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Mini-review

# Oligonucleotides as antivirals: Dream or realistic perspective?

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Dedicated to Prof. Erik De Clercq on the occasion of reaching the status of Emeritus-Professor at the Katholieke Universiteit Leuven in September 2006.

**Abstract**

Many reports have been published on antiviral activity of synthetic oligonucleotides, targeted to act either by a true antisense effect or via non-sequence specific interactions. This short review will try to evaluate the current status of the field by focusing on the effects as reported for inhibition of either HSV-1, HCMV or HIV-1. Following an introduction with a historical background and a brief discussion on the different types of constructs and mechanisms of action, the therapeutic potential of antisense oligonucleotides as antivirals, as well as possible pitfalls upon their evaluation will be discussed.


*Keywords:* Antisense oligonucleotides; Antiviral activity; HSV-1; HCMV; HIV-1

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## 1. Introduction

Nowadays, antisense oligonucleotides (ASOs) are widely known for their potential inhibitory effects on gene expression in vitro as well as in vivo. From theoretical standpoint, these magic bullets should allow selective inhibition of gene expression by virtue of their sequence-selective hybridization on the complementary target strand (Crooke, 1999; Uhlmann and Peyman, 1990). This way invading aggressors or excessive cell proliferation could be kept under control. Especially in the previous decade, large efforts were undertaken to fur-

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ther evaluate and document these effects, and the biological stability and the hybridization potential of the synthesized constructs was also greatly improved by introducing and studying many different types of backbone modifications (possible suggestions for a review are Manoharan, 1999; Herdewijn, 1999). However, progress has been relatively slow as numerous new challenges rose while trying to implement this entirely new therapeutic strategy into a viable treatment (i.e. synthetic feasibility and scale-up, cost of synthesis, uptake problems, selectivity, immunological effects, etc.). One has to keep in mind also the findings of Krieg et al. (1995) that unmethylated CpG dinucleotides induce murine B cells to proliferate and secrete immunoglobulin in vitro and in vivo. Therefore, careful analysis with control sequences is always mandatory for evaluation of possible antisense effects.

### 1.1. Beginnings and current status of the antisense domain

The earliest studies using antisense oligonucleotides as potential therapeutics aimed to inhibit viral growth, and date already from 1978 by the pioneering work of Zamecnik and Stephenson. They already documented in their seminal papers the use of a tridecamer oligonucleotide as a hybridization competitor to inhibit Rous sarcoma virus replication (Zamecnik and Stephenson, 1978; Stephenson and Zamecnik, 1978). However, the notion that gene expression could be inhibited through the use of exogenous nucleic acids ("hybrid-arrested translation" as they called it), was based on the pioneering work of Paterson et al. (1977), who were the first to use single-stranded DNA to inhibit translation of a complementary RNA, albeit in a cell-free system. A couple of years later, Miller and Ts'o published starting from 1985 several papers describing inhibition of herpes simplex virus type 1 (Miller et al., 1985) by oligodeoxyribonucleoside methylphosphonates in cell lysates and in reticulocytes. The discovery in the late 1980s and 1990s of naturally occurring antisense RNAs interfering with gene regulation (Simons, 1988; Dolnick, 1997) further showed the feasibility of the approach, leading to major developments in the field.

Historically, an interesting paper was published claiming phosphate-methylated oligonucleotides (ONs) to hybridize strongly and sequence specifically to natural DNA and RNA (Buck et al., 1990) and to selectively inhibit both transcription and HIV replication. Both antisense and sense phosphate-methylated eicosamers targeted to the transactivator responsive region or to the primer binding site were reported to completely inhibit viral replication. The manuscript was retracted shortly thereafter, because results could not be reproduced, and further analysis showed the ONs not to contain the expected pure methylated nucleotides (Moody et al., 1990). The results are mentioned here to highlight the care which is needed to analyze all synthetic ONs on their identity and purity (which was certainly not straightforward in those days!). These examples also emphasize the difficulty with which to meticulously carry out reproducible antiviral screening with ASOs. Nevertheless, methylphosphotriester DNA constructs remain an interesting study object from a biochemical point of view (Buck, 2004).

Traditional antiviral research has always been hampered with a shortage of viable targets, as for each virus only a limited number of proteins are available for selective inhibition. Therefore, in inhibition of viral growth, the antisense technique and/or more general genetic interference, offers the possibility of designing highly specific ligands targeted to any of the viral genes. The use of triple-helix forming oligonucleotides (Praseuth et al., 1999) or siRNA molecules (Colbère-Garapin et al., 2005) are promising alternatives to antisense oligonucleotides (ASOs), but due to space limitations they will not be discussed here. Although many oligonucleotides (ONs) reportedly demonstrated activity against various viral targets, no oligonucleotide drug has yet been approved for routine therapeutic use, except for Vitravene$^{TM}$ (fomivirsen, ISIS 2922), which is an ASO composed of 21 phosphorothioate-linked nucleosides (PS-ON) (Azad et al., 1993). It is administered intraocularly and is used for treatment of cytomegalovirus (CMV) retinitis in AIDS patients. However, it needs to be mentioned that this product has only relative importance and is only used in special cases, as CMV retinitis can be better treated with small molecules like HPMPC (Vistide$^{TM}$, recently approved by FDA and previously already available through an investigational new drug (IND) program). Nevertheless, the approval of fomivirsen in 1998 was important to antisense technology as a whole, because it demonstrated that antisense drugs can be effective in the treatment of a local disease. It also showed that antisense drugs can be manufactured for commercial use and can gain marketing approval by the regulatory agencies. However, it has been commented before that fomivirsen may largely act in a non-specific way (Field, 1998; De Clercq, 1999; Scholz et al., 2001).

Clinical trials for majority of ASOs, mostly concentrate and scrutinize the effect on various forms of cancer, multiple sclerosis, high cholesterol or cardiovascular problems, and not on viral inhibition. Recent clinical trial data for these compounds developed for treatment of chronic lymphocytic leukemia (Genasense$^{TM}$, Oblimersen; Genta Inc.; Tauchi et al., 2003) and for ulcerative colitis (Alicaforsen$^{TM}$, ISIS; van Deventer et al., 2004) have shown that parenterally or orally delivered PS-ONs possess specific therapeutic activity at their intended targets, giving further credibility to the field.

### 1.2. Phosphorothioates as main player

Evidently, oligonucleotide degradation by cellular nucleases, especially while using unmodified ONs, is a major concern for the antisense research, and therefore many backbone and sugar modifications have been introduced and investigated. While increasing enzymatic stability, the hybridization capacity and, if possible, the capacity to recruit RNase H catalysis for mRNA degradation should be retained. In general, most modifications of the backbone jeopardize the hybridization potential, because puckering changes for the sugar ring or steric constraints can hamper the normal Watson–Crick base pairing. For obvious reasons, heterocyclic bases therefore generally remain unaltered.

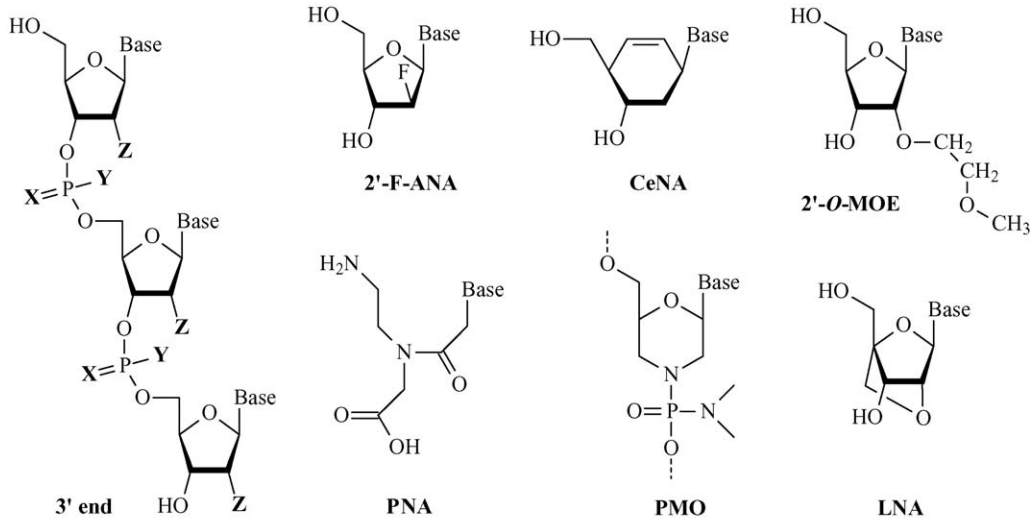
Initially, methylphosphonate modifications – where a non-bridging oxygen is replaced by a methyl moiety – seemed to be a preferred study object. The resulting oligonucleotides are

more hydrophobic (loss of a negative charge) but synthesis is less straightforward. In addition, it yields a mixture of isomers (with each methylphosphonate introduction, a new stereogenic centre is formed), causes a slight drop in hybridization affinity (lowered melting temperature or $T_m$) and also leads to a loss in RNase H activation. Therefore, targeting is basically restricted to essential control sequences corresponding to the start of translation or intron–exon junctions of mRNA sequences. Replacement of a non-bridging oxygen with sulfur otherwise yields phosphorothioates with strongly enhanced stability against enzymatic degradation. The loss in hybridization affinity (0.3–1 °C drop in $T_m$ per modification) is largely compensated for by preservation of the sensitivity to RNase H cleavage of the hybrid between a PS-ON and its RNA complement. Hence, most of the literature on ASOs is covering the use of PS-ONs, and all advanced clinical trials are focused on phosphorothioate compounds as well.

### 1.3. Second and third generation constructs

The second generation of ASOs with larger deviation from the natural structure, has taken longer to develop, and their mechanism is primarily based on steric blockage of the message, as these modifications, as represented by 2′-*O*-methyl derivatives or the strongly hybridizing 2′-*O*-methoxyethyl analogues (Manoharan, 1999), are no longer able to recruit the help of RNase H. There are only a few exceptions such as when using arabinonucleic acids (ANA) or better 2′-deoxy-2′-fluoro-arabinonucleic acids (2′F-ANA), whose mixed hybrids with RNA were shown to be substrates for RNase H (Wilds and Damha, 2000). This is also the case albeit to a lesser extent with cyclohexenyl oligonucleotides (CeNA) which demonstrated reduced RNase H activity with fully modified ONs (Verbeure et al., 2001) (Fig. 1).

Three more constructs with a backbone deviating more considerably from the natural (deoxy)ribose phosphodiester bridges have been thoroughly studied over the last 10 years, namely the peptide nucleic acids (PNA), the locked nucleic acids (LNA) and the phosphorodiamidate morpholino oligomers (PMOs). The PNAs are good structural mimics of DNA with a non-charged achiral polyamide backbone to which the nucleobases are attached. PNA oligomers are able to form very stable duplex structures with Watson–Crick complementary DNA and RNA oligomers (in addition to self-pairing with complementary PNA oligomers). Moreover, they even can bind to targets in duplex DNA by helix invasion. Numerous constructs have been pre-




| | X | Y | Z |
|---|---|---|---|
| deoxyoligonucleotides | O | $O^-$ | H |
| ribooligonucleotides | O | $O^-$ | OH |
| phosphorothioates | O | $S^-$ | H |
| methylphosphonates | O | $CH_3$ | H |
| 2'-*O*-methyl derivatives | O | $O^-$ | $OCH_3$ |
| 2'-*O*-methoxyethyl derivatives (MOE) | O | $O^-$ | O-MOE |

Fig. 1. Structure of different oligonucleotides. A trimer is depicted to delineate the internucleoside linkages and to show the main substitution possibilities at the non-bridging oxygens (*X*, *Y*) of the phosphodiester linkage. In addition, the main nucleoside modifications as mentioned in this review (2′-*O*-methoxyethyl (MOE) modified nucleosides, 2′-fluorinated arabinonucleosides (2′-F-ANA), cyclohexenyl nucleic acids (CeNA), peptide nucleic acids (PNA), phosphorodiamidate morpholino oligomers (PMO) and the locked nucleic acids (LNA)) are depicted.

pared in search for useful antisense effects (Pooga et al., 2001; Nielsen, 2004), and some of the antiviral findings will be discussed here as well.

PMOs are DNA-like oligomers with six-membered morpholine rings substituting for the deoxyribose, and with neutral phosphorodiamidate intersubunit linkages. Where the PNAs have a solubility disadvantage, the PMOs are claimed to be more easily water-soluble and they too base pair with RNA in a sequence-specific manner without inducing RNase H mediated degradation. However, the advantages and the potential of an RNase H-independent mechanism of action have been outlined before (Summerton, 1999). Very recently, the systemic delivery and in vivo efficacy of PMO constructs have been clearly shown as well (albeit in a different field) with the expression of functional levels of dystrophin in the muscles of dystrophic mice in an effort to find a cure for Duchenne muscular dystrophy (Alter et al., 2006). The antiviral potential was highlighted as well by blockage of either the viral translation or the viral RNA synthesis of Dengue virus, depending on the chosen sequence (Holden et al., 2006). As this review restricts itself however to HIV, HSV-1 and CMV, these results will not be further analyzed.

The LNAs finally, are one of the most studied constructs over the last year. These are ribonucleotides containing a bridging extra methylene carbon between the 2′- and 4′-positions of the ribose ring, and they were first described independently by the groups of Wengel and Imanishi (Koshkin et al., 1998; Obika et al., 1998). The supplementary ring imposes a strong constraint on the analogue and preorganizes the nucleosides in a 2′-exo conformation affording strongly increased affinity for RNA oligomers. Like other 2′ modifications, the supplementary linkage eliminates the possibility for recognition of the hybrid LNA–RNA duplex by RNase H, followed by mRNA target cleavage. However, the incorporation into oligonucleotides via standard DNA synthesis protocols, allows the design of gapmers with central DNA portions, restoring the possibility for recruitment of the cleaving enzyme, as reviewed by Kurreck et al. (2002).

As outlined before, most of the antisense constructs are unable to recruit RNase H and therefore are limited to steric inhibition of translation. However, one possibility with analogues which are assembled via standard phosphoramidite chemistry, is the creation of gapmers, as already mentioned. Such a strategy is precluded with PNA or PMO constructs. However, this limitation was cleverly overcome through recruitment of 2–5A-dependent RNase L. Hereto, 5′-phosphorylated-2′,5′-linked oligoadenylate was conjugated to an antisense PNA oligomer, effecting selective and specific cleavage of the intended RNA target, both in cell-free systems and in intact cells (Verheijen et al., 1999). The same strategy was recently used to endow MPO antisense constructs with catalytic activity (Zhou et al., 2005).

## 2. Current status on viral inhibition with ONs

The number of reports on inhibition of viral cytopathic effects is countless, and therefore this short review will be restricted to results obtained with three different viruses, HSV-1, HCMV and HIV-1, as examples of possible interference strategies using ASOs. Nevertheless, it is recognized that many valuable contributions have been made to the antisense antiviral strategy by studies involving other viruses such as influenza, RSV, hepatitis B and hepatitis C and many others.

### 2.1. Herpes simplex virus type 1 (HSV-1)

The HSV-1 genome contains about 152,000 basepairs and most of the genes are catalogued into immediate-early, early and late genes, which expression is tightly and coordinately organized. Oligonucleotides with a variety of chemical modifications have been evaluated against different sites of the HSV-1 genome, and demonstrated differing success.

Methylphosphonates do not recruit RNase H, and therefore are targeted to key control sequences of regulatory genes like splice sites of mRNAs. In 1986, the research group of Aurelian used the acceptor splice junction of HSV-1 immediate-early (IE) pre-mRNAs 4 and 5 (Smith et al., 1986) to inhibit virus growth. Treatment of HSV-1-infected cells with an octamer methylphosphonate ON, before or at the time of infection was reported to cause a dose-dependent inhibition in virus replication with an $IC_{50}$ of 25 μM. A minimal reduction in protein synthesis and growth rates of uninfected cells was also demonstrated. Longer sequences (dodecamers) and psoralen derivatized ONs with potential of covalent binding to the target upon irradiation further decreased the dose required for selective inhibition (Kulka et al., 1989). The splice donor site for IE pre-mRNAs 4 and 5 and the translation initiation site of IE4 mRNA likewise could be targeted (Kulka et al., 1993). Combination of ribonucleoside 2′-*O*-methyl modifications with the methylphosphonate internucleoside connection afforded a five times lower $IC_{50}$ compared to the parent methylphosphonate sequence (Kean et al., 1995).

Poddevin et al. (1994) similarly used the IE4 pre-mRNA as a target for a series of regular phosphodiester (PO) ONs, constituting of a 5′-dodecameric sequence, which was complementary to the acceptor splice junction of HSV-1, flanked at the 3′-end by octameric sequences adopting hairpin-like structures. These minihairpins at the 3′-end or so-called pseudo-cyclic ONs afforded substantial protection to nucleases and conferred a 10-fold increase in activity compared to the control dodecamers. The use of minihairpins at the 3′-end for enzymatic stabilization hereafter has been studied and confirmed many times (i.e. Jiang et al., 1999).

In attempts to define and identify the optimal targets for antisense inhibition, Peyman et al. (1995) approached the problem from a different angle. Hereto, a total of 100 ONs designed to bind different target genes of HSV-1 and different locations within those genes were screened for antiviral activity. Minimal stabilization of the ONs was aimed to have only two phosphorothioate linkages at both the 5′- and the 3′-end, thus avoiding undesired sequence-independent effects. The most active compound showing 50% protection from the cytopathic effect caused by the virus at 9 μM, was targeted against the translation start region of the IE110 mRNA and was the only sequence demonstrating substantial effect. Introduction of a third thioate linkage further improved the antiviral activity by three-fold.

However, all sequences turned active ($IC_{50}$ from 1 to 10 μM) when fully thioated, in agreement with earlier observations (loss of sequence specificity). A follow-up paper showed that the anti-HSV-1 efficacy of PS-ONs targeted to IE110 mRNA was determined as a function of their chemical structure. A minimal protection strategy, consisting of end-capping and pyrimidine protection, proved to be particularly useful as it yielded nuclease-resistant ONs and minimized non-sequence-specific effects (Peyman et al., 1997).

### 2.2. Human cytomegalovirus (HCMV)

As for the treatment of herpes infections, control of HCMV cytopathology with AS-ONs has been mainly focusing on inhibition of HCMV IE gene products. In all research efforts phosphorothioate oligonucleotides complementary to mRNA of the HCMV DNA polymerase gene or to RNA transcripts of the major immediate-early regions 1 and 2 (IE1 and IE2) of HCMV were used (Azad et al., 1993). Oligonucleotides complementary to RNA of the IE2 region exhibited the most potent antiviral activity with a particular sequence, ISIS 2922 (fomivirsen), which was 30-fold more potent than the nucleoside analog ganciclovir, with an $IC_{50}$ of 0.37 μM. Fomivirsen furthermore reduced IE protein synthesis in HCMV-infected cells in a dose-dependent manner correlating with antiviral activity. A control ON showed no inhibition of virus production at concentrations 10 times higher but mismatches in ISIS 2922 which reduced the hybridization, did not affect the antiviral activity.

Follow-up reports confirmed the sequence specific inhibition of protein expression at nanomolar concentrations using U373 cells transformed with cDNA encoding for the CMV IE 55-kDa (IE55) protein. Inhibition of the IE55 expression correlated with a reduction of the mRNA levels (Anderson et al., 1996). The authors also commented that both antisense and non-antisense, as well as sequence-dependent and sequence-independent mechanisms contribute to the antiviral activity of ISIS 2922. Thus, a treatment of human fibroblast cells with ISIS 2922 resulted not only in reduction of IE2 levels but also in sequence-independent reduction of the IE1 levels. As explained in Section 1, other authors have cautioned for such non-specific effects as well. Moreover, a HCMV mutant has been isolated with a 10-fold resistance to the ASO fomivirsen, and exhibited cross-resistance to a modified derivative of fomivirsen with identical base sequence, but showed little or no resistance to an oligonucleotide with an unrelated sequence. No changes in the mutant's DNA corresponding to the fomivirsen target sequence were found, thus confirming the sequence non-specific effects (Mulamba et al., 1998).

On the other hand, the group of Pari and his associates used ASOs to identify sensitive antiviral targets in HCMV, and identified a PS-ON complementary to the intron–exon boundary of the HCMV genes UL36 and UL37 (UL36ANTI). Based on their antisense studies, these genes were proven essential for viral replication inhibiting HCMV DNA replication at a concentration of 0.08 μM (Pari et al., 1995). The UL36 (and UL37) genes encode both for a transactivator upregulating viral and cellular transcription. The ability of these PS-ONs to prevent DNA replication was not due to inhibition of virus adsorption, since Northern blots confirmed that this ASO specifically reduced UL36 mRNA in treated cells to undetectable levels (Smith and Pari, 1995). GEM132, a chemically modified PS-ON Version of UL36ANTI was further pursued by Hybridon (now Idera Pharmaceuticals, WO9733992) but, apparently, was abandoned. The ON was metabolically stabilized by introducing three 2′-*O*-methyl nucleotides at the 3′-end and four 2′-*O*-methyl nucleotides at the 5′-end. In addition, the oligo possessed a cholesteryl moiety linked to the 3′-end, which resulted in superior antiviral potency, melting temperature and nuclease resistance, and displayed a more profound nuclear distribution in HCMV-infected human fibroblasts (Zhang et al., 1997).

In general, it has been reported that inhibition or modulation of HCMV IE expression and/or function is an important goal in treatment of HCMV-associated diseases. In the view of their transactivating functions, regulation of IE1 and IE2 gene expression is the key to either latency or to active replication. Likewise, IE1 and IE2 have the ability to transactivate cellular genes and to induce immunopathological effects in the absence of replicating virus (Scholz et al., 2001). In absence of small molecules interfering with the different HCMV IE-associated molecular interactions, largely unidentified so far, ASOs and analogous strategies for controlling gene expression seem the only option at the moment.

### 2.3. Human immunodeficiency virus-1 (HIV-1)

Inhibition of HIV-1 by different PS-ONs has been reported numerous times. While there might be some true antisense effects, most of these compounds most probably act via a non-sequence-specific mechanism as inhibitors of attachment, or as sequence-specific aptamers. Binding of the v3 loop of gp120 and preventing viral interaction with CD4 and hence attachment to the host cell is a well described phenomenon.

#### 2.3.1. The first antiviral findings

A first report on the use of phosphorothioates (PS-ONs) described potent antiviral activity for both complementary as well as non-complementary sequences, including some homo-oligomers in a de novo infection in ATH-8 by HIV. Indeed, a 28-mer phosphorothioate oligodeoxycytidine (S-$dC_{28}$) exhibited potent antiviral activity and inhibited viral DNA synthesis. However, no inhibition could be detected in chronically infected T cells. The authors therefore presumed that the mechanism of action is complex (Matsukura et al., 1987). The potent non-specific inhibitory effect of PS-ONs on de novo HIV infections was confirmed by the same group, by evaluation of a whole series of PS oligomers with mixed-base sequences. For all of them, a dose-dependent cytoprotective effect was established at an oligomer concentration of about 1–2 μM (Stein et al., 1989). On the other hand, a sequence-specific suppression of viral expression in T cells, chronically infected with human immunodeficiency virus 1 (HIV-1), could be established using a 28-mer PS-ASO by a translation arrest of the regulatory gene rev (anti-$rev_{AUG}$; Matsukura et al., 1989). Later on, the same

group was able to demonstrate that the sequence non-specific effects of S-$dC_{28}$ were due to a competing interaction with gp120 binding to CD4. In addition, S-$dC_{28}$ strongly inhibited syncytium formation resulting from HIV-1-induced cell fusion. The cytoprotective effects therefore were at least in part due to interference with the binding of HIV-1 to the target cells (Stein et al., 1991).

### 2.3.2. *Different mechanisms of action*

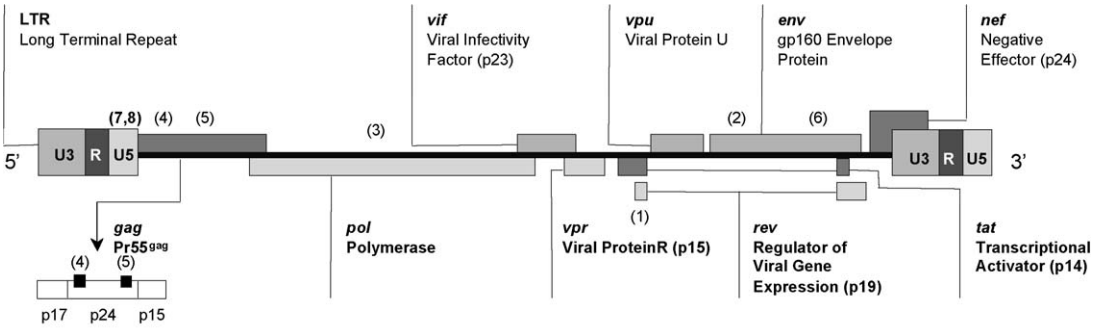
During the last two decades, it became known that the v3 loop of gp120 in HIV-1 strains is positively charged and its interaction with sulfated polysaccharides and other polyanions was studied. Further work of Stein et al. (1993) determined with the help of monoclonal antibodies that S-$dC_{28}$ appeared to bind specifically to this v3 loop. An increase in gp120 binding was seen for PS oligos with increase of their length with a maximum effect attained for the 18-mer.

The use of immunoliposomes containing either PO-ONs or PS-ONs permitted intracellular delivery and allowed distinction between inhibition of viral entry and intracellular ASO effects on viral RNA. Zelphati et al. (1994) were able, therefore, to define four general mechanisms of antiviral activity: (i) interference with virus-mediated cell fusion by free PS-ONs of any sequence; (ii) interference with reverse transcription by PS-ONs in a sequence non-specific manner; (iii) interference with the viral reverse transcription process by PO-ONs in a sequence-specific manner; (iv) sequence-specific RNase H cleavage of viral mRNA sequences by PS-ONs. This multiple inhibitory mechanism of action was later largely confirmed by evaluation of the PS-ON GEM 91, an anti-*gag* sequence. Sequence-dependent inhibition of virus entry/reverse transcription and a reduction in steady-state viral RNA levels were observed. In addition, sequence-independent inhibition of virion binding to cells and inhibition of virus production by chronically infected cells was noticed by the group of Yamaguchi et al. (1997) with this *gag* PS-ON sequence (Fig. 2).

### 2.3.3. *Efforts to reduce non-specific effects*

Since PS-ONs have reduced affinity for their complementary target, the antiviral activity of sequence-selective PS-ONs would be strongly increased, if a modification that could raise the hybridization potential is introduced. This goal was very nicely accomplished using C-5 propyne modifications on the pyrimidine bases, which via their enhanced stacking effects considerably raise the $T_m$ of mixed hybrids. Indeed, PS-ONs containing the C-5 propynylated pyrimidine analogs proved to be potent antisense inhibitors of gene expression, showing gene-specific antisense inhibition occurring at nanomolar concentrations of ONs (Wagner et al., 1993). A 15-mer C-5 propyne modified PS-ON antisense to *rev* also proved five-fold more effective in providing viral inhibition as compared to the previously described PS-ON 28-mer developed by Matsukura (anti-$rev_{AUG}$; Lund et al., 1995). By virtue of the shorter sequence and the presence of the lower total number of phosphorothioate modifications, the 15-mer product should be more specific and should have less unwanted side-effects.

Anazodo et al. (1995) also reported sequence-specific and dose-dependent inhibition of HIV-1 viral protein synthesis and significant inhibition at the mRNA level by an ASO construct GPI2A, that was directed against a non-regulatory but well conserved *gag* mRNA region of the HIV-1 genome (spanning from +1189 to +1208). Here, unspecific effects were largely avoided using a partially phosphorothioated oligo with only 7 PS linkages out of 19. Similar to the group of Zelphati et al. (1994), Selvam et al. (1996) used CD4 monoclonal-antibody-targeted liposomes containing anti-rev PS ONs to obtain sequence-specific suppression of HIV-1 replication. The potential efficacy of immunoliposomes to inhibit HIV replication was therefore




oligonucleotide targets most studied:
(1) Specific anti-$rev_{AUG}$ sequence (28-mer PS-ON + 15-mer propyne analog)
(2) Non-sequence specific interactions with v3 loop + aptameric G-tetrad targetting of the v3 loop
(3) Non-sequence specific interactions with RT
(4) Specific anti-*gag* sequence GEM91
(5) GPI2A anti-*gag* sequence of Anazode
(6) Non-sequence specific targetting of the heptadrepeat region of gp41
(7) Primer binding site, target for effective PNA constructs conjugated to membrane transducing peptides
Transactivation response element (TAR), encompassing +19 to + 42 relative to the transcriptional start site.
The complete TAR spans bases 453 to 614.

Fig. 2. Structure of the proviral genome with location of the main proteins and indication of the antisense targets discussed in this review.

further established. The choice of the oligo was guided by trial and error from a series of ONs.

*2.3.4. Antisense efforts with PNA constructs*

As outlined already, unspecific effects in many cases are due to hydrophobic interactions of phosphorothioates with a variety of proteins. For this and other reasons PS-ONs can cause multiple non-antisense effects. Different backbones, and especially non-charged backbones to a large extent prevent such constructs from binding to proteins, which normally recognize polyanions. Hence, many antiviral studies successfully have used PNA or LNA analogues with higher specific activity compared to PS-ONs. One of the targets among others for control of HIV-1 replication has been control of gene expression by modulating the transcriptional activation level. The latter is regulated via interaction of the transactivator protein Tat, with the transactivation response region TAR. The latter is endowed with a highly stable stem-loop structure with a bulge. Various TAR decoys have been studied to sequester Tat, but shielding the TAR region with different oligonucleotide antisense constructs interfering with either the loop or the bulge region, likewise inhibits HIV-1 reverse transcription (Boulme et al., 1998). While shorter PNAs of 12 or 13 bases efficiently could block in vitro reverse transcription, a 16-mer PNA was able to almost completely inhibit HIV-1 production (Kaushik et al., 2002).

Another interesting target for inhibition of replication can be found in the primer binding site (PBS). Cellular $tRNA^{Lys3}$ is packaged into HIV-1 virion particles, and is utilized as a primer for initiation of transcription. Its 3′-terminal 18 nucleotides are complementary to part of the long terminal repeat (LTR) sequence near the 5′ non-translated region of the viral genome. PNA molecules targeted to the PBS site prevent $tRNA^{Lys3}$ priming and in addition are able to invade the preformed duplex region of the $tRNA^{Lys3}$-viral RNA complex in in vitro studies. In a CEM cell line infected with luciferase reporter pseudotyped HIV-1 virions, a dramatic reduction of HIV-1 replication was noted by nearly 99% (Kaushik and Pandey, 2002).

While being electrostatically neutral is advantageous for abrogating most unspecific interactions with proteins, in contrast this generates serious problems concerning solubility and cellular uptake. The cellular delivery problem of PNA has been adequately reviewed by Koppelhus and Nielsen (2003). More recently, various membrane transducing peptides as delivery vectors have been conjugated via disulfide bonds to the previously mentioned anti-TAR PNA 16-mer. These conjugates provided effective uptake and displayed antiviral efficacy in CEM cells at around 30 nM (Chaubey et al., 2005; Tripathi et al., 2005). The exact uptake mechanism remains unknown, but it was shown to be neither receptor-dependent, nor to occur via endocytosis. In addition, the conjugates have potential anti-HIV virucidal activity, inactivating virions before they can infect healthy cells, and therefore having potential as prophylactic agents.

*2.3.5. Aptamer approaches*

Having noticed the specific interaction of S-$dC_{28}$ with the v3 loop gp120 found by Stein et al. (1993), and following up on the recent findings that synthetic ONs could be used as ligands to modulate the activities of proteins in the so-called aptamer approach, Wyatt et al. (1994) used a combinatorial technique to screen a library of ONs for anti-HIV activity in a cell culture. The starting mixture contained all possible octanucleotide sequences as stable PS analogs and the PS-oligodeoxynucleotide *ttggggtt* was identified as a strong inhibitor of HIV infection in vitro. It was found that this analog forms a parallel-stranded tetrameric guanosine-quartet structure, binds to the HIV envelope protein gp120 at the v3 loop, and inhibits both cell-to-cell and virus-to-cell infection, which is analogous to S-$dC_{28}$. Formation of the tetramer structure was a prerequisite for activity. Shortly thereafter, Rando et al. (1995) reported that ONs with natural phosphodiester (PO) linkages containing only deoxyguanosine and thymidine were equally apt to inhibit HIV-1 in a cell culture. The most potent analog was a 17-mer guanine-octet-forming ON with sequence 5′-*gtggtgggtgggtgggt*. According to NMR, the oligo appears to form a structure in which two stacked guanine tetrads are connected by three two-base loops. The formation of these guanine tetrads is necessary to achieve maximum antiviral activity. Only for reasons of biological stability the same 17-mer sequence was then assembled with a single PS linkage at both the 5′- and the 3′-ends (later named AR177 or zintevir; Ojwang et al., 1995), for which no toxicity could be observed. This product prevented viral adsorption and, in addition, proved to be a strong HIV-1 integrase inhibitor. The research group of De Clercq studied the compound in details and was able to generate mutants in a cell culture. DNA sequence analysis revealed mutations in the envelope glycoprotein gp120 but not in the integrase gene. Therefore, it was clearly established that gp120 is the main target for the anti-HIV-1 activity of all G-quartets (Cherepanov et al., 1997).

*2.3.6. Yet another mechanism of action*

The options for preventing virus attachment by ON analogues also seem to expand continuously. In analogy with S-$dC_{28}$, the anti-reverse transcriptase activity of a long homo-oligomer (35-mer) of 4-thio-deoxyuridylate [$(s^4dU)_{35}$] as well as its potent binding to CD4 receptors preventing virus attachment with an $IC_{50}$ of about 0.002 μg/ml was recently reported (Horváth et al., 2005). Finally, Vaillant et al. (2006) have found that PS-ONs are potent inhibitors of membrane fusion through interactions with the N-terminal heptad repeat region of gp41. This mechanism of action is analogous to the one for certain peptides, for instance by enfuvirtide. Using monoclonal antibodies, it was proved that especially very long hydrophobic PS-ONs (above 30-mers) have the ability to block gp41 six-helix bundle formation and consequential fusion as well as the attachment inhibition similar to shorter PS-ONs. Thus, this new mechanism of action for amphipathic oligos could have new therapeutic implications.

## 3. Conclusion

Having reviewed the different options for inhibition of viral cytopathicity on three different viruses, HSV-1, HCMV and HIV-1, with application of oligonucleotide derivatives, it must be clear that from a therapeutical standpoint several possibili-

ties to combat these viral infections are open. Sequences and modifications have to be carefully studied and chosen, since true sequence-specific antisense effects as well as sequence-unspecific hydrophobic effects can be obtained. In addition, inhibition via a sequence-specific or aptameric effect is possible targeting either processes related to adsorption and fusion or other specific viral proteins necessary for viral multiplication. While most advanced studies at the moment are ongoing with phosphorothioate sequences, the second generation ONs with backbone modifications takes longer to develop, and aptameric sequences with defined three-dimensional structure to interfere with different viral processes will be making their way as well. However, progress remains slow and the development cost altogether with different biological problems such as mentioned here like cellular uptake and long-term safety, will certainly keep oligonucleotides in a disadvantageous position as antivirals of the future as compared to small molecules. In addition, this review pointed out that even though ASOs are probably not very useful for acute viral infections, they can make their way for the chronic treatment, albeit the cost even with further optimization of the synthetic protocols remaining relatively high.

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