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Mini-review

Viral targets for antisense oligonucleotides: a mini review

A. Kirk Field [a,b,*]

[a] *Hybridon, Inc., Cambridge, MA, 02139, USA*
[b] *Department of Pharmacology and Molecular Toxicology, University of Massachusetts Medical Center, Worcester, MA, USA*

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Over the past three decades, antiviral researchers have participated in the discovery and development of an impressive array of drugs having efficacy against members of the herpesviruses, retroviruses and myxoviruses. These successes need not be chronicled here, except to refer to the adequate summaries available (Galasso et al., 1990; Jeffries and De Clercq, 1995). The approach which has led to this success entails screening and modifying compounds which interfere with virus replication through inhibition of viral proteins such as essential viral enzymes like herpesvirus DNA polymerases, human immunodeficiency virus reverse transcriptase and protease and influenza virus neurominidase; or proteins involved in virus adsorption like the picornavirus capsid proteins and influenza hemaglutinin. As successful as these approaches have been and as promising as the newer antiviral drugs may be, there are limitations to the present approaches. For each virus there are only a limited number of proteins that are readily amenable to the inhibitor screening process. These are usually enzymes required for replication. Nucleoside and nucleotide inhibitors of herpes simplex virus (HSV) DNA polymerase are the classic examples of successful screening and compound modification through structure/activity relationship studies. Occasionally a novel virus target is identified by the discovery of an agent that selectively inhibits replication, with the novel mechanism defined subsequently. For instance, certain D-ribofuranosyl benzimidazoles inhibit cytomegalovirus (CMV) replication, the result of interfering with DNA maturation (Townsend et al., 1995; Drach and Biron, personal communication).

However, a great opportunity would unfold with the capacity to design selective inhibitors targeted to any or all of the virus genes involved in replication, virulence or latency. Consider that HSV encodes for 71 open reading frames (orfs)

* Present address: 376 Meadowbrook Rd. North Wales, PA 19454, USA; Tel. +1 215 6995563; Fax: +1 215 6997976.

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and CMV encodes for over 200 orfs. Indeed, could we design inhibitors that would alter the expression of those virus genes for which we have not yet defined an assayable function? And what about the viruses such as the papilloma and certain hepatitis viruses for which there isn't a virus replication assay readily available?

Novel approaches are necessary, and one approach is just now being recognized as both versatile and practical for identifying novel antiviral drugs and drug targets. The approach is the use of antisense oligonucleotides complementary to the viral RNAs to block mRNA translation or genome replication. In this mini-review I will define both the opportunities and the cautions for the use of antisense oligonucleotides to probe for novel antiviral targets. To illustrate the opportunities, I will use selected recent examples rather than attempt to catalog the use of oligonucleotides as antiviral agents. Recent reviews will provide the reader with that more in-depth accounting (Field and Goodchild, 1995; Temsamani and Agrawal, 1996; Kilkuskie and Field, 1997).

1. What is Antisense and RNA Targeting?

The principle of antisense is simple and quite elegant. If the nucleotide sequence of an mRNA or genome RNA is known, then one should be able to design a complementary oligonucleotide that will hybridize to the RNA target and inhibit its function (Fig. 1). This principle was first documented in 1978 by the observations of Zamecnik and Stephenson using a phosphodiester oligodeoxynucleotide composed of 13 nucleotides (a 13-mer) to block Rous sarcoma virus replication (Stephenson and Zamecnik, 1978; Zamecnik and Stephenson, 1978). However, the path from these initial observations to the practical use of antisense oligonucleotides for defining novel antiviral agents has been a long one during which we have had to learn to:

(a) choose preferred viral RNA target sequences for optimal antisense impact, (b) to alter the antisense oligonucleotides to enhance stability and retain hybridization capacity and favorable biological characteristics and (c) to recognize that

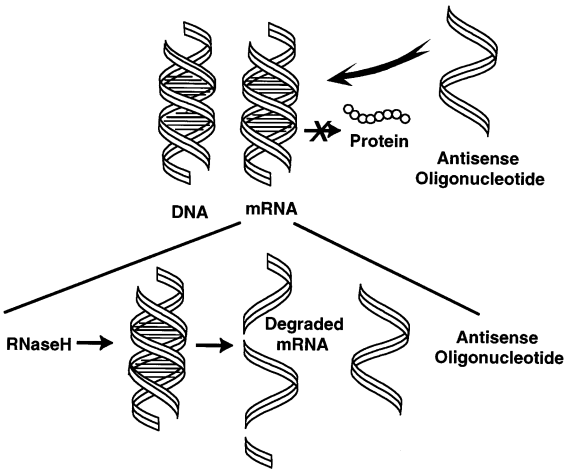


Fig. 1. Antisense oligonucleotides may bind to target viral precursor mRNA, processed mRNA, or genomic RNA, preventing protein synthesis by translation arrest. In addition, DNA/RNA heteroduplexes formed by oligodeoxynucleotide binding to RNA may be recognized by cellular RNase H, cleaving the target RNA, and potentially freeing the antisense oligonucleotide for binding to another RNA target.

oligonucleotides may display an array of antiviral activities unrelated to any antisense function for which they were designed.

(a) Since in theory the array of RNA targets for antisense is limited only by nucleotide sequence information, it follows that without developing some knowledge of preferred oligonucleotide hybridization sites, one is faced

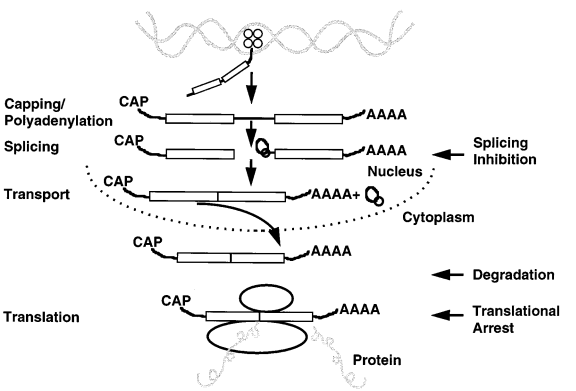


Fig. 2. mRNA transcription, processing, and translation are all potential targets for antisense oligonucleotide intervention. The arrows indicate potentially vulnerable antisense inhibition sites.

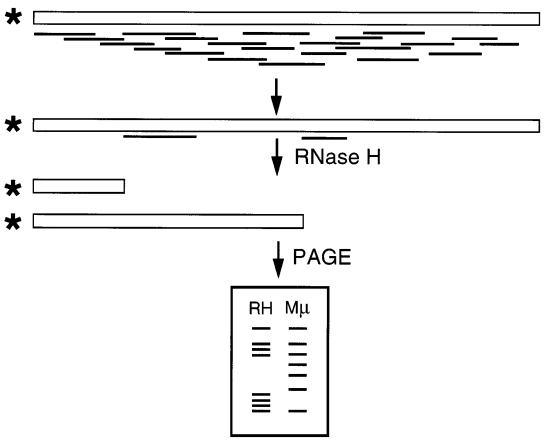
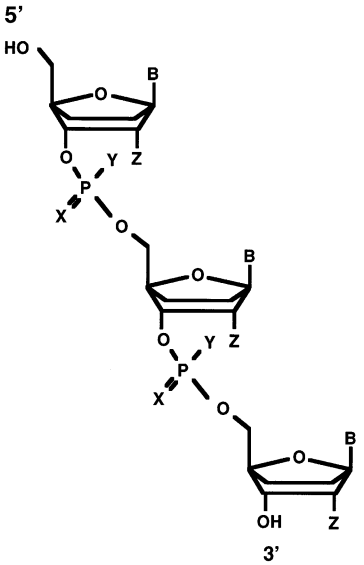


Fig. 3. Antisense oligonucleotide selection. Oligonucleotide libraries (thin lines) are incubated with 5′ end-labeled RNA (thick line), then treated with RNase H. RNA regions with bound oligonucleotide are digested, producing families of shorter labeled RNAs, which are separated on polyacrylamide gels. Discrete families of RNAs are detected on gels (lane RH) and compared to molecular weight markers (lane Mu) to identify regions of maximum oligonucleotide binding.

with a random screening process of potential antisense oligonucleotides which complement the entire RNA target sequence. But trial and error experience has taught us that there are in fact RNA sequences that are vulnerable to antisense oligonucleotide binding. Favorite targets have included RNA splice donor or receptor sites, 5′ capping, 3′ adenylation, translation start and termination sites, and ribozome entry. Thus as illustrated in Fig. 2, multiple opportunities during RNA processing may be vulnerable to antisense hybridization. But is there a fully rational approach to RNA target sequence selection? In a word—no, but there are multiple techniques which can impact on success. Computer models of RNA secondary structure (Sczakiel et al., 1993), nucleotide hybridization efficiency (Stull et al., 1992) and frequency (Han et al., 1994) have been compared to antisense oligonucleotide activity. Antisense activity correlates well with hybridization strength, while RNA secondary structure does not necessarily predict optimal antisense activity. In vitro translation inhibition has been used to screen potential antisense oligonucleotide inhibitors, and then the most active compounds evaluated in antiviral assays (Chen et al., 1996). Oligonucleotide libraries may also be screened for binding to target RNA in vitro using an assay described by Frank and colleagues (Frank and Goodchild, 1996; Frank et al., 1993) and further modified by Ho et al. (1996) (Fig. 3). Exposure of P^{32} end labeled target RNA to a random library of 20-mer oligodeoxynucleotides in the presence of RNase H, an enzyme that cleaves the RNA in an RNA/oligodeoxyribonucleotide heteroduplex, will result in a pattern of RNA fragments which are reflective of the preferred binding sequences on the RNA. The fragments and cleavage points can be identified by gel electrophoresis, and confirmed by repeating the technique using selected antisense oligodeoxynucleotides complementary to the predicted cleavage sites. This technique has been useful both in identifying vulnerable RNA nucleotide sequences for antisense oligonucleotides and in confirming the antisense activity of a given antisense oligonucleotide. But the predicted sites of preferred binding in vitro may be masked by RNA folding or associated proteins within the infected cell, so even this technique is not entirely predictive of in vivo success.

(b) Oligonucleotide degradation by cellular nucleases, particularly for unmodified phosphodiester compounds, has limited their use in antisense research. As a result practical application of antisense oligonucleotides has required modifications, with the aim of retaining the hybridization capacity and capacity to recruit RNase H catalysis of the RNA target while increasing stability. Some changes to the oligonucleotide are illustrated in Fig. 4, and some the resultant properties of these modifications are shown in Table 1. Generally those modifications that jeopardize the capacity of the oligonucleotide to participate in Watson–Crick base pairing are avoided, with most alterations focused on the phosphodiester backbone and/or the sugar moiety. Replacement of the non-bridging oxygen of the phosphdiester backbone with sulfur results in a phosphorothioate (PS) with enhanced stability to enzymatic degradation. Although the duplex formed with the target RNA has a lower melting temperature (T_m), it remains a substrate for RNase H. Replacement of the non-bridging oxy-



	X	Y	Z
Deoxyoligonucleotides	O	O^-	H
Ribooligonucleotides	O	O^-	OH
2'-*O*-methyl derivatives	O	O^-	OMe
Phosphorothioate	O	S^-	H
Methylphosphonate	O	CH_3	H
Phosphoramidate	O	NR^1R^2	H

Fig. 4. Structure of oligonucleotides. B = any of the heterocyclic bases found in DNA or in RNA. The terminal hydroxyl groups are distinguished as being at either the 5′ or 3′ ends. Some substitutions at the non-bridging oxygens (*X*, *Y*) of the phosphodiester linkage, or at the 2′ position on the sugar moiety (*Z*) are shown in the accompanying table.

gen by a methyl group results in greater hydrophobicity due to loss of the negative charge, but also a loss of RNase H activation. Alternatively, replacing the hydrogen at the deoxyribose 2′position with a hydroxymethyl group converts the sugar to a modified ribose. The resultant RNA/RNA duplex formed with the target RNA is more stable, as indicated by the elevated T_m. Thus, by these modifications one can synthesize tailored antisense oligonucleotides with a balance of characteristics of hybridization affinity, hydrophobicity and the capacity to recruit RNase H-mediated hydrolysis of the target RNA. Most of the literature on use of oligonucleotides as antiviral agents has used the PS-modified oligonucleotides, and the first generation of drugs which have progressed to clinical trials have been PS compounds (Agrawal and Temsamani, 1996; Field and Goodchild, 1995; Crooke and Bennett, 1996; Kilkuskie and Field, 1997). As we learn more about the pharmacological properties of modified oligonucleotides, a second generation of antisense oligonucleotides is now emerging. This includes combinations of nucleotide modifications incorporated within the oligonucleotide. *Hybrid oligonucleotides* may be defined as substituted at the 3′ and/or 5′ ends with 2′-OCH_3 ribonucoleosides, while maintaining the phosphorothioate backbone. An example of such a hybrid molecule which was chosen for further development is GEM 132, which is modified from the UL 36 ANTI described below, and is now in phase I clinical evaluation (Martin et al., 1997). Other advanced chemistry modifications include *chimeric oligonucleotides* which may have nonionic internucleotide linkages at the 3′ and/or 5′ ends, and *self-stabilized olgonucleotides* which are phosphorothioates or phosphdiesters that have two domains—a hairpin loop at the 3′ end to protect against degradation and a single-stranded antisense sequence. Agrawal and Temsamani, 1996; Crooke et al., 1996 have summarized the pharmacological characteristics of these and other modified oligonucleotides.

(c) Not unexpectedly, these modifications have also resulted in a variety of non-antisense antiviral activities of oligonucleotides which were designed for the selectivity expected of antisense. Thus, when evaluating an oligonucleotide which has been designed as antisense to a target RNA,

Table 1
Some properties of oligonucleotide modification

Oligonucleotide	Ribonuclease H[a]	Hybridisation affinity	Hydrophilicity	Nuclease resistance
Unmodified	++	++	Hydrophilic	−
Phosphorothioate	+	+	Hydrophillic	+
Methylphosphonate	−	+	Hydrophobic	+++
Phosphiramidate	−	+	Hydrophilic or hydrophobic	++
2′*O*-alkyl	−	+++	Hydrophilic	++(?)

[a]The ability of deoxyoligonucleotides to direct cleavage of the complementary RNA by ribonuclease H.

assaying the antiviral activity alone is insufficient and may be misleading. As shown in Table 2, numerous antiviral activities have been documented, and indeed others may be demonstrated in the future as new sequences and chemical modifications are assayed. For human immunodeficiency virus (HIV), modified oligonucleotides may be potent inhibitors of reverse transcriptase, RNase H and primer extension (Austermann et al., 1992; Bordier et al., 1992; Hatta et al., 1993), and direct inhibitors of virus adsorption to cells (Zelphati et al., 1994). Specific PS sequences such as T2G4T2 and poly dC bind to the V3 loop of gp120 and prevent virus infection (Stein et al., 1993; Wyatt et al., 1994). An oligomer composed only of dG and dT residues with some PS linkages inhibits HIV integrase (Ojwang et al., 1995) and blocks virus adsorption (Bishop et al., 1996). For HSV, PS oligonucleotides may be potent inhibitors of virus adsorption and DNA synthesis (Gao et al., 1989; Fennewald et al., 1995). Suffice it to say that chemical modifications and sequence motifs of oligonucleotides may contribute to non-antisense antiviral activities which may be direct (as in the case of the G-quartet's potential to block virus adsorption and the inhibition of key replicative enzymes), or indirect (as in the capacity of CpG motifs to enhance B-cell proliferation as described by Krieg et al. (1995)).

Thus, it is essential to define an antisense activity with solid supporting data when using an antisense-designed oligonucleotide to probe new antiviral targets. That definition should include a demonstration of both oligonucleotide sequence-

Table 2
Antiviral activities of oligonucleotides[a]

Possible antisense inhibition of virus RNA processing or function

Direct inhibition of virus adsorption by phosphorothioate oligonucleotides
- G-quartets may block virus adsorption
- $T_2G_4T_2$ binds to gp 120 and blocks HIV binding to CD_4 cell receptor

Virus enzyme inhibition
- Phosphorothioate oligonucleotides may inhibit HIV reverse transcriptase—Ki values of 6–12 nM
- A 17-mer dG, dT blocks HIV integrase $IC_{50} = 50$ nM
- A 28-mer dC phosphorothioate oligonucleotide inhibits HSV replication $IC_{90} = 1\ \mu M$—competes as a template for HSV DNA polymerase

Immunomodulation
- –CpG– motifs may enhance cellular immune responses
- Interferon induction by dsRNA formation

Others?

[a]Or why measuring antiviral activity alone isn't good enough.

Table 3
Suggested criteria for antisense activity

Oligonucleotide sequence-specificity: controls are so important
- Reverse the sequence 5′–3′ flipped to 3′–5′—non-complimentary to RNA target, but base sequence intact
- Scramble the nucleotides-same nucleotide composition but poor sequence-specificity
- Introduce mismatches-decreases complementarity to target RNA
- Compare to other anitsense oligonucleotides of similar length/chemistry

RNA target selectivity
- Demonstrate lack of inhibition of parallel gene expression at RNA, protein, and functional levels

Gain direct evidence of target mRNA cleavage

Table 4
Inhibition of productive EBV replication by anti-BZLF1 antisense oligonucleotides in anti-IgG-stimulated Akata cells (Daibata et al., 1996)

Oligonucleatide	Mean ± S.E. (%inhibition)			
	0.5 μM	2.5 μM	12.5 μM	25 μM
Phosphorothioate				
Anitsense	34 ± 6 (3)[b]	48 ± 13 (3)	68 ± 9 (4)	69 ± 5 (4)
Random	5 ± 5 (3)	21 ± 10 (3)	24 ± 9 (4)	32 ± 10 (4)
Sense[c]			11 (1)	36 (1)
Reverse[d]			17 (1)	36 (1)
Phosphodiester				
Antisense	16 ± 13 (3)	25 ± 13 (3)	46 ± 10 (3)	56 ± 8 (3)
Random	14 ± 14 (3)	14 ± 7 (3)	21 ± 3 (3)	19 ± 6 (3)

[a] Oligonucleotide-treated Akata cells were stimulated with anti-IgG for 24 h. Inhibition was measured by the amounts of linear EBV DNA from Gardella gel analysis.
[b] The number of experiments (*n*) is listed in parenthesis.
[c] Sense is the sequence complementary to the Z-1 anti-sense.
[d] Reverse is the sequence with the identical nucleotide sequence as Z-1 but read in the opposite direction.

specificity of action and selectivity of inhibition of expression of antisense gene target. The promise of antisense is a specificity of design and function, and its utility in defining novel antiviral targets is built on fulfilling the demonstration of an antisense mechanism of action. The criteria will continually undergo refinement as ever more sophisticated molecular techniques elucidate intracellular mechanisms of action. Table 3 lists some criteria, based on the suggestions of Stein and Krieg, 1994. Oligonucleotide sequence-specificity and RNA target selectivity are fundamental expectations of complementary base-paired hybridization, while more direct evidence for in situ antisense activity such as target RNA cleavage may be more elusive, but still attainable.

With the previous discussion and definitions as prologue, let us document some of the successes for which the antisense approach has been used to identify novel antiviral agents and novel antiviral targets. By the nature of this restricted definition (oligonucleotide sequence-specificity **and** gene target specificity) and the confines of a mini-review, the examples cited here will be relatively few, and even with these examples there may also be multiple mechanisms in play in the antiviral efficacy. The more inclusive literature concerning oligonucleotides as antiviral agents has recently been reviewed by Kilkuskie and Field, 1997.

2. Antisense and novel antiviral targets

2.1. Herpes simplex virus (HSV)

The splice donor/acceptor sites of the immediate early pre-mRNA of IE 4 have been the focus of studies by Aurelian and her colleagues (Kulka et al., 1989, 1993, 1994; Kean et al., 1995; Kulka and Aurelian, 1995) In the earlier studies a 12-mer methyl phosphonate oligodeoxynucleotide targeted to the IE 4 mRNA splice donor site reduced virus replication by 80% at 100 μM, whereas the same compound in which two central nucleotide residues were inverted was inactive. The same active oligonucleotide applied locally reduced HSV1 ear infection in mice. The studies were suggestive of the sequence-specificity of an antisense mechanism. In the more recent studies (Kean et al., 1995), the group targeted a 12-mer methyl phosphonate with 2′-OCH_3U substituted for dT to the intron/exon junction of the splice acceptor. They achieved greater affinity for the RNA target and a fivefold reduction in effective dose against HSV1, with no inhibition of HSV2. Mismatched oligonucleotides had less HSV1 an-

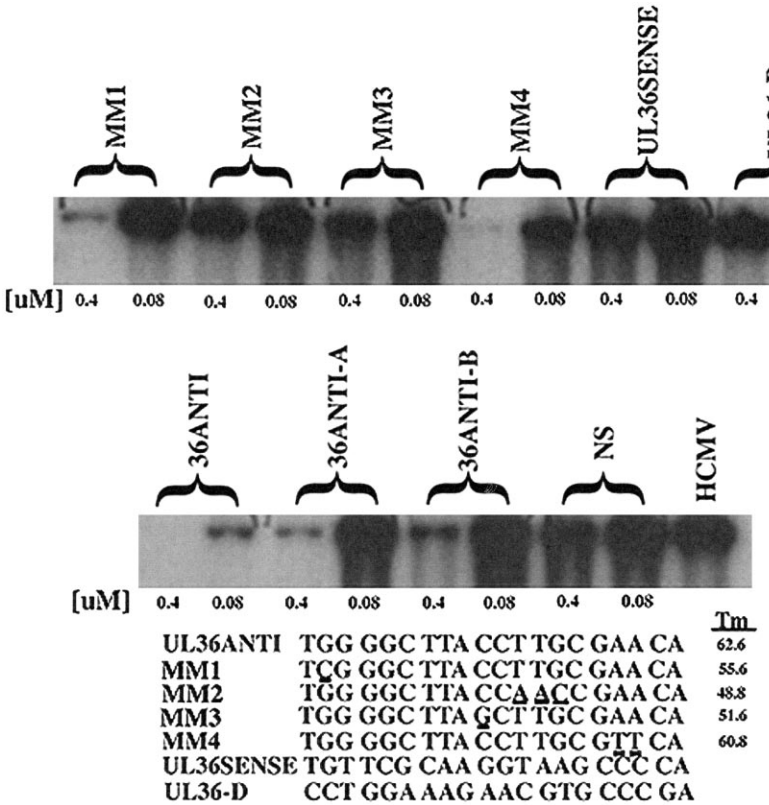


Fig. 5. UL36ANTI base-pair mismatches and their effect on inhibition of HCMV DNA replication are depicted by Southern analysis. Relative band intensities were calculated by scanning analysis. Positive control samples are assigned a value of one, with all other band intensities reported as a decimal fraction. UL36ANTI and various other oligonucleotides are shown below, with underlined bases indicating the substitutions. Relative differences in T_m values are shown at the right of the oligonucleotide sequences (Pari et al., 1995).

tiviral activity. The IE 4 pre-mRNA has also been the target of a series of phosphodiester oligodeoxynucleotides designed with 12-mer regions complementary to the RNA target and 3′ non-complementary flanking sequences that formed hairpin structures to stabilize against degradation. The studies were well controlled for non-sequence dependent antiviral effects and fair potency versus HSV-1, $IC_{50} = 1.5\ \mu M$ (Poddevin et al., 1994)

Peyman et al., 1995 targeted the translation start site of IE 110 mRNA. To minimize nonspecific effects they evaluated an array of 20-mers that were phosphodiester oligodeoxynucleotides except for two phosphorothioate nucleotide residues at both the 5′ and 3′ ends. The most potent compound inhibited virus cytopathology at 9 μM, and a 2-nucleotide shift in sequence reduced efficacy by about nine-fold. Mismatched oligonucleotides were inactive at 80 μM.

In all of these examples, attention has been paid to the need to demonstrate sequence-specificity of the oligonucleotides. Unfortunately, a relative lack of attention was given to gene target specificity, with no demonstrations of selective down-regulation of target mRNA translation.

2.2. Marek's disease virus (MDV)

MDV causes lymphoproliferative disease, with maintenance of the tumorigenic state in MDV-derived lymphoblastoid cells dependent on the

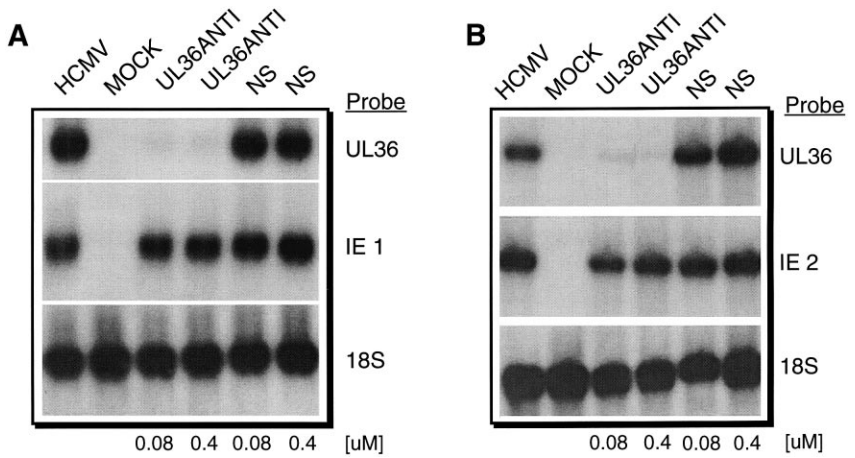


Fig. 6. Selective inhibition of UL36 mRNA. (A; UL 36 and IE 1) Northern analysis of infected total cellular RNA, harvested 6 h post infection from samples treated at the indicated concentrations with oligonucleotides, shows that UL36ANTI specifically decreases the steady-state level of UL36 RNA, using a UL 36-specific probe. The blot was also hybridized with a probe specific for IE1 mRNA, indicating that the steady-state level of IE1 RNA is unaffected by either UL36ANTI or nonspecific (NS) PS oligonucleotides. (B; UL 36 and IE 2) Northern analysis of immediate-early RNA in the presence of specific and nonspecific oligonucleotides. An autoradiogram of a Northern blot hybridized with a probe specific for IE2 mRNA indicates that the steady-state level of IE2 is unaffected in the absence of UL36 mRNA and in presence of UL36ANTI or nonspecific PS oligonucleotides (Smith and Pari, 1995).

expression of a 1.8 kb gene family. Kawamura et al. (1991) evaluated a phosphodiester 18-mer complementary to a splice donor sequence. They demonstrated an oligonucleotide sequence-specific inhibition of 1.8 kb mRNA expression and inhibition of colony growth in soft agar, while multiple copies of the MDV DNA remained in the lymphoblastoid cells. Sense and unrelated oligonucleotides were not active. Thus, it appeared that this controlled study provided evidence for the requirement for the 1.8 kb region for MDV-tumorigenicity in transformed cells.

2.3. Epstein–Barr virus (EBV)

EBV is the etiologic agent for most cases of infectious mononucleosis and can cause latent infections resulting in polyclonal B cell lymphoproliferative diseases in immunocompromised individuals, and nasopharyngeal carcinoma (Huang, 1991; Pagano, 1995). Two key genes, EBNA-1 which is required for maintenance of cell transformation, and BZLF 1 which is required for reactivation of latently infected cells, have been the subjects for design and evaluation of antisense oligonucleotides. Pagano et al., 1992 evaluated unmodified phosphodiester oligonucleotides complementary to the coding region just 3′ of the AUG on the EBNA-1 mRNA, and observed that prolonged treatment of Raji cells using relatively high oligonucleotide concentrations (40 μM) resulted in a progressive reduction of EBNA-1 proteins and in EBV DNA copy number. Similar treatments with the sense control oligonucleotide were ineffective. When PS oligonucleotides were used in similar studies, antisense specificity was also observed at lower doses (5 μM), but scrambled and sense control sequences were partially effective. Roth et al., 1994 also used unmodified oligonucleotides complementary to sequences in the EBNA-1 RNA and observed inhibition of translation and cell proliferation, with no effect of these antisense oligonucleotides on the growth of EBV-negative cells.

Akata cells are latently infected with EBV and are inducible to the lytic cycle, a reactivation that requires transcription and translation of the BZLF1 gene to produce the Zebra protein. Sequence-specific and gene target specific antisense inhibition of BZLF 1 expression was documented

by Daibata et al. (1996). 25-mer oligonucleotides complementary to the translation start codons inhibited the production of Zebra and replication of virus, as determined by the lack of production of replicative linear DNA, early antigen (EA-D) and virus capsid antigen (VCA). Sense, reverse sequence and random oligonucleotides were less effective. (Table 4) No inhibition of cellular DNA synthesis or expression of the B cell protein CD 19 occurred at the effective concentrations of antisense oligonucleotides.

The experience with antisense oligonucleotides to EBV targets illustrates both the phenotype resulting from selective inhibition of gene expression and the critical need to run the oligonucleotide control sequences and evaluate for gene target selectivity. If the aim were to develop an antisense drug candidate to inhibit either EBNA-1 or BZLF 1 expression, a rigorous evaluation of both target RNA sequences and oligonucleotide chemical modifications would be in order.

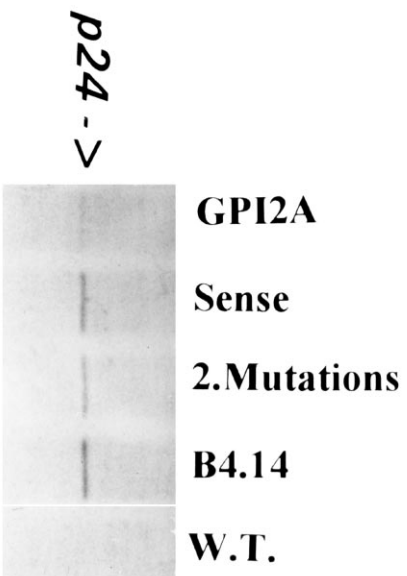


Fig. 7. Inhibition of HIV p24 production. B4.14 cells, stably transfected to express HIV-1p24, were treated with an antisense oligonucleotide (GP12A) spanning bases 1189–1208 of HIV. Two control oligonucleotides containing either two mutations (2.mutations) or the sense sequence were also tested. p24 production, measured by immunoprecipitation using a rabbit polyclonal antibody, was decreased only in cells treated with the antisense oligonucleotide. Control and 2.mutation oligonucleotides did not alter p24 levels. Cells that were not transfected (W.T.) did not express p24 (Anazodo et al., 1995).

2.4. Cytomegalovirus (CMV)

Two laboratories have focused on the use of oligonucleotides to inhibit CMV. A series of oligonucleotides complementary to the translation start sites, coding regions, intron/exon region and 5′ caps in RNAs including the DNA polymerase, and immediate early genes IE 1 and IE2 was evaluated by Azad et al., 1993. The most potent of these is a 21-mer (ISIS 2922) against the coding region of IE2, with an IC_{50} of about 0.1 μM. Whereas unrelated oligonucleotides were less active in both the reduction of IE2 and virus replication, mismatches in ISIS 2922 which substantially reduced hybridization did not alter antiviral effects. This suggested that the antiviral activities may be mainly or in part due to a non-antisense mechanism of action.

Pari and his associates used antisense oligonucleotides to identify particularly sensitive antiviral targets in CMV, with the resulting identification of a potent 20-mer PS oligoncleotide complementary to the splice donor site of the immediate early gene, UL 36. This gene is essential for human CMV origin of replication-dependent DNA synthesis (Pari and Anders, 1993), and based on the antisense studies it was proven essential for virus replication (Pari et al., 1995; Smith and Pari, 1995). Sequence specificity for inhibition of viral DNA replication was established by comparison of the efficacy of UL 36 ANTI with the sense, reverse, and unrelated sequences. (Fig. 5) Mismatches in the UL 36 ANTI sequence reduced activity substantially and correlated with reduced T_m values. Shift of the antisense sequences four or eight nucleotides in the 3′ direction (UL36ANTI-A and UL36ANTI-B respectively) reduced activity compared to UL36ANTI. Antisense (UL36-D) to a sequence entirely within the intron of the UL36 splice site was inactive. (Fig. 5). Gene target selectivity was also demonstrated using Northern blots showing that use of UL36ANTI reduced UL36 transcript accumulation without affecting accumulation of either IE 1 or IE2 (Fig. 6). Furthermore, infectious virus yield was reduced

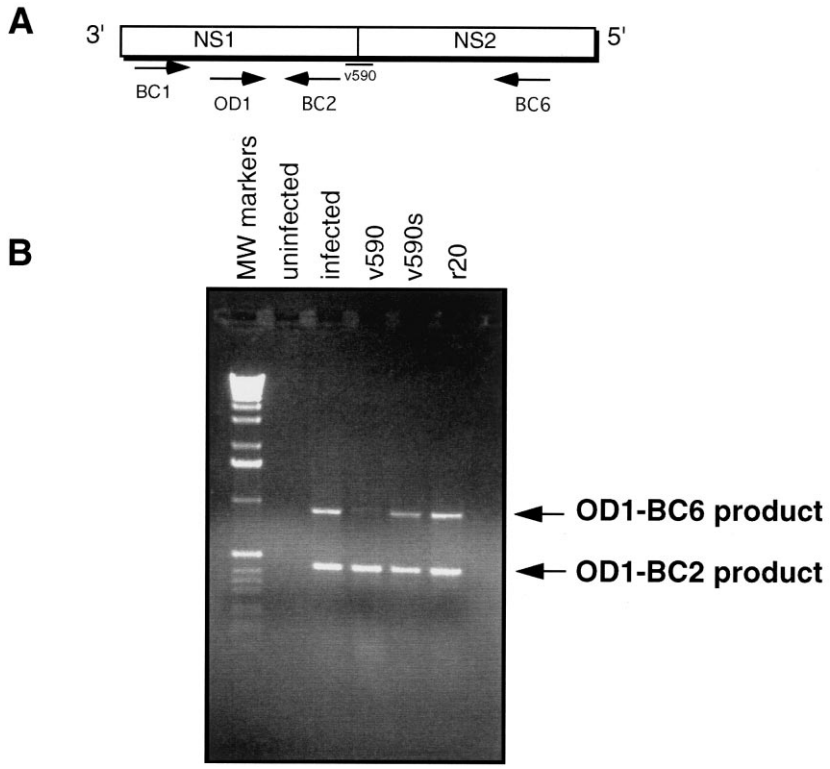


Fig. 8. Evaluation of genomic RSV RNA by reverse transcriptase polymerase chain reaction (RT–PCR). RT–PCR scheme (A) and results (B) are shown. RSV RNA was reverse transcribed using primer BC1. PCR was then conducted using OD1 and BC2 or OD1 and BC6. (B) shows the 410-base pair product generated from OD1 and BC2 in all samples; the 940-base pair product was generated from OD1 and BC6 and was missing from the antisense (v590) treated cells, but not from those treated with the reverse sequence oligonucleotide (590s) or a random oligonucleotide (r20) (Jairath et al., 1997).

by greater than 99% using UL 36 ANTI at 0.08 μM (Pari et al., 1995). It should also be noted that the sequence of UL36ANTI contains a G-quartet motif which could contribute to the antiviral activity. However, by adhering to the criteria outlined previously, use of antisense oligonucleotides has been effective in identifying a novel antiviral target and a potent and selective antiviral agent which acts primarily through an antisense mechanism of action. The nucleotide sequence of UL36ANTI has now been the basis for the chemically modified hybrid GEM 132 which is presently in clinical evaluation for intravitreal and non-ocular treatment of CMV diseases (Martin et al., 1997).

2.5. Human immunodeficiency virus (HIV)

Using cells stably transfected with the *gag-pol* region of HIV, Anazodo and colleagues demonstrated that a partially phosphorolated 20-mer targeted to a conserved *gag* mRNA region inhibited accumulation of the precursor p55 protein, the p55 protein and the p24 cleavage product. Inverse oligonucleotide sequence and double mismatch control oligonucleotides were less effective. (Fig. 7). The antisense 20-mer (at 1 μM) inhibited viral replication in a sequence-specific manner and showed selectivity by not altering the biosynthesis of cell ribonucleotide reductase or cell growth (Anazodo et al. 1995).

Family:	**Papovaviridae**
	> 60 types recognized
ORF	**Function**
E1	**DNA replication**
E2	**Regulation of transcription, DNA replication**
E4	**Abundant of cytoplasmic protein, interacts with cytokeratins**
E5	**Membrane protein, alters response of receptor tyrosine kinases**
E6	**Binds p53 tumor suppressor gene**
E7	**Binds retinoblastoma gene product**
L1	**Major capsid protein**
L2	**Minor capsid protein**

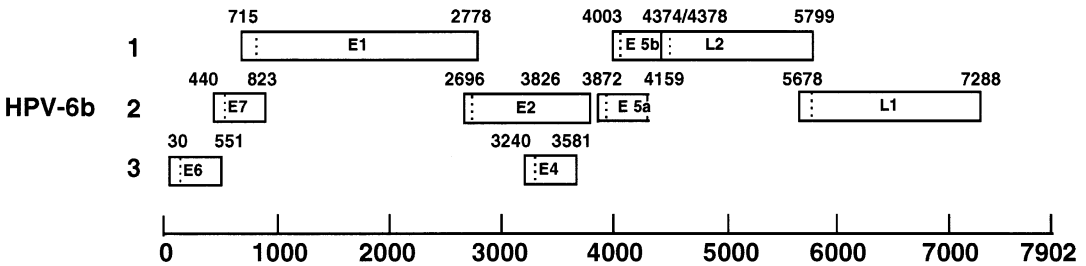


Fig. 9. Human Papillomavirus scheme showing the open reading frames (orfs), their suggested functions, and the relative locations on the HPV-6b genome.

2.6. Respiratory syncytial virus (RSV)

An antisense PS 20-mer targeted to the intergenic region/start site for the NS2 and P genes of the RSV genomic RNA was the subject of a rigorous characterization of antisense mechanisms of action (Jairath et al., 1997). This oligonucleotide was a more potent inhibitor of virus replication than the reverse sequence, oligonucleotides targeted to various RSV mRNAs, or a random mix of unrelated 20-mers. Most importantly, the mechanism of antisense activity was more directly observed by demonstrating the sequence-specific depletion of the RSV genomic RNA around the NS1-NS 2 region using reverse transcriptase–polymerase chain reaction (RT–PCR) (Fig. 8). Quantitation of the depletion indicated that whereas in RSV-infected cells treated with random oligonucleotide or the sense 20-mer the amount of RSV cDNA was about 3 pg/ml, in the antisense-treated cells RSV cDNA was not detected (less than 0.2 pg/ml). These results suggested RNase H cleavage in the antisense target region, and prompted identification of the cleavage sites by rapid amplification of cDNA ends (5′ RACE). The sequence-specific cleavage occurred within the antisense binding site, providing strong evidence that the antisense oligonucleotide interacted with the target RNA in situ and induced RNase H-mediated cleavage.

2.7. Hepatitis B virus (HBV)

HBV is a small DNA virus which contains four open reading frames (orfs) encoding two predominant transcripts and two minor transcripts. These transcripts are translated into three surface antigens, two nucleocapsid proteins, the polymerase

and a transactivator-the X gene product. For antisense intervention these transcripts and the short encapsidation signal, an RNA sequence with a well-defined secondary structure, have been the targets of antisense research. Korba and Gerin (1995) published studies using hepatocellular carcinoma-derived cells stably transfected with HBV DNA. Oligonucleotides targeted to transcripts encoding the S gene and C gene inhibited virion production, which correlated with protein reduction. The inhibition was oligonucleotide sequence-specific. Furthermore, the targeting was selective; inhibition of the S gene message inhibited only production of the HBsAg production, without altering HBeAg and HBcAg levels. Oligonucleotides targeted to the encapsidation signal inhibited virion production and viral DNA replication, consistent with this region's purported functions. Offensperger et al. (1993) reported that a PS oligonucleotide targeted against duck hepatitis B virus pre-S gene inhibited virus replication in vivo. Inhibition was dose dependent and sequence-specific, with treatment of infected ducks (20 mg/kg over a 10 day period) resulting in decreased HBV DNA in the liver and reduction in viral antigens in the serum and liver. Sense and random synthesis PS oligonucleotide controls were not effective. This represented the first reported successful use of antisense oligonucleotides to treat virus infection in vivo.

2.8. Human papillomaviruses (HPV)

Perhaps one of the most challenging issues for an antiviral researcher is identifying a selective inhibitor of a virus for which neither a cell culture replication assay nor a readily accessible animal infection model of disease is available. HPV provides such a challenge, and an opportunity for the antisense approach.

Based on DNA sequence diversity, there are over 65 types of HPV, with each type having preferred anatomical sites of replication (Shah and Howley, 1996). The circular DNA genome encodes for eight orfs which are translated from families of alternatively spliced mRNAs (Fig. 9). Two groups have successfully identified effective antisense oligonucleotides against HPV types 6 and 11 by employing cells transfected with the target HPV sequences which transactivate a reporter gene. Cowsert et al. (1993) reported that a PS oligonucleotide (ISIS 2105) targeted to the translation initiation site of the E2 mRNA for HPV types 6 and 11 inhibited transactivation of chloramphenicol acetyltransferase. Other control oligonucleotides were ineffective at the same concentration (5 μM). Furthermore, using a bovine papillomavirus cell culture focus formation model, ISIS 2105 inhibited virus-induced cell transformation.

More recently a combined research effort from Hybridon and the Roche Research Center successfully identified a PS oligonucleotide targeted to HPV E1 helicase transcript, and active in cell culture studies and in a mouse xenograft model of HPV replication (Lewis et al., 1997; Roberts et al., 1997). Roberts and colleagues used the in vitro RNase H assay described earlier and cell based HPV E1-luciferase fusion assays. The most active 20-mer PS oligonucleotide, targeted to the E1 translation start site has an EC_{50} of 30 nM in the cell based assay, and is both sequence-specific and gene target selective. Scrambled sequence, sense sequence and mismatched control oligonucleotides were inactive, whereas the antisense 20-mer reduced mRNA levels and luciferase activity. As expected, this antisense 20-mer did not alter luciferase expression from treated cells which were missing the E1 target RNA. A 2′-OCH_3 hybrid of the 20-PS antisense oligonucleotide was fully active in the cell culture assay and was chosen for in vivo studies employing the kidney xenograft nude mouse model implanted with HPV-infected human foreskin fragments. Compared to both saline-treated and mismatched hybrid oligonucleotide-treated mice, the antisense compound reduced the size of condylomas by up to 95%. Histological analysis of the tissue suggested that HPV was inhibited, with HPV-characteristic koilocytosis and thickness of the human epithelium greatly reduced (Lewis et al., 1997). Thus, a sequence-specific and target selective antiviral agent appears to have been identified by the antisense oligonucleotide approach.

3. Opportunities

The previous few examples have been chosen to illustrate that antisense oligonucleotides can be used to identify vulnerable antiviral targets in relatively obscure viral gene products, and in viruses that are not readily responsive to more traditional antiviral screening. But the antisense oligonucleotide studies may not be without difficulties of interpretation, reminding us of the critical importance of proper experimental controls and thoroughness in defining the antisense mechanisms of action. With this caution in mind, the opportunities for antisense oligonucleotides as probes of viral gene function and as a means to identify vulnerable antiviral targets are only limited by the knowledge of RNA target sequences, an ever expanding data base. Armed with new generations of chemically modified oligonucleotide structures and an increasingly sophisticated approach to identifying vulnerable antisense target sequences, the antisense researcher has never been in a better position to bring added novelty to antiviral research.

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