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Biochimica et Biophysica Acta 1758 (2006) 290 – 300
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
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Review

Targeting the HIV-1 RNA leader sequence with synthetic oligonucleotides and siRNA: Chemistry and cell delivery

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Received 12 August 2005; received in revised form 27 October 2005; accepted 28 October 2005
Available online 18 November 2005

Abstract

New candidates for development as potential drugs or virucides against HIV-1 infection and AIDS continue to be needed. The HIV-1 RNA leader sequence has many essential functional sites for virus replication and regulation that includes several highly conserved sequences. The review describes the historical context of targeting the HIV-1 RNA leader sequence with antisense phosphorothioate oligonucleotides, such as GEM 91, and goes on to describe modern approaches to targeting this region with steric blocking oligonucleotide analogues having newer and more advantageous chemistries, as well as recent studies on siRNA, towards the attainment of antiviral activity. Recent attempts to obtain improved cell delivery are highlighted, including exciting new developments in the use of peptide conjugates of peptide nucleic acid (PNA) as potential virucides.

Keywords: Oligonucleotide; siRNA; HIV-1; AIDS; Virucide; Antiviral agent

Contents

1. Introduction

HIV-1 infection continues to spread at an alarming rate. In 2004, some 5 million new cases of infection worldwide were reported (http://www.unaids.org/wad2004/EPIupdate2004_html_en/epi04_02_en.htm). This demonstrates the current

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doi:10.1016/j.bbamem.2005.10.013

inability to control viral spread. The lack of a suitable and widely applicable vaccine or other effective control measure means that the only weapons available to combat HIV-1 infection and AIDS are a combination of anti-retroviral agents (HAART, highly active anti-retroviral therapy) or virucidal agents that can be used in topical formulation for use during sexual contact, which is the main route of new infections. Unfortunately, no topical virucides have yet been approved for clinical use and sadly HAART is still only able to reach a small fraction of those that are infected. Nevertheless, new anti-retroviral agents continue to emerge [1,2]. There is a pressing need to find new anti-HIV-1 agents and combinations that are more effective, better tolerated, cheaper, and which may also have virucidal activity suitable for worldwide use.

Intense efforts are being made to understand the complex steps in the HIV-1 replication cycle. Almost all of these steps and gene products represent potential targets for the development of inhibitors, yet most clinically available drugs are small molecules aimed at only a very limited number of such targets, predominantly the viral proteins reverse transcriptase (RT) and protease. There are numerous other molecular strategies that are being investigated to interfere with virus replication (reviewed in [3]) many of which are aimed at gene therapy (reviewed in [4]). These include HIV-1 RNA targeting strategies such as antisense RNA, ribozymes, decoy RNAs (mimics of known RNA structures), aptamers (selected sequences that recognise RNA structures) and small interfering RNA (siRNA), as well as protein-based techniques such as transdominant negative proteins, nucleases, single-chain and whole antibodies. The aim is to deliver such reagents to haematopoietic progenitor cells to protect their differentiated progeny from HIV-1, to render HIV-1-susceptible cells resistant to HIV-1 infection, to inhibit HIV-1 replication in cells and in discrete organ target sites, or to immunize against HIV-1 for treatment or prophylaxis. An advantage of gene therapy is that there is an inherent and consistent antiviral protection maintained against virus challenge, whereas with pharmacotherapy a patient must take anti-retroviral drugs regularly to maintain sufficient therapeutic action. However, gene therapy approaches are unlikely to be applicable to a large enough number of patients worldwide to meet the perceived needs and may have safety issues.

Many of the RNA sequences that are considered targets in gene therapy and the entities that interact with them (proteins and other RNAs) are also potential targets for anti-HIV-1 drug development. The genome of HIV-1 is small (about 9000 nucleotides) [5] and hence molecular function is packed densely into the RNA sequence, with functionality sometimes overlapping or coincident. Although much of the RNA sequence is able to mutate rapidly (because of the infidelity of the HIV-1 RT during replication) and thereby gain resistance to drug action, a few highly functional regions have short sequences that are well conserved between viral isolates and rarely or never mutated. The HIV-1 RNA leader sequence is particularly rich in such functional elements.

Synthetic oligonucleotides and their analogues complementary to HIV-1 RNA have great potential for the sequence-specific targeting of functional RNA elements as probes for understanding the molecular biology of HIV-1 and for development of possible antiviral or virucidal agents. We review here the various oligonucleotide-based strategies that are currently under study and point to some recent exciting results in targeting the HIV-1 RNA leader sequence where antiviral and virucidal activities within cell culture assays have been attained. We also discuss some of the challenges of developing oligonucleotides as potential anti-HIV-1 therapeutics, particularly in obtaining efficient cell delivery. A more general review on antiviral activities of oligonucleotides has been published recently [6].

2. Targets in the HIV-1 RNA leader sequence

There are three classes of RNA produced during the expression of the HIV-1 proviral genome. The shortest class (~2 kb) are doubly spliced transcripts that are produced early in gene expression coding for the regulatory genes *tat*, *rev* and *nef*. Later in expression, a singly spliced transcript (~4 kb) encoding the genes for structural proteins *env*, *vif*, *vpr* and *vpu* is seen. Finally, full-length unspliced RNA (~9 kb) coding for *gag*/*pol*, leading to expression of the viral enzymes is produced. This acts also as the genomic RNA that is dimerized and incorporated into the HIV-1 virion. All of these RNAs contain the first 290 residues of RNA, up to the first splice donor (SD) site, which includes several functional sites that are important at distinctive stages of the viral replication cycle. Full-length genomic and partially spliced RNAs contain also additional essential sites in the next 50-nucleotide stretch including the initiator AUG of *gag*.

A model of secondary structure of the RNA leader sequence, based on phylogenetic and in vitro conformational studies, shows that the functional sites are commonly, but not exclusively, located within apical loop regions [7–9] (Fig. 1). The region of the HIV-1 genome between the Poly(A) loop and the primer binding site (PBS), known as U5 is thought to form a duplex with the AUG region [9]. Although the secondary structures of the first two stem–loops, the *trans*-activation responsive element TAR and the Poly(A) site, are well established, there are alternative folding proposals for other parts of the RNA leader sequence, for example the region including the dimerization initiation site (DIS), SD and packaging signals (psi, ψ) [10]. Models that involve longer distance interactions have also been presented, such as an alternative helix between the poly(A) site and the DIS sequences [11]. Of particular importance is that RNA structures alter as a result of interaction with proteins (e.g., the interaction of psi with the Gag protein [10]) or with other RNA (e.g., the PBS region with $tRNA^{Lys3}$ that occurs during initiation of reverse transcription [12]).

2.1. The trans-activation responsive element TAR

The most well-studied region of the HIV-1 leader RNA is the extreme 5′-end 59-residue stem–loop, the *trans*-activation responsive element TAR (Fig. 2). The apical part of TAR acts

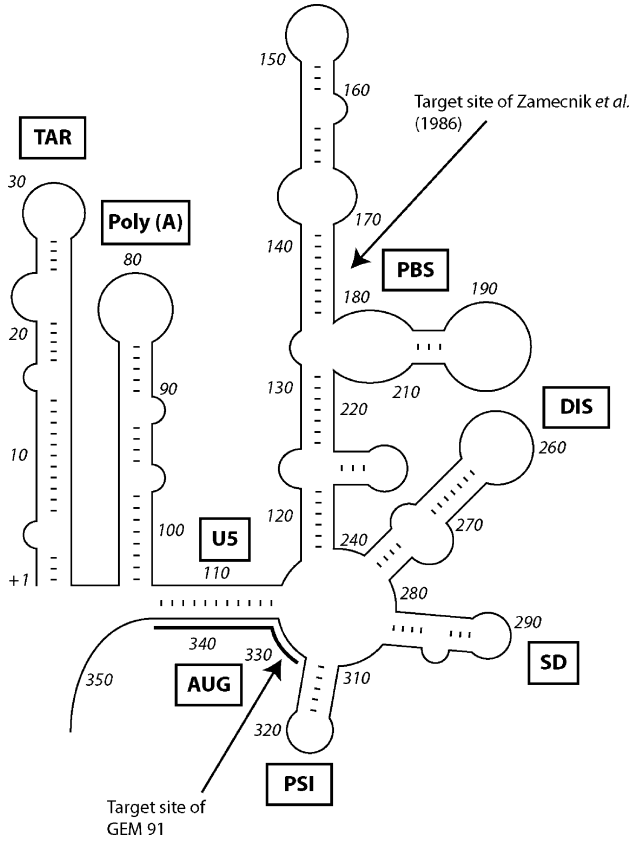

Fig. 1. Secondary structure schematic model of the HIV-1 leader sequence showing the regions of functionality and the sites of interaction of an early anti-HIV-1 oligonucleotide [30] and the clinically investigated phosphorothioate oligonucleotide GEM 91 [36].

as a binding site for the HIV-1 *trans*-activator protein Tat and some host cellular factors that together form a complex that triggers a massive boost in transcriptional elongation by stabilization of the transcription complex as it passes through the TAR region [13,14]. Both Tat and TAR are also thought to play a role in initiation of reverse transcription [15]. The apical loop and stem is highly conserved between viral isolates. Since Tat *trans*-activation is essential for viral replication, inhibitors of Tat or TAR have been sought for many years, Sadly, despite the numerous small molecule inhibitors that have been discovered (reviewed in [16]), no clinical anti-HIV-1 candidates have emerged. Many such inhibitors prove not to have TAR-binding specificity in cells or their antiviral activity is subsequently found not to correlate with TAR binding or Tat-dependent *trans*-activation inhibition. However, the TAR stem–loop has been an important target for oligonucleotide-based approaches since the early 1990s where it has been found that a high level of target binding specificity can be obtained [17].

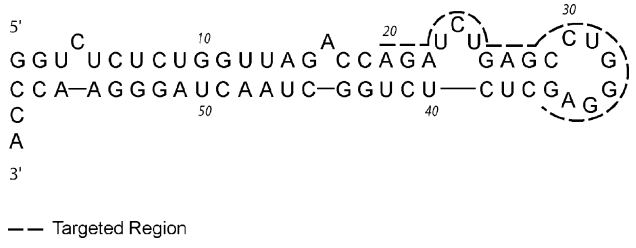

Fig. 2. Sequence and secondary structure model of the HIV-1 TAR element (subtype B, LAI strain) showing the region targeted by synthetic oligonucleotides.

2.2. The primer-binding and dimerization initiation sites

Another much studied region is the initiation site for reverse transcription. Following the essential enabling interaction between the 3′-end of the host $tRNA^{Lys3}$ and the PBS, there are rearrangements of the RNA structure involving additional interactions with other nearby regions (pre-PBS) such that the conformation of the RNA is radically altered [12] (Fig. 3). Not surprisingly, because of the 18-nucleotide PBS–tRNA interaction, the PBS sequence is highly conserved and thus has been an obvious target for oligonucleotides for the blocking of the initiation of reverse transcription, since the early 1990s [18] and as an anti-HIV-1 agent as far back as 1989 [19].

The DIS was identified as a stem–loop structure that is self-complementary and forms a loop–loop 'kissing complex' as a first step in the dimerization mechanism [20,21] (Fig. 4). Oligonucleotides in both antisense and sense forms were shown to form complexes with the DIS site, thus opening up alternative strategies for inhibition [22]. There is strong

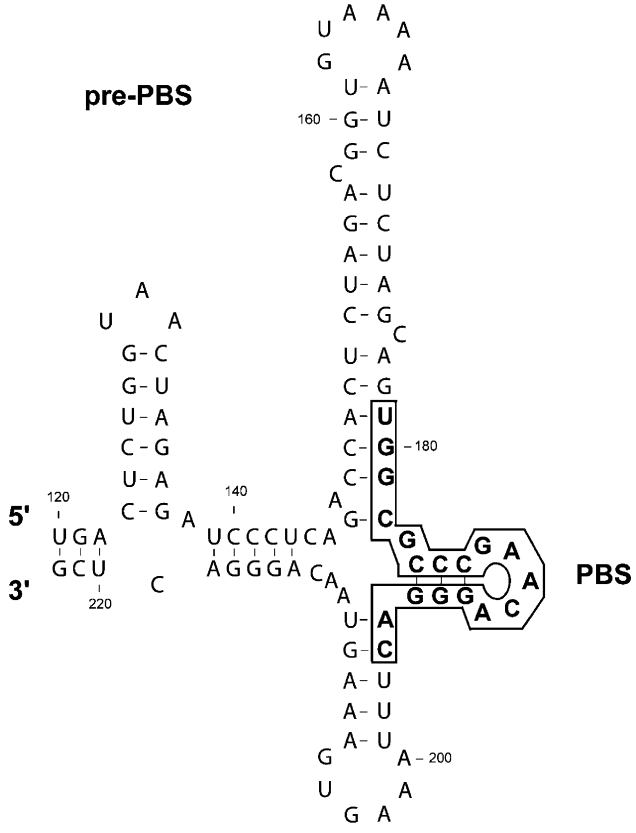

Fig. 3. Sequence and secondary structure model of the pre-PBS, PBS and part of the U5 region in the folded conformation believed to be present after formation of the RNA–$tRNA^{Lys3}$ reverse transcription initiation complex [11]. The 18-nucleotide sequence complementary to the 3′-end of the tRNA and highly conserved between viral isolates is boxed.

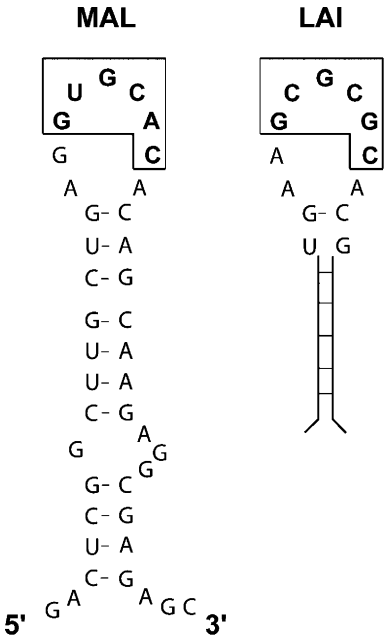

Fig. 4. Sequence and secondary structure model of the dimerization initiation site DIS (subtype A, MAL strain and subtype B, LAI strain).

selective pressure for the virus to maintain the dimeric nature of genomic RNA. However, the DIS loop sequence varies between viral isolates and thus, for example, homologous oligonucleotides display vastly different dimerization inhibition efficiencies between two strains that differ only modestly [23]. Nevertheless, oligonucleotides targeting this site have been shown to inhibit HIV-1 replication [24].

2.3. The major HIV-1 packaging signal

The major HIV-1 packaging signal ψ has been shown to fold in vitro into an extended stem–loop structure that contains three arms (Fig. 5). SL1 is the DIS stem–loop, SL2 stem–loop contains the major splice donor and SL3 is the major site for Gag interaction. Gag binding leads to an alteration of the RNA structure [10]. Use of high affinity oligonucleotides has helped to map Gag binding to the SL3 loop (Brown, D., Gait, M.J. and Lever, A., manuscript submitted). In addition, such oligonucleotides have been shown to elicit antiviral activity [25]. The overlapping nature of functionality in the HIV-1 leader is exemplified well in that the HIV-1 Rev protein, which binds primarily to an RNA structure known as the Rev Responsive Element within the *env* gene, also binds to a purine-rich loop in the SL1 region [26].

2.4. Mapping of accessible sites on the HIV-1 RNA leader

Throughout the viral cycle, it is very likely that RNA conformation alters significantly and thus accessibility to a particular RNA site would be expected to vary depending on the virus stage. Particularly little is known about the RNA structure in HIV-1 virions [27], where the RNA is both dimerized and complexed with other proteins such as reverse transcriptase, integrase and nucleocapsid as well as other structural proteins. Furthermore, there may be sites in the virion RNA that are occluded because of interactions, such as the PBS with tRNA and the DIS because of RNA dimerization. Therefore, in practice, the availability of any particular RNA

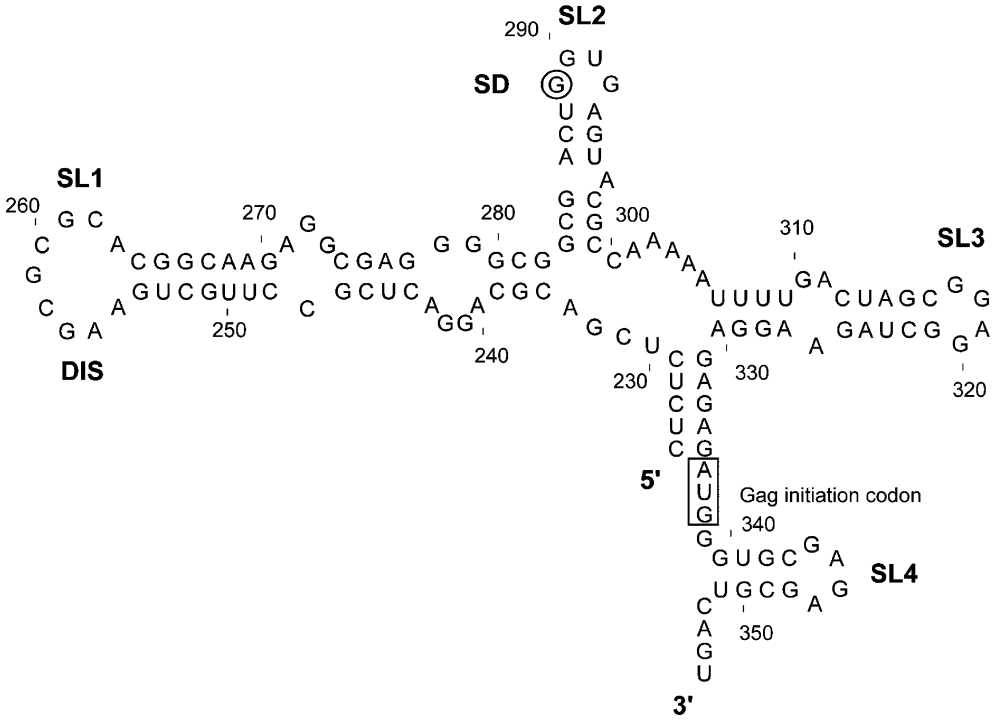

Fig. 5. Sequence and secondary structural model according to Zeffman et al. [9] of the major HIV-1 packaging signal region including SLI, the dimerization initiation site (DIS), SL2 (the splice donor, SD), SL3 (the psi region) that is targeted by oligonucleotides designed to block Gag binding [25], and a possible stem–loop at the AUG region (when not base-paired to the U5 region).

site for interaction with an oligonucleotide or siRNA reagent during HIV-1 infection cannot be predicted with reliability and must be tested by experiment.

An interesting approach to identifying accessible target sites for antisense oligonucleotides in highly structured RNA such as the HIV-1 leader has been developed, whereby a 20-mer library of RNA fragments generated from the HIV-1 genome is used as a probe for RNA binding and inhibition of dimerization [28]. Five distinct sites were identified, namely in the poly(A), PBS, DIS, SD and AUG regions. Surprisingly no sites in TAR or psi were found by this hybridization method employed, which may be because of biases introduced by use of a single 20-mer length. Strand invasion of highly structured RNAs is dependent on many complex factors, such that an increase in probe length does not always correspond to better RNA binding. Strand invasion of TAR, for example, is best accomplished by shorter oligonucleotides that lead to a pseudo-half knot structure [29]. Another limitation is that the conformations adopted by in vitro transcribed HIV-1 leader RNA may be different to those that actually occur in the viral cycle. In future, mapping of the HIV-1 RNA in the virion will be needed. Nevertheless, a library hybridization approach is a useful starting point to identify vulnerable RNA structures and it is now clear that several sites of essential functionality within the HIV-1 leader are also sites of good invasion by oligonucleotides [25].

3. Oligonucleotide-based RNA-targeting approaches

3.1. RNase H-active antisense oligonucleotides

The concept of an oligonucleotide targeting HIV-1 RNA as a therapeutic agent is almost 20 years old [30]. The first experiments utilized 12–26 long unmodified oligodeoxyribonucleotides that were targeted just upstream of the PBS (Fig. 1), in a region we now know to be structurally altered when the $tRNA^{Lys3}$ binds [12]. The oligonucleotides showed inhibition of HIV-1 growth when added at high (20 μM) concentrations to either peripheral human blood lymphocytes or transformed T-cells at the same time as virus. Unmodified oligodeoxynucleotides are unstable to serum and cellular nucleases and significant improvements to activity were found when the oligonucleotide backbone was changed to phosphorothioate (PS) (Fig. 6), which has much higher stability to nucleases. This analogue is one of the very few that permits recognition of an oligonucleotide/RNA hybrid by RNase H that results in RNA cleavage. The first evidence that such a mechanism might operate in cells when an oligonucleotide is used to bind to mRNA was just becoming known at this time [31].

Virus inhibitory activity could be elicited at much lower concentrations (1 μM) with phosphorothioates than unmodified oligodeoxynucleotides in both simultaneous oligonucleotide addition/ HIV-1 infection models (acute infection) and in chronically infected cell models (where the virus has already integrated its genome into the host) [32]. Sequence specificity of inhibition was much higher in the chronic than in acute models, since for example a dC_{28} oligonucleotide was just as effective in an acute model [33].

Although many different sites on the HIV-1 genome were investigated over the following years and a number of preclinical oligonucleotide candidates developed, the only oligonucleotide to enter clinical trials was a 25-mer PS oligodeoxynucleotide, named GEM 91, targeted to the AUG initiator site of the Gag protein [34–36] (Fig. 1). Interestingly, this site is identical to one of the five selected recently by 20-mer library scanning of the HIV-1 leader RNA, a point not commented on by these authors [28]. The target sequence is highly conserved between viral isolates and GEM 91 was found to be very effective in long term culture using primary blood lymphocytes and macrophages infected with HIV-1 obtained from some patient isolates at 1 μM and at higher concentrations with chronically infected cell lines [34,36]. Viral escape in long term culture of up to 3 months was not seen [34]. Although GEM 91 was well tolerated by patients in Phase I studies, there were some side effects of thrombocytopenia, elevation of transaminases and prolongation of activated partial thromboplastin time. Another problem was a relatively short half-life in serum that necessitated extended i.v. perfusion treatment. Sadly, lack of efficacy forced the withdrawal of GEM 91 from later clinical trials.

GEM 91 was shown in 1997 to have multiple inhibitory mechanisms [37]. Sequence-dependent inhibition of virus

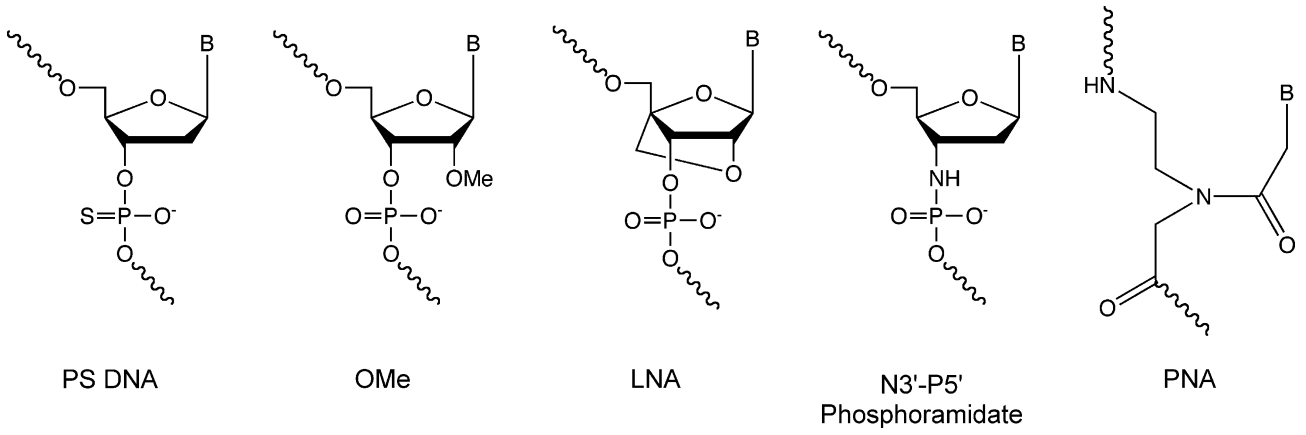

Fig. 6. Nucleotide analogues used in antisense oligonucleotide studies.

entry and reverse transcription were implicated as well as some effect on the steady state RNA levels. Sequence-independent effects were also seen of inhibition of virion binding and inhibition of virus production. The non-sequence specific inhibition of virus absorption was shown to play a predominant role in the total antiviral activity due to a polyanionic effect of the phosphorothioate backbone, similar to dextran sulfate. Steady state Gag mRNA levels, for example, dropped only 3-fold in treatment of chronically infected cells with 1 μM GEM 91, but p24 production was 10-fold lower [37]. This suggests that intracellular RNase H cleavage by GEM 91 is at best inefficient. This is not surprising since it had been shown clearly that free uptake of GEM 91 PS oligonucleotide was almost exclusively restricted to endosomal vesicles within the cytosol, for example in MOLT-3 cells [36]. By 1992, it had been shown that good delivery of PS oligonucleotides into cells in culture required formulation with cationic lipids for efficient nuclear delivery and such delivery induced a much higher antisense activity [38]. The RT enzyme also has an RNase H activity and it is likely that the effects of GEM 91 on reverse transcription might partly have been due to this [18] as well as to non-specific phosphorothioate inhibition of the RT template–primer complex formation [39].

Anazodo et al. noticed a more than 10-fold potentiation of antisense inhibition effects of a 20-mer PS oligodeoxynucleotide targeted to the extended stem of the DIS site when cells were pretreated with oligonucleotide/lipid formulation for 24 h [40]. Protection of MT-2 cells from the cytopathic effect of HIV-1 by the oligonucleotide in the absence of lipid transfection required 1 μM concentration. An anti-TAR 26-mer PS oligodeoxynucleotide, long enough to invade the extended TAR stem, inhibited viral replication in both acute and chronically infected models, but without sequence specificity [17]. Despite the widespread use of cationic lipids in cell culture experiments with synthetic oligonucleotides to enhance delivery and promote specificity, clinical trials proceeded with PS oligonucleotides in the belief that in vivo special cell uptake mechanisms may operate.

Improved versions of GEM 91 were studied later, that involved a phosphorothioate gapmer approach (mixed backbone oligonucleotides) where the flanking nucleotides are composed of 2′-*O*-methylnucleotides (OMe) (Fig. 6) but the central core maintained as deoxynucleotides (GEM 92). Such second-generation oligonucleotides maintain the ability to induce RNA cleavage by RNase H, yet are more greatly protected against degradation by serum and cellular nucleases, and also have higher RNA binding. Mixed backbone oligonucleotide gapmers carrying nucleoside sugar-modified nucleotides in the arms, in addition to the uniform PS backbone, have largely supplanted standard phosphorothioates for most applications and clinical trials [41,42]. However, no second generation and only a single first generation antisense oligonucleotide, for the treatment of CMV-induced retinitis in AIDS patients, have been clinically approved. For HIV-1 treatment, no clinical trial of an oligonucleotide is currently in progress.

Recently, a DNA oligonucleotide targeted to the DIS, where a section of the oligonucleotide had been modified by tight RNA-binding locked nucleic acid (LNA) nucleotides (Fig. 6) but which still directs RNase H cleavage, showed a modest inhibition of HIV-1 replication using a p24 immunoassay [24].

3.2. Steric block oligonucleotides targeted to HIV-1 RNA

Oligonucleotides that induce RNase H cleavage suffer from the disadvantage that binding to an incorrect RNA sequence may nevertheless induce cleavage, leading to the possibility of off-target mRNA inhibition effects. Such unwanted cleavage of a non-targeted RNA might lead to cellular and hence physiological side-effects. By contrast, oligonucleotides that merely bind an RNA target are much less likely to show off-target RNA inhibition effects, since incorrect binding will be generally of no biological consequence, although stoichiometric quantities are clearly needed for the desired activity.

The blocking of HIV-1 reverse transcription by oligodeoxyribonucleotides complementary to the region adjacent to the PBS (pre-PBS) or U5 region was shown many years ago [18]. This could be achieved efficiently for mutant HIV-1-RT lacking the RNase H activity in the case of the U5 oligonucleotide, but the pre-PBS oligonucleotide required a primer to bind to PBS, to alter the RNA conformation, similarly to the binding of $tRNA^{Lys3}$, and here an intact RNase H domain was also needed. Both oligonucleotides, when conjugated to poly-L-lysine, showed inhibition of HIV-1 infection in MOLT-4 cells in culture in the submicromolar range and it was shown that blockage was occurring at the level of proviral DNA synthesis [43].

Steric block oligonucleotide analogues containing uniform nucleotide modifications that cannot elicit RNase H activity, for example OMe/PS oligonucleotides, have been investigated as antisense agents to block translation by binding to the 5′-cap or AUG sequences in mRNA [44,45]. Potent effects in inhibition of translation of endogenous genes have been reported in cells by oligonucleotides complexed with cationic lipids [46]. More recently, steric block oligonucleotides having a variety of chemically modified backbones have found wide application for redirection of splicing pathways in cell nuclei by binding to intron–exon boundaries (reviewed in [47]). Several such steric blocking agents have been found to be active in altering splicing patterns in mice [48,49]. In addition, a steric block N3′–P5′-phosphorothioamidate (Fig. 6) oligonucleotide targeted to telomerase RNA has entered clinical trials [50].

In 1989, steric block 21-mer OMe/PS oligonucleotides targeted to the HIV-1 PBS or splice acceptor site were reported to block the cytopathic activity of freshly infected MOLT-4 cells, but the authors failed to see activity in a chronically HIV-1-infected cell model [19]. The detrimental effect of PS inclusion in 2′-*O*-methyloligonucleotides on binding RNA is exemplified well in the 10-fold drop seen in TAR binding experiments for 17-mers [29]. Such poor binding to structured RNAs may explain why little antisense activity is observed with PS/OMe oligonucleotides targeting the HIV-1 leader,

which has considerable secondary structure. On the other hand, we have shown by confocal microscopy that fluorescently labelled 2′-*O*-methyloligonucleotides lacking PS linkages are much more poorly taken up by HeLa cells in culture in the presence of cationic lipids than those with PS linkages or containing LNA [51]. Probably, the PS linkages or LNA additions help interactions with lipids or membranes. 2′-*O*-methyloligoribonucleotides without such additional modifications also have insufficient stability to serum nucleases to be considered for in vivo therapeutic use, although they are sufficiently stable for cell culture. Nevertheless, 20-mer 2′-*O*-methyl oligonucleotides were found to act as competitive inhibitors of $tRNA^{Lys3}$ in binding to the PBS and also showed significant inhibition of HIV-1 infectivity in a HeLa P4 cell line expressing CD4 receptors and the *lac*Z gene under the control of HIV-1 LTR [52]. 2′-*O*-methyl, N3′–P5′-phosphoramidate and peptide nucleic acid (PNA) (Fig. 6) steric block 16-mer oligonucleotides targeted to TAR (Fig. 2) were shown to be much more efficient at sequence-specific inhibition of reverse transcription than unmodified oligonucleotides with IC_{50} in the nM range [53]. Such oligonucleotides did not bind non-specifically to HIV-1 reverse transcriptase, in contrast to a PS oligonucleotide.

Mixmer steric block oligonucleotides containing OMe and locked nucleic acid units (LNA) have even higher binding to TAR RNA than OMe alone and showed sequence specific inhibition of in vitro Tat binding and Tat-dependent in vitro transcription [54,55]. A 16-mer OMe/LNA mixmer showed sequence-specific inhibition of Tat-dependent *trans*-activation in a HeLa cell reporter system involving stably integrated luciferase plasmids with IC_{50} of 120 nM when delivered by cationic lipids [51,56]. More recently, this oligonucleotide, and another similarly composed OMe/LNA mixmer targeted to the SL3 loop recognised by Gag protein, showed sequence-specific inhibition of syncitia formation induced by HIV-1 infection in a HeLa T4 LTR-β-galactosidase model, again when delivered by cationic lipids [25]. The introduction of LNA units into the steric block OMe oligonucleotide enhances nuclear delivery [51] as well as resistance to nucleases. Some inhibition of *trans*-activation in a transient cell reporter assay has also been obtained with OMe oligonucleotides targeted to TAR that contain some methylphosphonate linkages [57].

Key to the possible use of steric block oligonucleotides as anti-HIV-1 agents is to be able to enhance free cellular uptake and delivery. An interesting approach is prefaced by the early discovery that poly-L-lysine conjugates of oligonucleotides were delivered into HIV-1-infected cells [43]. These studies developed into the concept of using one of a number of cell-penetrating peptides (CPP) as conjugates to oligonucleotides to enhance their delivery (reviewed in [58–60]). This subject has been controversial as to the mechanisms of uptake of such peptides and how their use might lead to the internalization of attached cargoes. Few examples of significant intracellular activity have been documented for peptide conjugates of negatively charged oligonucleotides following free delivery [61]. We found recently that CPP conjugates of a fluorescein-labelled, 12-mer OMe/LNA oligonucleotide targeted to TAR were trapped in endosomal compartments of HeLa cells following free delivery and accordingly were unable to inhibit Tat-dependent *trans*-activation in a HeLa cell assay involving stably integrated luciferase plasmids [62]. By contrast, we have found recently that certain CPPs (for example, Transportan and R_6-Penetratin) disulphide-linked to a 16-mer PNA targeted to TAR were able to elicit significant inhibition on free delivery for 24 h in the HeLa cell *trans*-activation assay [63]. We showed also that in general fluorescently labelled CPP-PNA conjugates are trapped in endosomal or membrane-bound compartments and their release (and subsequent *trans*-activation inhibition activity) can be enhanced by co-administration of the lysosomotropic agent chloroquine.

Sequence-specific targeting of the PBS and TAR regions of HIV-1 by PNA to block reverse transcription was established several years ago [53,64]. Inhibition of Tat-dependent *trans*-activation in cell culture with transiently transfected plasmids [65] and of HIV-1 replication [66] was then achieved by electroporation of 16-mer PNA into cells in the laboratory of Pandey. A particularly important discovery was made in which a disulphide-linked conjugate of 16-mer PNA with the CPP Transportan was able to elicit anti-HIV-1 activity in chronically infected CEM or Jurkat cells by free delivery at 1 to 5 μM concentrations [67]. An even more exciting recent development was that in addition to its antiviral activity this Transportan-PNA conjugate (rather similar to the conjugate that we have been investigating [63]) has very high activity (IC_{50} 66 nM) as a virucidal agent by pre-treatment of HIV-1 virions before cell infection [68]. The conjugate has been shown to inhibit reverse transcription within virions at 500 nM, which may account for part of this activity. Similar levels of virucidal activity were found for several other CPP conjugates of the 16-mer PNA, but their antiviral activities varied significantly from 700 to 1100 nM IC_{50} [69]. At present, there is insufficient evidence to know how efficiently such CPP-PNA conjugates enter virions or whether part of the virucidal activity can be explained by blocking of virus uptake into cells or some other effect on infectivity of pretreated HIV-1 virions. Effective virucidal agents are badly needed and this approach has fascinating prospects in this respect and thus merits further virological studies.

3.3. Aptamers targeted to HIV-1 RNA loops

Loop–loop interactions are well known structural features within folded RNAs and also form the basis of the dimerization of HIV-1 RNA [22]. A novel approach to oligonucleotide-type reagents that can bind specifically to HIV-1 RNA has been described based on the in vitro selection from oligonucleotide libraries of ‘aptamers’ that recognise the apical loop of TAR. Both RNA aptamers [70] and DNA aptamers [71] were first selected. Such aptamers have remarkable specificity for the TAR RNA apical loop sequence, even more than the equivalent antisense oligonucleotides. More recently, chemically modified DNA aptamers containing N3′–P5′-phosphoramidates [72] or LNA residues [73] have been selected that have much tighter binding to TAR and much better serum nuclease resistance.

Such molecules are good candidates for testing in more biological assays and as anti-HIV-1 agents.

3.4. Short interfering RNA (siRNA)

RNA interference is a natural cell mechanism for control of gene expression and is induced in particular as a defensive response to long double-stranded RNA. One part of the RNA interference pathway has been utilized for mRNA degradation by introduction into cells of short RNA duplexes of 19–23 residues, often containing 2-nucleotide overhangs at the 3′-end (siRNA). Such siRNAs can either be expressed from vectors (as individual strands or a single hairpin) or exogenously delivered (e.g., cationic lipid-mediated). The siRNAs are recognised by the RISC complex, which is found in the cytoplasm of cells, and one strand (antisense or guide strand) is directed to form a duplex with the RNA target, which is subsequently cleaved by an RNase III-like activity within a component of RISC.

HIV-1 RNA was an obvious early target for siRNA and reviews on gene silencing of HIV-1 are now available [74–76]. Many different targets on HIV-1 RNA have been explored and compared, particularly with respect to the genes coding for essential proteins. But there are serious difficulties associated with the ability of HIV-1 to generate mutations that give rise to resistance [77]. This can include altering the folding of the HIV-1 RNA genome [78]. As a result gene therapy approaches are focussing on targeting several regions of the HIV-1 genome simultaneously, in the hope that the virus may be less able to escape from such a challenge.

Surprisingly, few papers have described targeting of the HIV-1 RNA leader. Although an early report appeared promising on TAR targeting of a 19-mer siRNA duplex in blocking HIV-1 infection in an acute assay [79], a more recent study suggests that the TAR target has too tight a secondary structure for efficient strand invasion and siRNA activity, based on an expression activity of an adjacent luciferase reporter gene [80]. An siRNA targeted to the U5 region, which also tails back into the bottom part of the stem of the poly(A) region, was one of the least effective in inhibition of HIV-1 in transfected and infected cells [81]. The siRNA was expressed intracellularly from a plasmid.

Perhaps, the most encouraging article describes the evaluation of an siRNA targeted to the highly conserved 18-residue PBS sequence. The well-known availability of this RNA sequence for interaction with tRNAs as well as its extremely high sequence conservation of all 18 residues makes this a prime target for siRNA. Delivery of the PBS-targeted siRNA by lipofection into SupT1 cells resulted in substantial reduction in p24 levels when subsequently infected by HIV-1 NL4-3 strain, resulting in a lag of HIV-1 production over a 15-day period [82]. Similarly, an anti-PBS siRNA expressed in an adeno-associated virus vector inhibited virus production almost completely for 2 weeks during extended cell culture. Interestingly, the virus that eventually broke through did not show any mutation in the PBS target sequence [82].

One advantage of siRNA over antisense approaches is that within cells the siRNA is thought to be able to remain within RISC complexes for multiple rounds of RNA cleavage and therefore effects are often seen for several days in cell culture. However, not enough is known at present about what off-target effects might be seen with siRNA if used as an anti-HIV-1 therapeutic. Further, systemic delivery of unmodified siRNA is unlikely to be an option because of rapid degradation and clearance. Highly chemically modified RNA would undoubtedly be needed [83], in order to allow a sufficient chance to reach enough target T cells and macrophages to reduce HIV-1 replication. To date there has been very little work published on the design of chemically modified siRNA suitable for anti-HIV-1 use with systemic treatment, or on trying to improve cellular uptake, for example conjugate synthesis. Both of these topics will need to be addressed before there would be any hope of proceeding towards possible clinical application.

4. The challenges of oligonucleotides as antivirals and virucides

The failure of GEM91 in clinical trials was a body blow to those dedicated to evaluation of oligonucleotides and their analogues as anti-HIV-1 agents. This failure has undoubtedly led to the current reluctance by industry to consider such reagent types again in the context of antiviral agents. Yet all indications are that GEM 91 was nowhere close to optimal as a therapeutic chemical entity, particularly because of its non-sequence-specific effects, even though the target RNA sequence at the AUG initiator codon was well chosen. There are now very promising results in several areas that suggest that the time is right for further serious efforts to study the potential of newer oligonucleotide-type materials and their derivatives as anti-HIV-1 therapeutics.

Steric block oligonucleotides have been shown to have considerable activity in vivo [48,49]. Further, a steric block PNA-peptide conjugate appears to have favourable pharmacokinetics in an animal model for Burkitt's lymphoma and low or no toxicity [84]. It is not clear as yet as to how PNA-peptides may enter tumour cells or whether such conjugates will have sustainable anti-cancer activity, but there appears to be no intrinsic pharmacological barrier to their further development as therapeutics. Key issues for use of steric block oligonucleotides as anti-HIV-1 agents are a) to determine the most effective viral targets where viral escape is minimal, b) to be able to enhance free cellular uptake and delivery, in order to obtain high efficacy levels without the non-sequence-specific effects previously observed with oligonucleotides composed of PS linkages, such as GEM 91 [37]. As shown in this article, the viral leader of HIV-1 RNA contains several highly conserved sequences that are very good candidates for future development of steric block oligonucleotide, aptamer, or siRNA approaches. These targets now need to be evaluated head-to-head in long-term cell culture studies in models of acute and chronic HIV-1 infection with these new oligonucleotide reagent types.

The issue of enhancement of cell uptake and delivery to the desired intracellular compartment is another crucial aspect.

There is now general acceptance that the entry pathway for most oligonucleotides into cells is by endocytosis. Although several types of endocytosis have been delineated, it seems that entrapment in vesicular compartments of some type is the most likely fate of an oligonucleotide when it is taken up by a cell. Finding better ways to release the oligonucleotide, at least in part, from endocytic vesicles to enhance intracellular activity is now a major preoccupation for those working in this field. For example, we are currently investigating the anti-HIV-1 activities of a range of steric block oligonucleotides targeted to TAR containing different chemical backbone types, especially with a view to obtaining activity via free delivery [63]. PNA–peptide conjugates with their demonstrated antiviral and virucidal activities look particularly promising in this respect [68]. SiRNA delivery is still in its infancy and it is hoped that the promise of the early results against the PBS target [82] can be transformed into a potential clinical lead by the use of chemically modified siRNA having good pharmacological parameters when delivered systemically in vivo. This re-emerging field, to date mostly involving a small number of research groups, is now worth more serious consideration by virologists and pharmacologists interested in developing lead anti-HIV-1 compounds suitable for clinical evaluation.

Acknowledgements

This work is funded in part by a grant from EC Framework 5 (contract QLK3-CT-2002-01989). We thank Bernard Lebleu for helpful comments on the manuscript.

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