficiency for plasmid DNA and also enhances the cellular uptake of ODNs.[165] A phosphodiester ODN was covalently attached to poly(L-lysine) and found to have greater cellular association compared with the control ODNs and was also reported to have greater anti-HIV activity than the nonconjugated ODNs.[166] Enhanced cellular association has been demonstrated for cholesterol-conjugated ODNs (chol-S-dC-10)[56] and also a cholesteryl-conjugated antisense ODN.[167] These results may indicate that there are some simple solutions to enhance the cellular association of ODNs, but further studies are clearly necessary to demonstrate that ODN conjugates enhance both delivery of ODNs to the cytoplasm of the cell and that the ODNs cause specific inhibition of gene expression through an antisense mechanism.

Conclusions on Antisense Therapeutics

Antisense technology was once viewed as an opportunity for straightforward drug design, since anyone with access to a DNA synthesizer could create molecules to potentially manipulate gene expression. The literature provides ample evidence that the addition of ODNs to tissue culture media does cause phenotypic changes in cells through multiple mechanisms, not necessarily including antisense hybridization. The literature also provides evidence that, under the appropriate conditions, ODNs can specifically inhibit gene expression in cells in a manner consistent with an antisense mechanism. The task for those striving to develop therapeutic antisense molecules is to design the proper ODN derivatives which have the required properties of stability, affinity, permeation, and, ultimately, favorable pharmacokinetics. None of the currently available ODN analogues contain all of these properties, and advancement in this field depends upon the devel-

opment of new, potent antisense agents. The current advances in antisense technology we have described give promise that new and exciting analogues will be forthcoming for both research and therapeutic uses.

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