Aptamers in the virologists’ toolkit

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Aptamers are artificial nucleic acid ligands that can be generated in vitro against a wide range of molecules, including the gene products of viruses. Aptamers are isolated from complex libraries of synthetic nucleic acids by an iterative, cell-free process that involves repetitively reducing the complexity of the library by partitioning on the basis of selective binding to the target molecule, followed by reamplification. For virologists, aptamers have potential uses as tools to help to analyse the molecular biology of virus replication, as a complement to the more familiar monoclonal antibodies. They also have potential applications as diagnostic biosensors and in the development of antiviral agents. In recent years, these two promising avenues have been explored increasingly by virologists; here, the progress that has been made is reviewed.

Introduction

The term aptamer (Ellington & Szostak, 1990) was coined from the Latin word aptus, meaning ‘fitting’ or ‘fastened’, and the Greek suffix -μέρος (-meros), meaning ‘portion’, which was itself first used in English in this sense in the word ‘polymer’, adopted from German in the 1860s. The process through which most aptamers have been identified is often termed SELEX (‘systematic evolution of ligands by exponential enrichment’; Fig. 1) (Tuerk & Gold, 1990). This involves repetitively reducing the complexity of the library by partitioning on the basis of selective binding to the target molecule, followed by reamplification. The key variables in the process are the target molecules, the partitioning methodology and the nucleic acid chemistry (reviewed by Fitzwater & Polisky, 1996; James, 2000).

In this review, I shall first explore the uses that virologists have made of aptamers as tools for studying the fundamental molecular biology of virus replication and pathogenesis. In doing so, I shall not present the material in the sequence used commonly in virology textbooks, beginning with virion attachment to cellular receptors and concluding with budding or release. After all, the virus replication cycle is cyclical and descriptions can begin as logically at one point as at any other (Fig. 2). Rather, I shall commence with virus polymerases, historically the first targets for aptamers. I shall proceed to discuss aptamer targets in a sequence broadly in line with the historical development of the subject, which has tended to move first from targeting proteins that naturally bind nucleic acids, through those that bind other polyanions, such as glycosaminoglycans, and those that have no such partners, to particularly challenging targets, including polyanions such as RNA itself. By chance, this sequence broadly follows the post-replication stages of the virus life cycle.

In the second part of the review, I shall examine selected applications of antiviral aptamers and indicate how these may develop by analogy with commercialized aptamers in non-virological fields. More general reviews of aptamer discovery and exploitation are available (James, 2001; Bunka & Stockley, 2006) and the possibility of using aptamers for in vivo imaging is reviewed elsewhere (Pestourie et al., 2005). In Fig. 3, I outline some of the typical steps that are taken to convert a raw lead aptamer from SELEX into an optimized reagent for applied use.

Analysis of virus molecular biology using aptamers

Genome replication

The earliest targets to be chosen for aptamer technology were virus polymerases. These make attractive targets for aptamers, as they have an innate capacity to interact with nucleic acid. The landmark study by Tuerk & Gold (1990) exploited this interaction to elucidate the mechanism by which T7 DNA polymerase inhibits the translation of its own message. Rather than screening the binding of the enzyme to the 65,567 possible mutant versions of the translation control region individually, the authors used SELEX to pull out the highest-affinity sequences from a mixture of all mutants. The two sequences that emerged from the selection process were the wild-type version of the element and a closely related sequence, giving a strong indication that the sequence found in nature is nearly optimal.

The same group went on to isolate aptamers to a nucleic acid polymerase of a mammalian virus, the reverse transcriptase (RT) of Human immunodeficiency virus 1 (HIV-1) (Tuerk...
et al., 1992). The RNA aptamers that they isolated had affinities in the nanomolar range, a consensus pseudoknot structure and conserved sequence elements that were not found in the HIV genome. The fact that the aptamers were strongly inhibitory of reverse transcription, but only for the RT of HIV and not those of other retroviruses, suggested that it might be possible to develop more selective RT inhibitors than those based on substrate analogues. They isolated further inhibitory aptamers to the RTs of HIV-1 and other retroviruses, using a range of chemistries (Chen & Gold, 1994; Schneider et al., 1995; Burke et al., 1996; Chen et al., 1996). The story has recently come full circle, with SELEX used once more to identify the substrate-sequence preference of the polymerase (DeStefano & Cristofaro, 2006). Although 12 rounds of SELEX only produced a 10-fold improvement in affinity over random-sequence templates, the most strongly binding sequences strongly resembled the 3’ portion of the polypurine tract found in the HIV-1 genome. This sequence is used to prime second-strand synthesis during reverse transcription, and the aptamer sequences indicate that the function of this tract is not only to act as an RNase H-resistant remnant of HIV virion RNA (vRNA), but also to bind and align RT for efficient reverse transcription.

RT is a multifunctional enzyme, an essential activity of which is as an RNase H. Targeting this, as opposed to its polymerase activity, by a SELEX approach was first attempted by using an N35 DNA library (one with a 35 nt randomized sequence) (Andreola et al., 2001). The best aptamers isolated by using this approach, which appeared to have a common ‘G-quartet’ structure, inhibited both RNase H and DNA polymerase activities in vitro with an IC50 of approximately 0.5 μM. More surprisingly, in a Tat transactivation reporter assay, the same oligonucleotides showed an ability to inhibit HIV infection at an IC50 of 10 nM when added to cells, even in the absence of transfection reagents. Non-specific effects of control DNAs, which demonstrated IC50 values of 300 nM in this assay, might be partly responsible for the strength of inhibition observed with the aptamer. In an elegant enzymic dissection of the anti-RT activity of the RNA-pseudoknot

![Fig. 1. The SELEX process.](image-url)
Aptamers first described in the mid-1990s, it was shown that all RNA-dependent steps of the enzyme’s activity were susceptible to inhibition by the same aptamer, suggesting that their antiviral effect might stem from the cumulative inhibition of sequential steps (Held et al., 2006). Oddly, although the IC₅₀ values for aptamer inhibition of polymerase and RNase H activities were similar, the latter effect was very transient. In an alternative approach, by directly screening a limited library of chemically synthesized RNAs designed to adopt hairpin and ‘dumb-bell’ configurations, aptamers were isolated that inhibited the RNase H activity of RT with an IC₅₀ of approximately 3 μM, without measurable inhibitory effect on polymerase activity (Hannoush et al., 2004).

A second virus polymerase to be the subject of intense aptamer-based investigation was that of Hepatitis C virus (HCV), NS5B. This enzyme has a weak preference for binding to the structured terminal untranslated regions (UTRs) of the vRNA over random sequences, but binds still better to a structure within the coding region. One ambition was to use SELEX technology to elucidate the basis for this selectivity. RNA aptamers raised to this protein inhibited enzyme activity in the nanomolar range, but did not bind the homologous enzyme from the related hepatotropic flavivirus GB virus B (Biroccio et al., 2002). Similarly potent were DNA aptamers isolated independently to the HCV polymerase (Bellecave et al., 2003), which were able to compete with vRNA terminal UTRs for interaction with the enzyme. On the other hand, an independent collection of RNA aptamers to NS5B, with structures different from those described above (Biroccio et al., 2002), were only inhibitory to transcription on the natural template at equimolar or higher ratios (Vo et al., 2003).

**Regulation of gene expression**

An early application of aptamer technology was in the elucidation of the binding-site requirements for vRNA-binding regulatory proteins, such as HIV-1 Rev, which promotes the nuclear export of incompletely spliced virus mRNAs. The Szostak group used a very large pool of randomized RNAs based loosely on the known minimal Rev-binding element (RBE) to isolate new ligands with affinity for Rev equal to or greater than that of the natural sequence (Bartel et al., 1991). Analysis of the winning sequences confirmed that the RBE was a stem–bulge–stem structure with a central, homopurine non-canonical base pair. By using a smaller degree of randomization and stringent affinity selection, the Ellington and Tuerk groups developed this approach independently to isolate improved RBEs with affinities several-fold higher than that of the wild type, shedding more light on the essential sequence requirements of this region (Giver et al., 1993; Tuerk & MacDougall-Waugh, 1993; Jensen et al., 1994), particularly the superiority of an A:A over a G:G pair in the central bulge. Detailed structural analysis showed that the aptamer’s widened major groove was able to accommodate the Arg-rich basic region of Rev in a manner reminiscent of the natural Rev-response element (RRE) (Ye et al., 1996). It was to be anticipated that the aptamer’s structure would alter as it interacted with its target protein, but the Ellington group were able to show that two different aptamers to the same target could induce alternative structural conformations in the Rev peptide (Ye et al., 1999). More recently, the basis for

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**Fig. 2.** Virus targets for aptamers. The categories of structural components of viruses to which aptamers have been raised are indicated in lower case. The text follows the sequence indicated by the dashed arrow, and the stages of the generic virus life cycle to which they broadly relate are indicated in upper case.
the higher affinity of the artificial aptamer over the natural RBE has been revealed to be the greater side-chain flexibility of the peptide in the aptamer-bound state, presumably reducing the entropic penalty faced by the latter on binding to Rev (Wilkinson et al., 2004). These aptamers have been investigated actively as potential gene-therapeutic agents (see below).

Aptamer technology was also instrumental in helping to elucidate the critical features of the binding site for the Rev equivalent in human T-lymphotropic virus 1 (HTLV-1), Rex (Baskerville et al., 1995). The core of the Rex-binding element (XBE) had already been established, but its precise structure and essential residues were only revealed by Rex-binding aptamers, isolated by the Ellington group from an
XBE-based randomized library (Baskerville et al., 1995). The studies revealed a striking similarity to the Tat-binding site on the HIV trans-activation response (TAR) element, suggesting an analogous mode of binding to an Arg-rich motif (ARM) of Rex. Nevertheless, when the structure of a Rex ARM-containing peptide bound to the aptamer was solved by nuclear magnetic resonance (Jiang et al., 1999), several novel and unanticipated features were revealed, including internal, loop-closing base triplets, and the peptide was found to adopt a conformation very different from those of the equivalent ARMs of HIV-1 Rev (above) or Tat of Bovine immunodeficiency virus (BIV) (Puglisi et al., 1995). The artificial aptamer XBE functioned in vivo to promote export of RNAs via Rex interaction (Baskerville et al., 1999).

The HIV-1 trans-activator protein Tat binds to TAR, a conserved, bulged hairpin structure at the 5′ end of all HIV-1 mRNAs, and thereby recruits cellular factors that permit efficient elongation of the transcripts. Early attempts to use SELEX to identify TAR analogues with enhanced activity have produced TAR-like elements with affinities for Tat of the same order as the wild-type element, but which have lost the ability to recruit cellular factors (Marozzi et al., 1998). However, more recently, using a library with a 120 nt randomized sequence (120N), giving access to a theoretical sequence space of $>10^{12}$, P. K. Kumar's lab were able to identify Tat ligands with affinity for Tat 100-fold higher than that of the natural TAR element (Yamamoto et al., 2000). Although this property was dependent on two TAR bulge-like elements (one on each strand of the aptamer), the binding stoichiometry was still 1:1. This was later shown to be the result of simultaneous coordination of two different Tat arginine residues (Matsugami et al., 2003).

Although one might expect nucleic acid aptamers to be particularly able to target proteins that are adapted naturally to interact with nucleic acids, in practice, a much wider range of proteins can be targeted. The earliest transcription factor to be analysed in detail by using aptamers was HTLV-1 Tax, a virus protein that does not interact directly with nucleic acid (Tian et al., 1995). The Ellington group isolated an RNA aptamer, YT1, that competed with NF-κB for binding to Tax, but which did not inhibit the interaction of Tax with its other known transcription-factor partner, serum response factor (Tian et al., 1995). Although it had been suspected from mutagenesis studies that Tax had separate binding sites for these two transcription factors, the aptamer proved a useful confirmatory tool.

Integration

A single-stranded DNA oligonucleotide comprising just G and T residues had been found serendipitously to have activity against HIV-1 integrase as a form of accidental aptamer (Jing et al., 1997). Its architecture is based on the K⁺-stabilized G-quartet motif, identified previously in an aptamer selected for binding to thrombin, and it has an IC₅₀ of approximately 30 nM (Jing & Hogan, 1998).

Genome encapsidation

The specificity of interaction between the core structural proteins of viruses and the packaging signals within their nucleic acid genomes is amenable to analysis using aptamer technology. The earliest example of this approach was in the single-stranded RNA coliphage R17/MS2 (Schneider et al., 1992). The story of the elucidation of the MS2 packaging signal is an example of the way that the aptamer approach can be incorporated as a powerful technique within a programme of conventional molecular genetics and synthetic chemistry. In an elegant series of experiments in which the crystal structure of packaging signal–capsid protein complexes was compared with those complexed with a range of aptamers, it became clear that the packaging signal identified previously by conventional genetics could be modified in important ways to achieve the same functional interactions (Convery et al., 1998; Rowsell et al., 1998; van den Worm et al., 1998). In spite of these sequence differences, chemical-modification analysis showed that the natural and artificial RNAs interacted with the capsid protein in essentially analogous ways (Parrott et al., 2000), but that a carefully designed substitution of a single adenine for a 2-aminopurine could generate additional bonds between the interactors, thereby enhancing affinity (Horn et al., 2004). The approach has also been used to investigate the packaging signals (psi or ψ) of eukaryotic viruses. For example, RNA aptamers to HIV-1 nucleocapsid (NC) protein were isolated independently by the Polisky group (Lochrie et al., 1997) and the Rosbash group (Berglund et al., 1997), and these showed homology both to each other and to the third of the four stem–loops (SL3) of psi. A minimized version of the aptamer could replace SL3 in a packaging assay (Clever et al., 2000), suggesting a particular importance of this region in genomic packaging. Interestingly, the Jeong group later isolated rather different NC-binding aptamers that had an obligatory pseudoknot structure (Kim et al., 2002; Kim & Jeong, 2003) and these inhibited interaction of the packaging signal in virus RNA with NC in an in vitro binding assay (Kim & Jeong, 2004).

Virion maturation

The NS3 protein of HCV comprises an N-terminal serine protease domain and a C-terminal DEAD-box RNA-helicase domain, and is considered a prime target for the development of antiviral therapies. The Nishikawa group undertook a series of SELEX experiments to isolate aptamers to NS3 that proved to be useful analytical tools (Fukuda et al., 1997; Kumar et al., 1997; Urvil et al., 1997). The most useful aptamers, G6-16 and G5-19, which derived from a N120 library, bound to the protease domain rather than the helicase (Kumar et al., 1997). This was unexpected, as one might have anticipated the latter to be the easier target for RNA aptamers, as it naturally binds nucleic acid. Nevertheless, these aptamers inhibited not only protease
activity, but also helicase activity in the full NS3 protein (Kumar et al., 1997), indicating some cross-talk between the domains of potential regulatory importance. The $K_i$ for these aptamers lay in the micromolar range, prompting a search for higher-affinity aptamers (G9 series) from an N30 library. The resulting aptamers bound with submicromolar affinity (Fukuda et al., 2000) and required two arginine residues within the protease binding domain (Hwang et al., 2000). These aptamers have since been characterized structurally (Sekiya et al., 2003) and investigated for their potential as agents for gene therapy (see below; Fukuda et al., 2004; Nishikawa et al., 2004; Umehara et al., 2005).

Virus entry

The work reviewed above shows that aptamers can be isolated that bind to virus core proteins, even to domains that did not have intrinsic nucleic acid-binding propensity. The possibility that this raised was that one might be able to generate aptamers to virus envelope glycoproteins, whose ectodomains have evolved to operate in compartments that are essentially devoid of nucleic acid. Accordingly, we isolated aptamers of 2′-F-pyrimidine-substituted RNA chemistry to the gp120 (SU) glycoprotein of HIV HXB2, a clade B, CXCR4-utilizing (X4) virus (Sayer et al., 2002), and of HIV BaL, a clade B, CCR5-utilizing (R5) virus (Khati et al., 2003). The latter neutralized the infectivity of a wide range of R5 viruses from representative clades and geographical locations (Khati et al., 2003). This property was explained by the finding that aptamer competed with the N terminus of CCR5 for binding to gp120 (Dey et al., 2005b). Structural characterization of these aptamers (Dey et al., 2005a) paved the way for their development as potential antiviral compounds, as discussed below.

To target influenza virus entry, a globular fragment of an H3-type haemagglutinin (HA) was used by the Arnon group as the SELEX target to generate DNA aptamers (Jeon et al., 2004). This region of HA, which is not well-exposed in the virion spike, contains the receptor-binding pocket and is capable of eliciting cross-reactive neutralizing antibodies. DNA aptamers to this region were able to reduce titres of influenza virus in challenged mice by at least 1 log and were effective against H1N1, H3N2 and H2N2 strains (Jeon et al., 2004). Soon afterwards, the Kumar group used the BlAcore-based surface plasmon resonance (SPR)–SELEX technique described previously by Khati et al. (2003) to isolate RNA aptamers to the H3N2 HA (Misono & Kumar, 2005). They isolated a second group of aptamers to the H3N2 HA by using conventional filter binding-based SELEX. These were able to bind to the HAs of the original target virus (A/Panama/2007/99) and of H3 Wyoming, but not to that of the closely related H3 strains A/Sydney/05/97 and A/Wuhan/359/95 (Gopinath et al., 2006b), offering the possibility of rapid subtyping of influenza viruses by using aptamer-based biosensors. Similarly, the same group’s aptamers to HA of Influenza B virus were able to inhibit HA-mediated fusion and were type-specific (Gopinath et al., 2006a).

Nucleic acid

Given that Watson–Crick base pairing provides one with a rational tool for designing specific ligands to single-stranded nucleic acid sequences, it seems at first surprising that one would resort to in vitro selection from randomized libraries to achieve the same end. Nevertheless, it had become clear by the early 1990s that intramolecular structures of complementary nucleic acids compromised the kinetics of their interaction dramatically and unpredictably. Many groups, including our own, had contributed to this understanding, but it is perhaps illustrated most graphically by the fluctuating pattern of RNA hybridization revealed by oligonucleotide-scanning arrays (Southern et al., 1994).

The first in vitro selection of nucleic acid ligands to virus RNAs involved a nested library of antisense fragments, without the cyclic amplification steps of SELEX (Rittner et al., 1993), but nevertheless resulted in the isolation of favourable antisense reagents to HIV. The full potential of SELEX was exploited by the Toumlé group to isolate both DNA (Boiziu et al., 1999) and RNA (Duconge & Toumlé, 1999) ligands for the TAR stem–loop from HIV. In both cases, the winning sequences were better ligands than a simple antisense TAR and folded into imperfect stem–loop structures with loop complementarity to TAR, which generated a limited loop–loop ‘kissing complex’ upon mixing (Collin et al., 2000; Beaurein et al., 2003). A series of experiments designed to replace the original, nucleoside-sensitive ligands with more resistant derivatives culminated in the synthesis of derivatives of the original RNA anti-TAR aptamers, in which key positions were replaced with DNA and locked nucleic acid or with hexitol nucleic acid homologues, respectively, and which preserved the low-nanomolar affinity for the TAR sequence (Darfeuille et al., 2004; Kolb et al., 2005).

The mRNA of HCV has complex secondary structures in its 5′ and 3′ UTRs: the internal ribosome entry site (IRES; for initiation of translation) and SL1, respectively. The Toumlé group isolated aptamers against components of both structures, and these showed unusual apical loop–internal loop (‘ALIL’) interactions (Aldaz-Carroll et al., 2002). Both the Nishikawa and Toumlé groups targeted domain II of the HCV IRES specifically (Kikuchi et al., 2003; Da Rocha Gomes et al., 2004), isolating apical loop-interacting and ALIL-interacting aptamers as a result of the use of structurally constrained versus unconstrained libraries, respectively. Aptamers to the IRES are of interest as potential antiviral agents, and the most potent inhibitors in vivo described to date target its IIId subdomain (Kikuchi et al., 2005). In an imaginative combination of approaches, the Berzal-Heranz group have isolated chimeric ribozyme–aptamers to the HCV IRES (Romero-Lopez et al., 2005). The factor limiting the effectiveness of ribozymes in vivo is the generally very slow rate of association between the flanking complementary regions of the ribozyme catalytic motif and the target RNA. By undertaking sequential selection for molecules with IRES-binding and IRES-cleaving activity from a library comprising a ribozyme coupled to a
randomized segment, they were able to isolate aptamer–
ribozyme chimeras that inhibited translation of IRES-
containing mRNAs by >90% in reticulocyte lysates.

Applied research

Purification and detection of biomolecules

The ability of aptamers to bind specific proteins with high
affinity makes them attractive reagents for applications in
which one needs to detect the presence of a rare protein in a
complex mixture. They have advantages in some contexts
over analogous ligands, such as antibodies, because of their
chemistry, which permits a wide range of controlled
chemical derivatizations and greater reusability. Aptamers
can be used as affinity tags in a range of applications by
exploiting their ability to bind to convenient molecules such
as biotin, streptomycin or streptavidin (Wilson et al., 1998;
Bachler et al., 1999; Srisawat & Engelke, 2001; Tahiri-Alaoui
et al., 2002). In a recent application of this approach,
Dangerfield et al. (2006) were able to purify the host nuclear
protein that interacted with an important, highly structured
RNA motif encoded within the U3 region of the genome of
Mouse mammary tumor virus. The protein was identified by
mass spectrometry with a karyophilic protein known
variously as RoBPI/PUF60/FIR, which was implicated
previously in splicing and transcriptional regulation. In
order to concentrate and purify HCV replicase, as a
preparatory step before mass spectrometry, the B.-G. Kim
group conjugated a replicase-specific aptamer to micro-
beads via a photocleavable linker (Cho et al., 2004). This
approach was sufficient to enhance detection sensitivity to
an estimated limit of 10 fmol polymerase (the equivalent of
6 × 10^9 molecules).

Aptamers have been used to generate biosensors in a range of
formats. An early example used an RNA aptamer to HIV RT
as a probe in a system involving affinity capillary electro-
phoresis and laser-induced fluorescence (Pavski & Le, 2001).
Although detection of RT was possible, it was quenched
strongly by normal medium, making it of limited usefulness.
More recently, however, the approach was used successfully
to show that RT, a heterodimer, could bind two DNA
aptamers simultaneously (Fu et al., 2006). In the first head-
to-head comparison, in a quartz crystal-based biosensor, an
aptamer to IgE proved to be equally sensitive, but more
robust with a greater dynamic range, biosensor than the
equivalent anti-IgE antibody (Liss et al., 2002). A biosensor
for the Tat protein of HIV has been described by using
similar aptamer–quartz crystal technology (Tombelli et al.,
2005).

An elegant approach to the generation of a detectable signal
from aptamer–target interaction is the allosteric activation
of a ribozyme by the binding of aptamer to its target protein,
triggering the uncoupling of a fluorochrome and a quencher
in the ribozyme’s substrate oligonucleotide. This approach
was used by the Kim group in developing a biosensor to the
replicase of HCV (Cho et al., 2005). The results were
promising, but not yet practicable, with signal : noise ratios
of about 10-fold and sensitivity in the high-nanomolar
range. The biosensor field is one of active and varied
development and the search for platforms giving both high
sensitivity and selectivity continues. The most sensitive
aptamer-based biosensor to date has been used to detect
multiple targets simultaneously at as low as attomolar
concentrations (Hansen et al., 2006). In this method
(Fig. 4), the analyte, such as thrombin or lysozyme,
Aptamers are not expected to be able to penetrate epithelia and cellular membranes because of their size and hydrophilicity. Consequently, aptamers whose targets are found within the infected cell are unlikely to be practicable as antiviral drugs, but might be exploited as agents of gene therapy (see below). However, when the aptamer target is potentially extracellular, such as the envelope glycoproteins or capsid proteins of virions, direct antiviral applications can be envisaged. Aptamers that bind to envelope glycoproteins and thereby neutralize virus infectivity, such as those against HIV-1 gp120 SU (Sayer et al., 2002; Khati et al., 2003; Dey et al., 2005a, b) or influenza virus HA (Jeon et al., 2004; Misono & Kumar, 2005; Gopinath et al., 2006a, b) could, in principle, be used to prevent infection or to restrict virus spread.

How might this work in practice? First, neutralizing aptamers of suitably nuclease-resistant chemistry would be analysed structurally to determine the minimal functional element suitable for complete chemical synthesis. Reduction below approximately 60 nt is almost essential for efficient chemical synthesis and a size of 40 nt or below is desirable on grounds of cost of goods. Next, systematic mutagenesis (to optimize aptamer folding and stability), modification of additional 2’ positions (to increase nuclease resistance; pyrimidines are usually modified in the starting library) and further chemical derivatization (to improve pharmacokinetics) would be undertaken to produce a molecule suitable for pilot-scale synthesis and preclinical trials. An agent of this sort might be employed in a number of modalities, depending on the route of infection and pathogenesis of the virus. A respiratory virus, such as influenza, might be tackled with an aerosol preparation of neutralizing aptamer, to block incoming infectious particles. A sexually transmitted virus, such as HIV-1, might be blocked by using an aptamer-containing microbicide, delivered on condoms or in pessaries and creams. In life-threatening, persistent infections, where antiviral drugs are either unavailable or have failed, for example in people infected with drug-resistant HIV, neutralizing aptamers could be envisaged as a form of injectable ‘salvage therapy’. In this context, they might be expected to compete favourably with peptide-based therapies, such as Enfuvirtide (Fuzeon, T-20), which require twice-daily administration and are associated with significant adverse reactions. Finally, a neutralizing aptamer offers a useful tool for the discovery of more conventional drug-like molecules in two mutually reinforcing ways. On the one hand, the high-resolution structure of the aptamer–target protein complex (determined most probably by X-ray crystallography) will provide the basis for in silico design of lead compounds or the refinement of those identified through other means. On the other hand, the aptamer–target interaction can be exploited to produce a high-throughput screen of drug-like molecules to identify those that compete for target binding with the aptamer. Such molecules would potentially mimic the essential contacts between the relatively high-molecular-mass aptamer and the target protein in a manner that would often be hard to design ab initio, and would be useful leads for further structure-based refinement. These approaches are under way in a number of laboratories, including our own, and one hopes that they will produce clinically useful agents in the coming years.

Gene therapy

The notion that virus infections could be prevented or treated by delivering vectors that express ligands for virus gene products into host cells has been around since the late 1980s, but practical application has been fraught by issues of vector safety and efficiency in vivo. Nevertheless, a range of effective, RNA-based inhibitory ligands have been demonstrated in model cell systems, the first of which was our description in this journal of the use of antisense RNA-expressing retroviral vectors to inhibit HIV replication in T-cell lines (Rhodes & James, 1990). Shortly afterwards, the Sullenger and Gilboa groups described the use of ‘Tat decoys’ based on the TAR stem–loop of HIV-1, expressed within tRNA cassettes, to knock down HIV replication in cell lines by two orders of magnitude (Sullenger et al., 1990). Since then, such decoys have been rebranded ‘aptamers’, and the RBE-based ‘Rev aptamer’ proved to be particularly effective in one expression system using cell lines (Good et al., 1997). More recently, in a step closer to the potential clinical setting for such gene-therapy approaches, the Akkina group used lentiviral vectors encoding anti-HIV ribozymes together with Tat aptamers to transduce primary human CD34+ haematopoietic stem cells and transferred them into the severe combined immunodeficiency (SCID)-hu mouse model for HIV. Although some evidence for inhibition of virus replication was evident, the specific contribution of the aptamers to the effect was inconclusive (Bai et al., 2002). More recently, these authors have differentiated the transduced stem cells in vitro to generate macrophages (Banerjea et al., 2004). Encouragingly, compared with mock-transduced cells, the decoy-expressing macrophages showed very limited ability to support HIV replication.

SELEX-derived aptamers have also been used to inhibit virus replication in vitro. For example, the expression of the aptamers to RT in Jurkat cells described above resulted in substantial inhibition of HIV replication (Chaloin et al., 2002; Joshi & Prasad, 2002). The Burke group have been able to measure in vivo activities of these aptamers by using an
elegant expression system in mutants of *Escherichia coli* carrying a temperature-sensitive DNA polymerase. This approach showed that they are surprisingly effective at concentrations equimolar to those of their targets (Nickens *et al.*, 2003). Reassuringly, the drug-resistant mutants of RT found commonly *in vivo* following antiviral therapy were found to be fully sensitive to inhibition by RT aptamers (Fisher *et al.*, 2005). Recently, the Prasad group directly compared the efficacy of potent anti-HIV silencing hairpin RNA (shRNA)-encoding and anti-HIV RT aptamer-encoding cassettes expressed from U6 promoters on the minus strand of an integrated retrovirus vector (Joshi *et al.*, 2005). They found that, although shRNAs and aptamers produced comparable levels of inhibition of HIV replication and challenged m.o.i.s of 5.0 or less, only aptamers showed significant inhibition at higher multiplicities. Given that much cell-to-cell transmission *in vivo* is believed to occur through intercellular synapses, in which large numbers of virions are evident (Piguet & Sattentau, 2004), it is possible that a high effective m.o.i. is pathologically relevant. Moreover, unlike shRNAs, aptamers were shown to be encapsidated in virions produced by aptamer-expressing cells, enabling them to inhibit replication in a second round of infection (Joshi *et al.*, 2005).

Until recently, it has been very difficult to study inhibitors of HCV replication in cultured cells. Nevertheless, the Nishikawa group have explored the possibility that the anti-NS3 aptamers (described above) might have potential as therapeutic agents. For example, when expressed intracellularly as tandem repeats with signals for cytoplasmic localization and *Hepatitis delta virus*-derived processing signals, the G-9-II aptamer was able to inhibit NS3 protease activity significantly in transfected cells (Nishikawa *et al.*, 2003). In a further refinement, they expressed an aptamer to the NS3 protease domain linked via oligo(U) to one against the helicase domain and obtained convincing inhibition of an HCV replicon (Umehara *et al.*, 2005).

**Macugen: aptamers in the clinic**

Thus far, none of the antiviral aptamers described above have become fully licensed pharmaceutical agents. It might be helpful, therefore, to illustrate the process of aptamer commercialization by reference to a non-infectious clinical indication, macular degeneration. This common disease of the elderly results in loss of vision through a process of neovascularization of the retina. It had been known for some time that vascular endothelial growth factor (VEGF) is required for this pathological process, and so VEGF was an obvious therapeutic target. Accordingly, scientists at NeXagen Inc. isolated RNA aptamers to VEGF whose affinity compared favourably with those of contemporary VEGF antagonists (Jellinek *et al.*, 1994). However, the extreme sensitivity of RNA to RNases prompted NeXstar Pharmaceuticals (who inherited SELEX technology rights following commercial restructuring) to search for aptamers that were RNA-resistant by exploiting the ability of T7 polymerase to incorporate non-hydrolysable nucleotide analogues, such as 2′-deoxy, 2′-aminopyrimidine nucleotides (Aurup *et al.*, 1992). These ligands had adequate affinity and nuclease resistance and could be improved still further by substituting most of the remaining purine ribonucleotides with 2′-deoxy, 2′-O-methyl nucleotides and protecting both 5′ and 3′ ends against exonucleases with phosphorothioate linkages (Green *et al.*, 1995). However, the chemical reactivity of the 2′-amino group required shielding during oligonucleotide synthesis by an additional protecting group, making the cost of goods of the VEGF aptamer commercially prohibitive. The approach to this problem was to undertake a third cycle of *in vitro* selection, using 2′-deoxy, 2′-fluoropyrimidine nucleotides (Ruckman *et al.*, 1998). The advantage of this chemistry is that, when it comes to solid-phase synthesis, although the monomers are relatively expensive, the side group is chemically unreactive and so does not require expensive protection. The lead aptamer was minimized, synthesized chemically and modified to replace all but two of the remaining ribopurines with 2′-O-methyl groups, protecting the 3′ end with an inverted dT cap and adding a 40 kDa polyethylene glycol (PEG) moiety to the 5′ end to prevent renal clearance *in vivo* (Fig. 5).

This PEG-derived, chemically synthesized aptamer, then coded NX1838, proved to inhibit vascular permeability in an intradermal guinea-pig assay (Ruckman *et al.*, 1998), prompting the initiation of clinical trials. Over 1000 patients were recruited to the first trial, in which this aptamer, now given the generic name pegaptanib, was administered by intracocular injection at 6-weekly intervals. The results showed remarkably few adverse reactions and clear efficacy at the lowest dose used (300 μg) (Gragoudas *et al.*, 2004). By this stage, the intellectual property rights to the SELEX process in relation to *in vivo* uses had been acquired first by Gilead Sciences Inc. and then by Archemix Corporation,

**Fig. 5.** Macugen (pegaptanib). The composition and secondary structure of a commercialized aptamer (see text for details). The nucleotide bases are annotated to indicate the 2′-substituent: *f*, fluorine; *m*, O-methyl; *OH*, hydroxyl; *d*, deoxy.
who granted EyeTech Pharmaceuticals an exclusive licence for its use in the ophthalmic field. The commercialization of this product, under the name Macugen, was undertaken as a partnership between Eyetech and Pfizer, who obtained FDA approval for its use for all types of neovascular age-related macular degeneration (AMD) in December 2004. At this point, Macugen was the only approved treatment for such a wide spectrum of AMD. Macugen showed effectiveness in a phase II trial against diabetic oedema [a very common cause of blindness in type 2 diabetics (Cunningham et al., 2005)], could reverse the effects of earlier neovascularization episodes in AMD (Adamis et al., 2006), continued to show safety and effectiveness after 2 years continuous use (Chakravarthy et al., 2006a; D’Amico et al., 2006) and is currently the treatment of choice for the widest range of ‘wet’ AMD conditions (Chakravarthy et al., 2006b). Eyetech Pharmaceuticals were acquired by OSI Pharmaceuticals for a total valuation of $650 million in late 2005, largely on the basis of the expected earnings of Macugen.

Hard on the heels of Macugen, Genetech has been developing antibody-based therapy for AMD, also targeting VEGF. Avastin (bevacizumab) is a humanized monoclonal antibody (mAb) to VEGF licensed for use in colorectal cancer. Earlier work had shown that whole Ig of this sort could not penetrate the eye’s limiting membrane following intravitreal injection, whereas Fab fragments could. Accordingly, Genetech developed a Fab derivative of the same antibody for ophthalmic use, called Lucentis (ranibizumab). This agent has proved to have excellent effects in clinical trials (Heier et al., 2006; Rosenfeld et al., 2006a, b). It now appears that the much cheaper, full-sized Avastin might be as effective in the eye as Lucentis (Avery et al., 2006) and, if this holds up in full-scale trials, antibody therapy may well triumph.

The parental, humanized antibody, and a high-affinity derivative obtained through mutagenesis of the complementarity-determining regions and in vitro selection for slow off-rate in a phage-display system, bind to the receptor-binding domain of VEGF (Muller et al., 1998; Chen et al., 1999), in contrast to Macugen, which binds to the heparin-binding domain (Lee et al., 2005). This difference means that Lucentis and Avastin can bind to all active forms of VEGF, unlike Macugen, and makes them much more appealing for widespread clinical adoption. One worry is whether the well-known tendency of aptamers to target the positively charged, heparin-binding patches of their target proteins will result in similar issues arising in the virological field.

**Closing remarks**

It is clear that aptamer technology has matured considerably in recent years and can now be considered a practical alternative to mAb technology, particularly when ligand size, stability or antigenicity is an issue. It is still relatively early for the use of aptamers in diagnostic biosensors and in gene therapy. However, it has been mooted that aptamers could even replace virus vectors, with all their associated limitations in gene therapy, by coupling aptamers for internalized cell-surface molecules to therapeutic RNAs, such as small interfering RNAs, or antiviral aptamers (Guo et al., 2005).

The power and flexibility of in vitro selection techniques seem to stimulate a great deal of productive inventiveness, ensuring that aptamers are likely to figure increasingly in virology, one way or another, for some years to come.

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