Arch Immunol Ther Exp, 2004, 52, 307–315
PL ISSN 0004-069X

WWW.AITE–ONLINE.ORG
Review

Received: 2004.03.29
Accepted: 2004.06.17
Published: 2004.10.15

Nucleic acid aptamers in human viral disease

Zhiren Zhang[1], Michael Blank[2] and Hermann J. Schluesener[1]

[1] Institute of Brain Research, University of Tuebingen, Tuebingen, Germany
[2] NascaCell IP GmbH, München, Germany

Source of support: self financing

Summary

Nucleic acid aptamers are short, single-stranded oligonucleotides or their modified analogues which avidly and specifically interact with targeted ligands through their 3-dimensional structure. Aptamers can be selected out of a large combinatorial oligonucleotide library through an *in vitro* evolution process termed SELEX. Since 1990, a wide variety of aptamers targeted to ligands ranging from small molecules to complex mixtures have been isolated. Most selected aptamers have shown high specificity to and affinity for their ligands and are potential detection and/or diagnostic reagents. Furthermore, some aptamers specifically inhibit biological functions of targeted proteins, resulting in potent therapeutic candidates in disease models. Some recent advances to increase the stability of aptamers, extend their *in vivo* circulation time and their *in vivo* expression have pushed aptamers closer to therapeutic applications. This review presents recent developments in the field of aptamer research and focuses on their applications to human viral diseases, particularly HIV induced diseases.

Key words: **aptamer • SELEX • therapeutic applications • analytic applications • human viral diseases • HIV • HCV**

Full-text PDF: http://www.aite-online/pdf/vol_52/no_5/6336.pdf

Author's address: Zhiren Zhang, Institute of Brain Research, University of Tuebingen, Calwer Str. 3, D-72076 Tuebingen, Germany, tel.: +0049 7071 2984882, fax: +0049 7071 295456, e-mail: zhangzhiren@yahoo.com

INTRODUCTION

DNA and RNA are often considered as large molecules of information storage, but they might also have great potential in the development of novel therapeutic agents based on the vast structural and conformational topology of single-stranded (ss) polynucleotides. ssDNA and RNA can fold into a variety of conformations and directly interact with cellular proteins and other ligands. Such studies have opened a new research field, the selection and design of "aptamers". The name was derived from the Latin word "aptus", meaning "to fit", and these aptamers are agents to fit basically any given structure. In 1990, Szostak and Gold pioneered an *in vitro* evolutionary process termed SELEX (systematic evolution of ligands by exponential enrichment) to identify nucleic acids (aptamers) specific to organic dyes and T4 DNA polymerase[27, 80]. Since then, great effort has been made to improve this method, and many aptamers have been selected for ligands ranging from small molecules to complex mixtures.

Different from antisense nucleic acid molecules, which are considered linear molecules and block transcription according to the Watson-Crick base-pairing model, aptamers are molecules with complex 3-dimensional structures and they function through affinity-binding to targeted molecules, somehow similarly to antibodies. Aptamers can fold into 3-dimensional structures as a result of intramolecular interaction and bind to targets through a small number of contact points which confer high specificity. They are more stable than antibodies and can undergo denaturing and renaturing. Aptamers (8–15 kDa) are smaller than antibodies (155 kDa) and therefore have higher permeability and can penetrate targets more easily. Reporter molecules, such as fluorophores, biotin, or radionuclides, can be easily attached to aptamers by chemical synthesis. Aptamers are derived from an *in vitro* process (described in detail below), so toxins or molecules that do not elicit immune responses can serve as targets. The *in vitro* process also permits non-physiological selection conditions. Additionally, SELEX confers aptamers with high specificity, discriminating targets of subtle structural difference, such as isoforms of protein enzymes[41]. Aptamer have typical dissociation constants ranging from the low picomolar to low nanomolar[33, 40].

SELEX is an effective technology for the *in vitro* selection of aptamers that have high specificity to and affinity for a particular target. Therefore, SELEX has clear applications in the pharmaceutical industry for prime investigation. A large variety of aptamers were selected against various targets, such as organic dye[28], amino acids[35], biological cofactors[12], antibiotics[83], peptides[86], proteins[17], cell-surface epitopes (i.e. CD4[20] and selectins[42]), and even complex mixtures (whole virus particles[66, 84], red blood cell membrane[61], the membrane-bound nicotinic acetylcholine receptor[36], live African typanosomes[38], and mammalian endothelial cells[6]). This indicates that aptamers can be selected for almost any target. Many aptamers not only show high specificity and affinity, but also interfere with biological functions of target molecules, thus presenting themselves as potential drug candidates[9, 65]. The aptamer database is an online resource of all selected aptamer sequences that may have diagnostic or therapeutic utility. The database is updated monthly and is publicly available at "http://aptamer.icmb.utexas.edu/".

Despite the obvious benefits, aptamers are also known to have some drawbacks. Aptamers are easily degraded by nucleases and rapidly cleared *in vivo*, mainly through the kidney. Fortunately, many efforts have been made to solve these problems. This review will introduce recent advances in SELEX technology, developments to solve the problems of instability and rapid clearance of aptamers, and general applications of aptamers in the analytical, therapeutic, and diagnostic fields, with special attention to aptamer applications to human viral diseases.

SELEX

SELEX is a technique to identify, from a huge random sequence, nucleic acid library sequences that have high specificity to and affinity for targets. In the past decade, many developments have been made to increase the versatility and power of this technique. In general, the SELEX process includes the following steps: the first step is to construct a random, single-stranded DNA library. This library is chemically synthesized with a centralized random sequence (typically 40 nucleotides) flanked by fixed sequences at either end. Theoretically, such a library can be composed of 10^{24} ($4^{40} = 1.2 \times 10^{24}$) distinct molecules and, consequently, the same number of distinct structures. The longer the randomized sequence, the more complex the library. The second step is to incubate the initial library (typically adjusted to about 10^{15} molecules) with the target. Usually, small target molecules are immobilized on solid supports to generate affinity matrices. The third step is to separate bound oligonucleotides from unbound. If the small target molecules are immobilized on solid supports, a washing step is enough to remove the unbound oligonucleotides. For protein targets, a nitrocellulose filter partition is most widely used. The fourth step is to

dissociate bound nucleic acid from the target. Generally, standard RNA or DNA precipitation and purification is sufficient to accomplish this goal. The fifth step is to amplify and enrich the resultant nucleic acid pool for the next selection cycle. Reverse transcription polymerase chain reaction, *in vitro* RNA transcription, and single-stranded DNA separation may be used according to the DNA or RNA aptamer selection procedure. After reiterating these steps over several cycles, nucleic acids with high specificity to and affinity for target molecules may be isolated. For each cycle, more stringent selection conditions may be used to increase the enrichment efficiency. The resultant nucleic acid is subjected to DNA cloning and sequencing. Then, binding affinity and specificity of nucleic acids with different motifs to target molecules can be tested and compared, and the aptamers with the highest affinity and specificity or other desired features selected[45, 57]. Figures 1 and 2 present the SELEX processes for isolating RNA and DNA aptamers. Besides this classical SELEX, many modifications have been made to satisfy different demands[7, 41, 48, 58, 73].

APTAMERS *IN VIVO*

Many aptamers were isolated aiming at possible therapeutic applications, but there are several barriers to *in vivo* applications.

Aptamer stability

Insufficient aptamer stability is a major limitation of *in vivo* applications. RNA and DNA aptamers are rapidly cleaved in biological fluids and tissues by nucleases. Efforts have been directed toward increasing aptamer stability by a variety of strategies.

During nuclease cleavage, the ribose 2'-OH engages in nucleophilic attack on the neighboring 3' phosphodiester bond. Therefore, 2' modifications that diminish reactivity can significantly affect nuclease resistance in plasma[67]. Furthermore, the most abundant nucleases in biological fluids are pyrimidine-specific endonucleases. An amino (NH_2), fluoro (F), or 2'-*O*-alkyl group introduced at the 2' positions of pyrimidine is sufficient to produce nuclease-resistant oligonucleotides in biological fluids[10, 37, 70, 81]. Most importantly, the 2'-NH_2 and 2'-F pyrimidines are compatible with the enzymes of the SELEX process. RNA containing 2'-NH_2 and 2'-F pyrimidines is 1000-fold more resistant than the unmodified RNA to nuclease in biological fluids[67]. Using a SELEX library containing 5-(1-pentynyl)-2'--deoxyuridine instead of pyrimidine, Latham et al.[55] isolated a nuclease-resistant aptamer against human thrombin. Also, attachment of a benzoyl group at the C-5 position of pyrimidine appears to be tolerated by the enzymes used in SELEX[23, 25].

Phosphorothioate linkage-modified oligonucleotides are the most widely used analogs in antisense research. Phosphorothioated oligonucleotides, dNTPs, and rNTPs are also compatible with SELEX. Though reports indicate oligonucleotides containing phosphorothioate linkage exert non-specific protein binding[43, 49, 60], a phosphorothioate aptamer to human immunodeficiency virus (HIV) reverse transcriptase with high affinity and specificity was selected[2, 26].

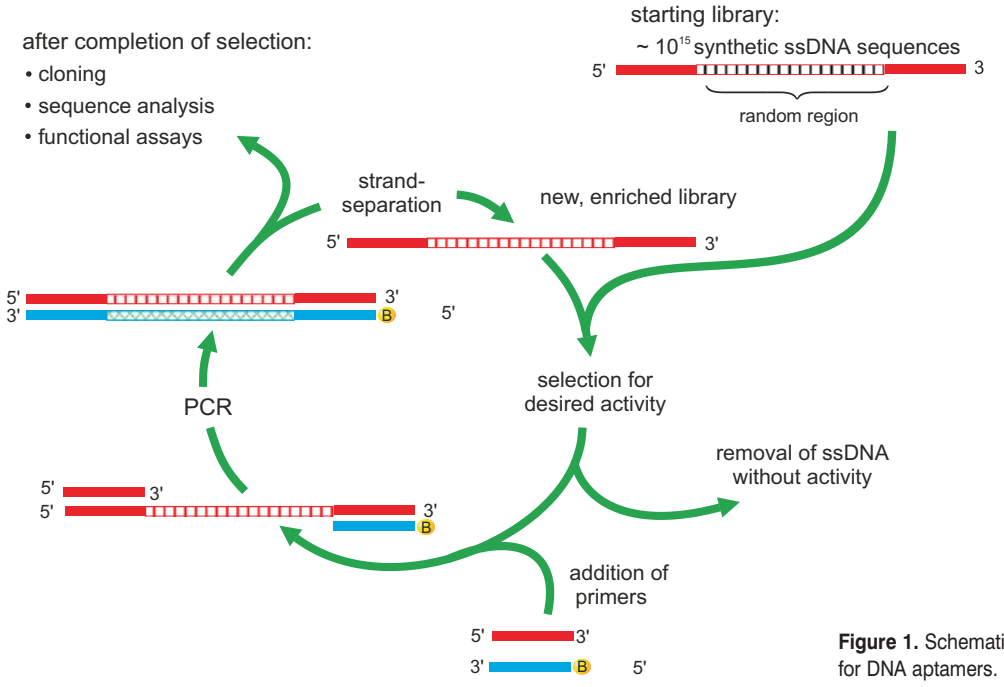

Figure 1. Schematic SELEX process for DNA aptamers.

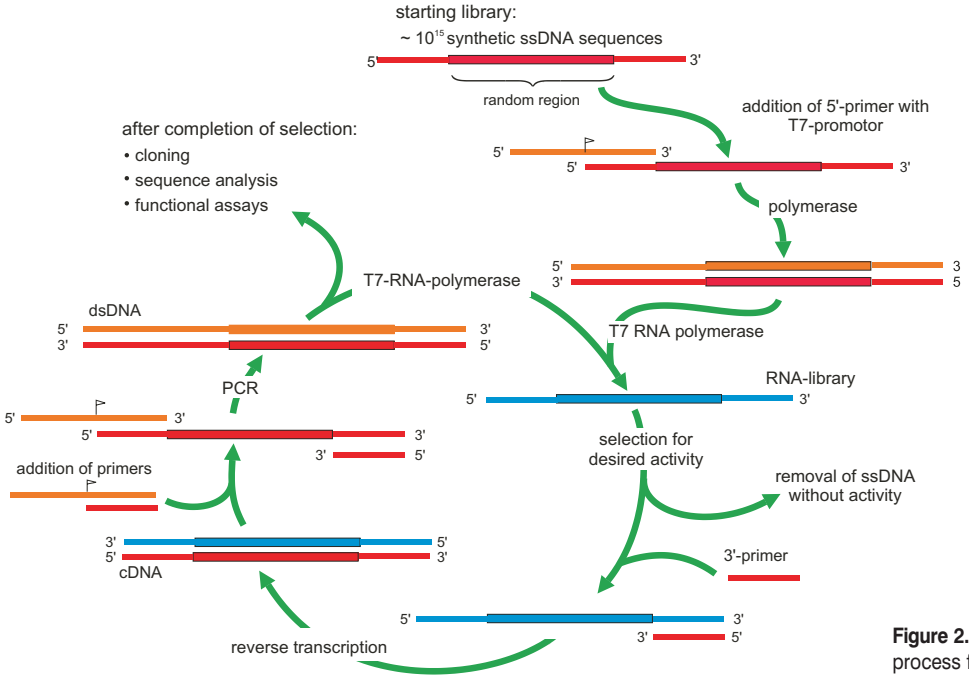

Figure 2. Schematic SELEX process for RNA aptamers.

Furthermore, by split synthesis a phosphorothioate aptamer against nuclear factor κB p50/p50 protein was identified from a one-bead, one-phosphorothioate oligonucleotide library[91].

Another method, termed Spiegelmers, is to replace natural D-ribose with L-ribose to create totally stable aptamers in a mirror-image configuration. However, L-ribose is not compatible with T7 polymerase, and one way to circumvent this problem is to select an aptamer that binds the enantiomer of the target, then synthesize the enantiomer of the aptamer as a nuclease-insensitive ligand of the normal target. Using this strategy, several nuclease-resistant aptamers have been isolated for different targets[50, 63, 86, 88].

Kidney filtration of aptamers

Due to their relative small size (25–40 nucleotides and 8,250–13,200 kDa), aptamers are cleared from the blood quickly by kidney filtration. Therefore, the bioavailability of aptamers is poor. To circumvent rapid clearance, aptamers are conjugated to high-molecular-weight molecules, such as polyethylene glycol (PEG), or attached to the surface of liposomes to increase the circulation time. The first aptamer in human clinical trials, NX1838, was a PEG-conjugated 2'-fluoropyrimidine aptamer that inhibits vascular endothelial growth factor (VEGF). Following intravenous injection, it had a half-life in rhesus monkeys of 9.3 h[79]. Similar results were obtained from a PEG-conjugated 2'-fluoropyrimidine aptamer against L-selectin, which had a half-life of about 11 h in a mouse model[85]. In a nuclease-resistant VEGF aptamer anchored to liposome bilayers through a lipid group on the aptamer, high-affinity binding to VEGF was maintained and the plasma residence time of the liposome-anchored aptamer was considerably improved in comparison with free aptamer[87].

Streptavidin was also conjugated to aptamers. 3'-biotin-streptavidin (SA)-conjugated DNA aptamers retained their affinity for thrombin, were resistant to blood nucleases, and had longer-bioavailability (10–20 times)[24].

Therapeutic applications

One of the most studied aptamers in the therapeutic field is the anti-VEGF aptamer EYE001 (formerly referred to as NX1838). EYE001 is a chemically modified RNA aptamer with high specificity, high affinity, and nuclease resistance. Promising *in vitro* and *in vivo* results against disease related with angiogenesis indicate EYE001 might be a potent therapeutic reagent. Recently, clinical phase IA and phase II trials have shown that EYE001 anti-VEGF therapy is a promising treatment for various forms of ocular neovascularization, including age-related macular degeneration. Single and multiple intravitreal injections of EYE001 were well tolerated in humans[13, 29, 30, 70].

A number of aptamers that can block the biological functions of protein targets are also well characterized, though not in clinical trial. A DNA aptamer against thrombin designed as an anticoagulant for use in surgical indications requiring regional antico-

agulation of an extracorporeal circuit successfully replaced heparin in a sheep model[21, 34]. A nuclease-resistant aptamer coupled to PEG was successfully used to specifically inhibit the interaction between platelet-derived growth factor and its receptor in rat renal disease and rat colon carcinoma[31, 68]. Using blended-SELEX, aptamers against human neutrophil elastase were isolated. An aptamer inhibited lung injury in a rat alveolitis model. In a rat model of reverse passive Arthus reaction, ^{99m}Tc-labeled aptamers achieved a significantly higher target-to--background ratio than a ^{99m}Tc-labeled antibody control when used as an *in vivo* imaging reagent[7, 15].

APPLICATIONS OF APTAMERS TO HUMAN VIRAL DISEASE

With the advances in genomics and proteomics and the completion of numerous viral genome sequences, the number of potential antiviral therapeutic intervention points has increased rapidly. For the efficient identification of the leading structures for these potential therapeutic targets, the large-scale application of combinatorial chemistry to drug discovery has developed quickly.

A number of aptamers that block the biological functions of target proteins which play pivotal roles in the viral life cycle have been isolated. Here we will review the applications of aptamers to viral disease in general, and to HIV- and hepatitis C virus (HCV)-induced diseases in particular.

HIV

A number of molecules which play key roles in the HIV life cycle have been selected as potential therapeutic targets.

Glycoprotein (gp) 120, embedded in the viral envelope, is a key component responsible for HIV entry through its interaction with chemokine receptors. James's group isolated a 2'-fluoropyrimidine-containing RNA aptamer specific to gp120 of HIV-1 strain R5. This aptamer, which partially targeted the conserved, chemokine-receptor binding site, not only bound gp120 with high affinity, but also neutralized HIV-1 infectivity in human peripheral blood mononuclear cells by more than 1,000-fold and showed to be a promising lead compound in developing efficacious anti-HIV-1 drugs[46, 72]. From an eight-nucleotide, randomized, phosphorothioate oligonucleotide library, a sequence, $T_2G_4T_2$, was identified to bind HIV gp120 at the V3 loop and which can inhibit cell-to-cell and virus-to-cell infection. This phosphorothioate oligonucleotide forms a parallel-stranded tetrameric guanosine-quart structure[11, 89]. A phosphorothioate oligonucleotide with a dimeric hairpin guanosine quadruplex (dG3T4G3-s) and its analogue (Gm3Um4Gm3-s) also blocked the interaction between gp120 and CD4 and inhibited the entry of HIV-1 into cells[54, 77]. This type of oligonucleotide also might be a potent anti-HIV drug candidate. Interestingly, using a photoaptamer developed by Golden and co-workers against HIV gp120, Smith et al.[74] detected target protein at subnanomolar concentration in 5% human serum using a microarray format.

Following HIV entry into host cells, reverse transcriptase (RT) reversely transcribes HIV RNA to DNA. RT is therefore an important target for HIV therapy. A number of RNA/DNA aptamers targeted to RT have been isolated and several of them have been tested in human cells. A transiently expressed RNA aptamer against HIV-1 RT in human 293T cells not only reduced HIV particle release by more than 75%, but also reduced virus production in human T lymphoid C8166 cells infected with viral particles released from co-transfected 293T cells by more than 75%[14]. Joshi and Prasad[71] also tested RNA aptamers in human cells. They observed that the virion particles released from 293T cells, which were infected by HIV and stably expressed by aptamers, encapsidated aptamers. The ability of these virions to infect LuSIV reporter cells was reduced by 90–99.5% compared with virions from cells not expressing aptamers[71].

Once the genetic material of HIV has been changed into DNA, this viral DNA enters the host cell nucleus and is inserted into the host gene by HIV integrase. HIV integrase is essential for virus replication and is therefore another attractive target for anti--HIV therapy. Two DNA aptamers, which have G--rich sequences and are able to form G-quartets, inhibited recombinant HIV integrase activity in a cell-free assay system and abolished HIV-1 replication in infected human cells[22]. Another type of interesting oligonucleotide is T30177, composed only of deoxyguanosine and thymidine. T30177 can inhibit a variety of laboratory strains of HIV-1 and multiple clinical isolates of HIV-1 replicating in human lymphocytes. The ability to suppress HIV replication can still exist for several weeks after T30177 was removed from infected cell cultures[64, 69]. T30177 forms intramolecular stacked guanosine quartets, which confer nuclease resistance[5]. T30177 is a potent inhibitor of HIV integrase and can also inhibit virus cell entry through interaction with gp120[16, 59].

After integration into a host gene, HIV DNA is transcribed. Regulation of HIV-1 transcription involves

a complex interplay between cis-acting DNA and RNA elements and regulatory proteins, especially Tat and Rev, which are expressed early in the viral life cycle. Interaction between Tat and *trans*-activation response region (TAR) can increase the processivity of RNA polymerase II. Also, interaction between Rev and Rev response element (RRE) can mediate the shift of the mRNA-production phase of the HIV life cycle from early to late phase[78]. Since Tat, TAR, Rev, and RRE play important roles in HIV replication, a variety of aptamers targeted to these molecules have been isolated[8, 44], and different modifications to increase their stability deployed[18, 19]. Several aptamers even blocked the interaction between Tat and TAR or between Rev and RRE and inhibited HIV replication in human cells. An aptamer termed RBE(apt) is an HIV-1 Rev-binding aptamer. Direct delivery to or expression of RBE(apt) in HeLa cells transfected with HIV-1 proviral clones significantly reduced HIV-1 production[52, 53]. Overexpression of sequences corresponding to the major Rev-binding site in the RRE HIV--1, called RRE decoys, in HIV-1-infected CEM SS cells inhibited HIV-1 replication by more than 90%. These RRE decoys inhibited HIV-1 replication in CEM SS cells by interfering with Rev function, presumably by competing for Rev binding to its physiological target[56]. A phase I clinical trial was performed to evaluate the safety and feasibility of the RRE decoy. Though no adverse effects were observed, this clinical trial emphasized the need for improved gene transfer techniques[51]. An RNA aptamer against the Tat protein of HIV-1 has a 133--times higher binding affinity for Tat than that of authentic TAR. This RNA aptamer can specifically prevent Tat-dependent trans-activation both *in vitro* and *in vivo*[90]. Overexpression of TAR-containing sequences in HIV-1-infected CEM SS cells significantly inhibited HIV-1 replication by more than 99%[76].

The nucleocapsid (NC) protein of the HIV-1 plays an important role in the encapsidation of viral RNA and assembly of viral particles. To study the RNA structure recognized by NC protein, high-affinity RNA ligands binding to NC were isolated by SELEX. Conserved sequences and predicted structures of these RNA ligands were analyzed and revealed a potential interaction site for NC protein[1, 3].

HCV

HCV is a major etiologic agent for chronic hepatitis world-wide, which often leads to the development of cirrhosis and hepatocellular carcinoma. Over the past decades, developments in HCV molecular biology have led to the identification of attractive targets for HCV inhibition. Nonstructural protein 3 (NS3) of HCV, which possesses protease, nucleotide triphosphatase, and helicase activity, and NS5B, which possesses RNA-dependent RNA polymerase activity, present as attractive therapeutic targets[75]. Hang et al.[39] isolated aptamers (named G9-I, -II, and -III) specific to the HCV-NS3 protease domain. G9-I, -II, and -III share a common sequence, 5'-GA (A/U)UGGGAC-3', and form stem-loop structures. Arg161 and Arg130 of HCV-NS3 protease play important roles in the interaction with these aptamers[39]. These aptamers bind to HCV-NS3 protease with a binding constant of about 10 nM and inhibit approximately 90% of the protease activity of HCV-NS3 protease[32]. G9-II aptamer, if stably expressed in HeLa cells, efficiently inhibited HCV--NS3 protease activity[62]. Two groups have developed RNA aptamers with high affinity for and specificity against HCV NS5B, and these aptamers also efficiently inhibit HCV NS5B activity *in vitro*.

The internal ribosome entry site (IRES), which is located at the 5' untranslated region of HCV RNA, directs the viral protein translation and is well-conserved in HCV strains[4, 82]. Kikuchi et al.[47] used a novel selection strategy to obtain RNA aptamers binding to the HCV IRES region. The isolated aptamers strongly inhibit IRES-dependent translation *in vitro* via RNA-RNA interaction.

Other viral diseases

Aptamers can also directly target viral particles. Using Rous sarcoma virus (RSV) particles as the target, Pan et al.[66] isolated RNA and ribonuclease-resistance 2'-fluoropyrimidine aptamers. At a concentration of 0.16 μM, the aptamers completely neutralized RSV infectivity. This effect is dependent on the interaction between the aptamer and RSV, but not on the interaction of aptamer and host cells[66]. Two ribonuclease-resistant RNA aptamers isolated against human cytomegalovirus (HCMV) particles specifically and effectively inhibited HCMV infection in cell culture. Using ultraviolet crosslinking studies, two HCMV-essential glycoproteins B and H were identified as targets[84]. These studies not only demonstrated the potential of aptamers as therapeutic reagents, but also showed the potential of aptamers as research tools.

CONCLUDION REMARKS

Since the first isolation of nucleic acid aptamers, considerable progress has been made in SELEX technology and preclinical application of aptamers.

Nucleic acid aptamers against a wide variety of targets have been isolated. Owing to high affinity and high specificity, aptamers might be used in analytical procedures or imaging. Furthermore, some aptamers show effective inhibitory activity on their target proteins *in vitro* and *in vivo*. Recent advances in increasing aptamer stability and prolonging aptamer bioavailability have further paved the road to the *in vivo* application of aptamers. Progress in aptamer selection in HIV and HCV suggests that both diseases are promising candidates for clinical studies.

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