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REVIEWS

Molecular Recognition Elements: DNA/RNA-Aptamers to Proteins

V. A. Spiridonova

A.N. Belozersky Institute of Physical and Chemical Biology, M.V. Lomonosov Moscow State University, Vorobievy Gory, 1, bld. 40, Moscow, 119992 Russia; tel.: (495)-9393149; fax: (495)-9393181; e-mail: spiridon@belozersky.msu.ru

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Abstract—The review summarizes data on DNA/RNA aptamers, a novel class of molecular recognition elements. Special attention is paid to the aptamers to proteins involved into pathogenesis of wide spread human diseases. These include aptamers to serine proteases, cytokines, influenza viral proteins, immune deficiency virus protein and nucleic acid binding proteins. High affinity and specific binding of aptamers to particular protein targets make them attractive as direct protein inhibitors. They can inhibit pathogenic proteins and data presented here demonstrate that the idea that nucleic acid aptamers can regulate (inhibit) activity of protein targets has been transformed from the stage of basic developments into the stage of realization of practical tasks.

Key words: DNA/RNA aptamers, proteins, the SELEX method.

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INTRODUCTION

During the last decade a significant break has been achieved in the use of basic knowledge on DNA in applied studies. The development of highly technological analytic methods employing immobilized DNA is one of rapidly developing directions. The major achievement of microarray technology (i.e. DNA-chips) consists in possibility of the use of various DNA libraries amplified by polymerase chain reaction (PCR) for the development of sets of DNA sequences. Using hybridization these sets can rapidly analyze and compare sequences of thousands genes, their mutant forms, DNA polymorphism and to discover new genes. The second direction consists in the development of irrational design of nucleic acids for studies of nucleic acid protein recognition. In 1990, two labs (the Gold and Szoztak Laboratories; USA) independently developed the SELEX method (Systematic Evolution of Ligands by Exponential enrichment) [1, 2]. Using this method it is possible to isolate targeted nucleic acid molecules (aptamers) from the large set of individual molecules (more than 10^{18}) known as the combinatorial library. Aptamers are small single stranded DNA or RNA molecules of 40–100 nucleotide residues in length with rather complex three-dimensional structure. Such complex structure determines aptamer ability to bind various molecules including proteins. Thus, such complex process of biosynthesis of protein recognizing elements, antibodies, which nature has been naturally creating for thousands years, is now modeled in vitro.

Selection begins with generation of a large RNA library with fixed 5'- and 3'-ends and a degenerated region of 30–60 nucleotides in length (Fig. 1). Such library contains 10^{14}–10^{15} variants of RNA molecules, which are folded in complex 3D-structure. The library is incubated with a protein and RNA molecules bound to the protein target are separated from unbound RNA molecules. The bound RNA molecules are separated from proteins and then amplified by means of reverse transcriptase and PCR to obtain a new pool of molecules with increased affinity. The procedure is usually repeated 10–15 times until maximal number of aptamers exhibiting affinity to the target will be clearly detectable in the enriched fraction. Aptamers are then cloned (usually into a bacterial vector) and sequenced. In the case of DNA the selection also begins with DNA library in which the randomized region is flanked by fixed sequences at 5'- and 3'-ends. To produce single stranded DNA molecules either asymmetric PCR or one of primers carries a biotinylated tag, which helps to separate one DNA strand from another on streptavidin columns are used. Many reviews on detailed description of all steps of this method have been published to date [3–10]. Now, the idea that nucleic acid aptamers can regulate (inhibit) activity of protein targets has been transformed from the stage of basic developments into the stage of realization of practical tasks. In this review the major attention is paid to the aptamers to proteins involved into pathogenesis of wide spread human diseases.

Tuer and Gold proposed to use combinatorial RNA libraries for creation of RNA-ligands selectively bound to T4 DNA polymerase [1]. RNA ligands were named as aptamers by Ellington and Szoztak [2]. They also introduced a name of this method, SELEX. Now convincing evidence exists that aptamers are a new effective group of therapeutics, which may represent

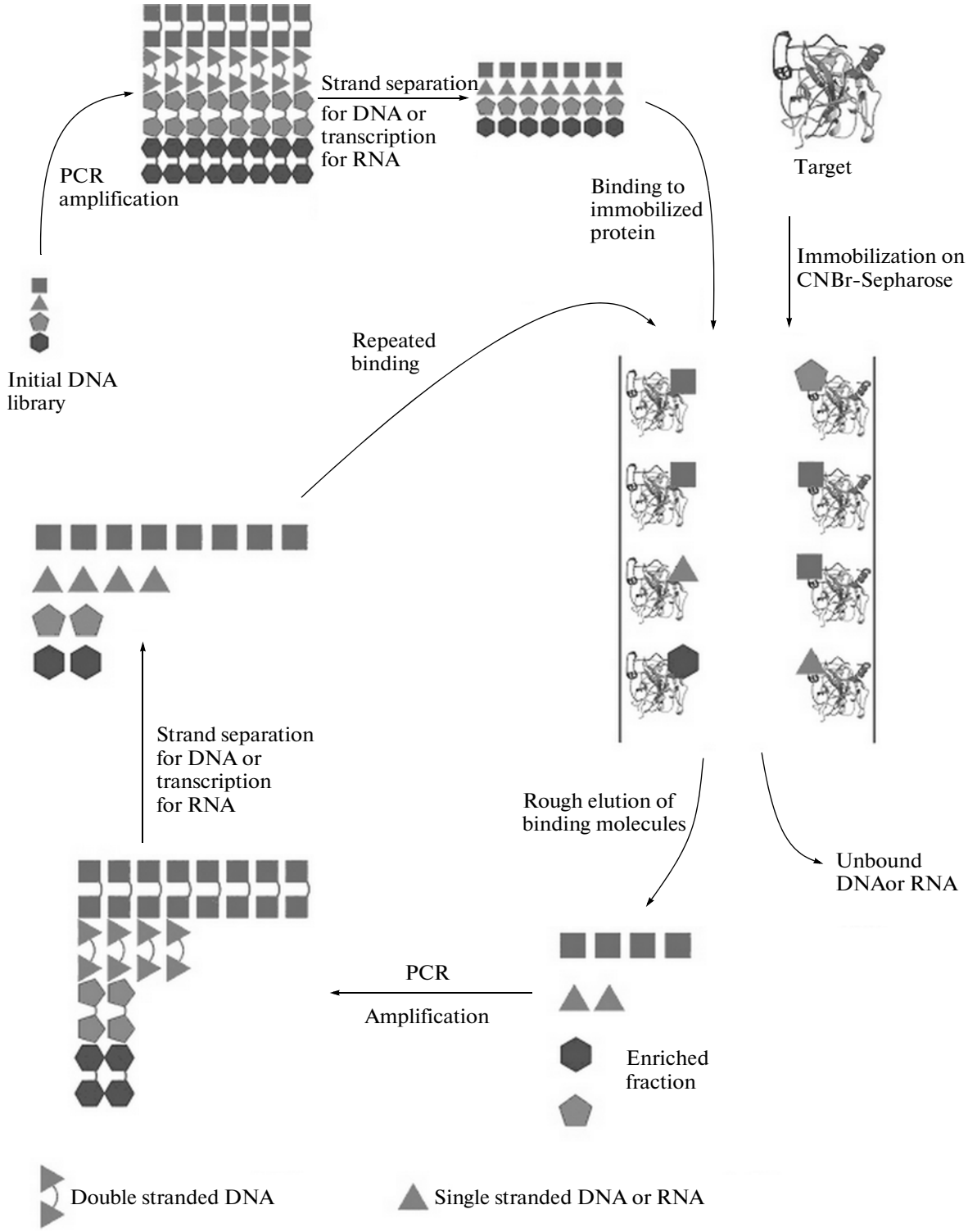

Fig. 1. The scheme illustrating the SELEX method for preparation of DNA and RNA aptamers. An initial randomized DNA library is transformed into single stranded DNA (ssDNA) and then introduced into binding reaction with a protein, pre-immobilized onto a column. RNA is obtained by transcription of the initial library; the latter carries introduced promoter of T7 RNA polymerase. RNA molecules are also exposed for binding with the protein immobilized onto the column. After removal of unbound molecules, DNA/RNA molecules that bind to the immobilized protein are separated from this protein by phenol-chloroform treatment and then subjected to alcohol sedimentation. RNA molecules are used for cDNA preparation by means of reverse transcriptase. All resultant molecules are then transformed into double stranded DNA (dsDNA). In the case of DNA ssDNA prepared using asymmetric PCR is used again for repeated binding. RNA molecules are subjected for transcription and resultant molecules are used for binding with the immobilized protein. The selection includes 10–15 rounds and this yields an enriched fraction of aptamers. The next step includes cloning into a plasmid vector and sequencing of resultant sequences.

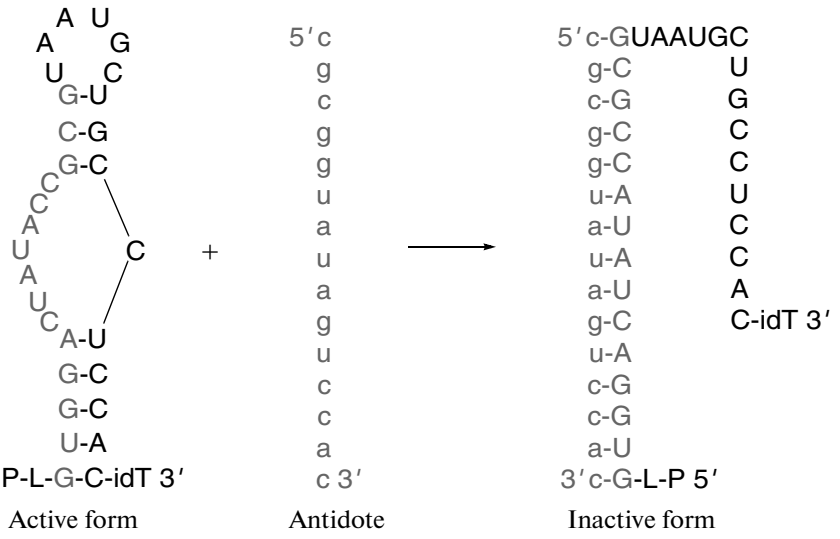

Fig. 2. The mechanism of antidote action in the pair aptamer-antidote (modified from [26]). The antidote is an oligonucleotide of 15 nucleotides in length; it forms an inactive complex with the aptamer to factor IXa involved into the blood coagulation cascade. Possessing a sequence complementary to the aptamer, the antidote forms a complex with the aptamer and thus alters its 3D-structure.

an important tool against many diseases. The drug Macugen has already been approved by US FDA (Food and Drug Administration) for the treatment of age-related macular degeneration (AMD). Some aptamers are now in different stages of pre- and clinical trials [11, 12].

1. PROPERTIES OF APTAMERS AS PROTEIN INHIBITORS

Highly specific (antibody like) recognition and binding of aptamers to their protein targets make them attractive therapeutics. Aptamers (as well as antibodies) are folded into complex three-dimensional structures and form hairpins and loops. The range of dissociation constants characterizing binding of DNA- and RNA-aptamers to their protein targets varies from nanomolar to subnanomolar levels. Aptamers can discriminate related proteins consisting of the same structural domains [13–16]. It should be noted that the use of 1000-fold excess of aptamer doses in animal models employed in preclinical trials and in therapeutic applications in humans did not cause allergic reactions [11, 17]. Studies on biocompatibility and pharmacokinetics of aptamers and investigations of various modifications of aptamer structures have been performed for their further applications as drugs [12].

Nuclease degradation is the major problem that complicates manipulations with oligonucleotides. Protection against nonspecific action of nucleases during selection includes modification of pyrimidine nucleotides at ribose C2' (amino- and fluoro-derivatives) [18–20], use of liposomes as carriers [21], post-selection ribose C2'-hydroxyl modifications by introducing methyl, allyl, amino groups, etc. [22–24].

The molecular mass of short polynucleotides is 8–14 kDa; this corresponds to 25–40 nucleotides. Such a small size facilitates rapid renal filtration within a few minutes. Aptamer modification by conjugation to polyethylene glycol or other agents and their attachment to the liposome surface prolonged the period of aptamer action [21, 25].

Usually, aptamers exhibit high specificity towards their targets and this should be taken into consideration in preclinical trials on animal models. However, protein orthologs may decrease efficacy of such compounds. Nevertheless, an improved selection process named as "toggle-SELEX" seems to overcome this problem. Toggle-SELEX has been proposed for RNA libraries incubated with the same protein from human plasma. During the follwing rounds of selection it was also exposed to binding with the animal ortholog used in subsequent preclinical trials [26].

Studies on pharmacokinetics of aptamers subjected to modifications should be accompanied by analysis of their excretion from the body. This is important because in addition to the main disease patients may have multiple dysfunctions including renal insufficiency.

For regulation of aptamer activity Rusconi proposed the antidote proof-of-concept (the method of rational design) [27]. Using Watson-Crick base pairing he designed an antidote structure as a complementary sequence that bound to the aptamer, altered its struc-

ture and, thus, prevented its complex formation with the protein target (Fig. 2). The proposed concept gave a unique possibility of aptamer regulation because it allows to control administration of an aptamer-based drug in any clinical use.

Considering aptamers as direct protein inhibitors it is very attractive to use them for studies of inhibitory mechanisms and also for therapeutic application. Possibility of aptamer preparation for any class of biomolecules allows to evaluate importance of their use and increases areas of their applications. Advantage of aptamers maintaining their activity in multicellular organisms significantly facilitates preclinical trials, saves time and reduces costs required for antibody preparation on animal models. Finally, design of an antidote that control aptamer activity possibly represents the most important contribution to the development of nuclei acid therapy because it can control drug dosage and, thus, determine required safety. It should be noted that in addition to biomedical studies aptamers are also used as a recognizing elements in microarray-based biosensors; this is another important and logic continuation of the development of the DNA-chip technology.

This review summarizes current knowledge on aptamers to various proteins, their affinity to protein targets; it describes inhibitory properties of aptamers and information about preclinical and clinical trials.

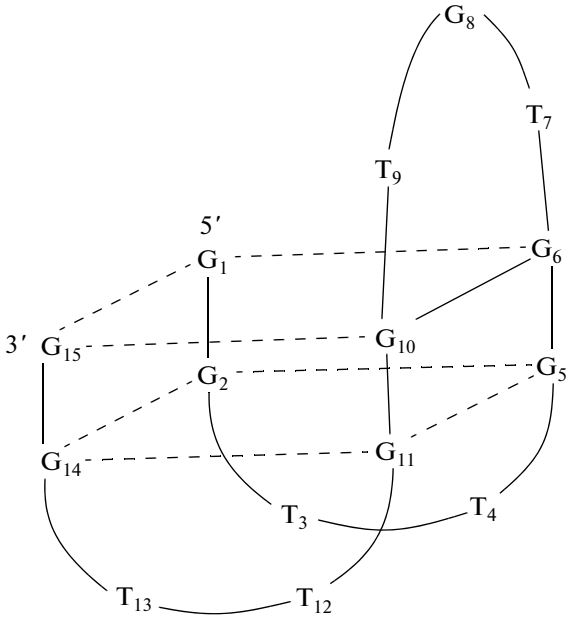

Fig. 3. The structure of the 15TBA aptamer to thrombin, which inhibits fibrinogen hydrolyzing activity. 15TBA has the oligonucleotide sequence dGGTTGGTGTGGTTGG. The G-quadruplex structure is a structure-forming element for DNA. Eight of nine guanines form two planar G-quartets with three loops; the loop TGT located in the center and two symmetrical loops TT. The presence of octa-coordinating calcium ion and stacking interaction between G-quartets of the duplex determine maintenance of the G-quadruplex. Calcium ion is located between parallel planes of the G-quadruplex.

2. SERINE PROTEASES

2.1. Thrombin

Thrombin is a key protein in blood clotting process. This serine protease is generated during a cascade of proteolytic reactions initiated by epithelial damage. Thrombin is produced from prothrombin by factor Xa. Active thrombin catalyzes the reaction of fibrinogen conversion into fibrin, which forms a fibrin matrix for the thrombus by "capturing" blood cells [28]. Thrombin also activates platelets via interaction with their PAR-receptors and regulates expression of some substrates and activation molecules such as P-selectin [29, 30].

Problem of hemostasis requires creation of such thrombin inhibitor, which would be specific for blood clotting process, does not cause allergic reaction and effectively regulates this process. An anti-thrombin aptamer was one of the first therapeutic aptamers obtained by the SELEX method. The single stranded DNA-aptamer was isolated from a pool of ~10^{13} oligonucleotide sequences containing a 60-nucleotide randomized region [31]. The 5-round selection resulted in identification of aptamers forming a complex with thrombin, which was characterized by the K_d values ranged from 25 to 200 nM. These aptamers were based on the 15-nucleotide sequence: dGGTTGGTGTGGTTGG (15TBA). The aptamer increased time required for clot formation from 25 to 170 s in vitro and from 25 to 43 s in human blood plasma. The 15TBA structure was investigated by means of NMR analysis, which included the study of 15TBA alone, in the complex with thrombin and the study of aptamer binding with the anion-binding site of thrombin, exosite 1 [32–35]. 15TBA forms a complex compact tertiary structure known as G-quadruplex (Fig. 3). Other laboratories also used the SELEX method to perform aptamer selection to thrombin [36, 37]. 15TBA also influenced platelet aggregation stimulated by thrombin. Thrombin also caused proteolytic activation of platelet PAR-1 receptor and its aptamer inhibited this activation in a dose-dependent manner [38].

The anticoagulant activity of the aptamer was tested on monkeys. The prothrombin time (PT) increased by 1.7-fold in 10 min and returned to baseline 10 min after aptamer administration. 15TBA also inhibited platelet aggregation and prolonged platelet activation induced by thrombin [39]. The aptamer was also investigated using an anticoagulation model of extracorporeal circulation in sheep. The PT values reached 40–45 s (versus 21.7 s of the baseline level), whereas control PT remained close to the baseline. In the other experiment 15TBA was investigated using a

cardiopulmonary bypass (CPB) model. The study included examination of anticoagulation activity, pharmacokinetics and renal clearance of the aptamer [40]. Animals were subdivided into two groups: one group received injections of heparin (300 U/kg) and protamine in boluses and was used as control of activity by CPB. The second group of animals received aptamer infusion (0.3–0.5 mg/kg per 1 min). These animals were characterized by increased PT, activated partial thromboplastin time (aPTT), and activated clotting time (ACT), which then returned to the baseline after infusion [39–41].

In the pharmacokinetic studies using the CPB model, the elimination half-life of the aptamer was 1.9 min; however, during the 60-min infusion this parameter increased up to 7.7 min. These results suggested that the aptamer would function in the animal model and that unmodified DNA-aptamers were rapidly eliminated from the body. Now this DNA-aptamer is under preclinical trials by Archemix Corp. for subsequent trials in humans.

Anti-thrombin RNA-aptamers were obtained using the library with a 30-nucleotide randomized sequence [42]. The anti-thrombin aptamers were isolated after 12 rounds of selection. The enriched fraction was then cloned in the plasmid pUC18 and sequenced; this yielded two classes of aptamers. The conservative motif in 22 clones of the class I RNAs was represented by the sequence UCCGGAUCGAAG-UUAGUAGGCGGA inside a variable zone. One of the best anti-thrombin aptamers was characterized by the K_d value of 9.3 ± 1.0 nM. Members of the second class exhibited lower affinity (the K_d value of 155 ± 9.0 nM). Competitive analysis with heparin and hirudin demonstrated that heparin but not hirudin displaced the RNA-aptamer from its complex with thrombin. This suggests affinity of this aptamer to thrombin exosite II. However, tests on functional activity in animal models have not been performed.

In preclinical trials highly specific aptamers to human proteins may demonstrate lowered affinity to protein orthologs in animal models. To overcome this problem so-called "toggle" approach has been proposed: 2'-fluoro-RNA aptamers were incubated with a mixture of human and porcine thrombin during the first round and then porcine and human thrombin were alternatively used in subsequent rounds of selection [43]. After 13 rounds of selection clones with the conservative sequence GGGAACAAAGCUGAAGUAC-UUACCC have been found; they exhibited cross-reactivity with porcine and human thrombin. The complex with human thrombin was characterized by the dissociation constant K_d of 2.8 ± 0.7 nM and the complex with porcine thrombin had the K_d value of 83 ± 3 pM. The aptamer increased clotting time (thrombin concentration was 10 nM) of blood plasma from 11.6 ± 0.2 to 22.6 ± 1.4 s. In porcine plasma Tog-25 increase clotting time from 15.7 ± 0.7 to 61.9 ± 1.2 s. Improvement of thrombin-dependent platelet aggregation by the aptamer occurred in the dose-dependent manner. The higher effect was achieved using porcine platelets: a 10-fold excess of Tog-25 inhibited thrombin activity by 90% [26, 43].

2.2. Factor VIIa

Factor VIIa (FVIIa) is a trypsin-like protease involved into the coagulation cascade. In combination with the tissue factor (TF) FVIIa plays a critical role in thrombin formation and thus promotes active clot formation. Aptamers to FVIIa have been isolated from an RNA-library using the SELEX method. These aptamers inhibited activation of factor X (inactive precursor in the coagulation cascade) by FVIIa [15]. After 16 rounds of selection from the 2'-amino modified RNA library the isolated aptamers formed a complex with FVIIa characterized by the K_d value of 11.3 ± 1.3 nM. Specificity of some aptamers was investigated in binding reactions with other protein factors (FXIa and FXa). The micromolar range of K_d values determined for these complexes suggested nonspecific binding of these aptamers with protein factors. Addition of the anti-FVIIa aptamer inhibited an initial rate of FX activation by about 95%. Experiments on dilution of the reaction mixture revealed a dose-dependent mode of inhibition. The aptamer prolonged clotting time up to 175% in the PT test.

2.3. Factor IXa

Factor IXa (FIXa) is a serine protease that plays an important role in formation of critical mass of thrombin required for coagulation. The complex TF/FVIIa performs proteolytic cleavage of the protein factor FIX into its active form FIXa; the latter binds to FVIIIa on the platelet surface and activates factor FX to FXa, which catalyzes conversion of prothrombin into thrombin [28]. Rusconi et al. performed RNA selection to FIXa; after eight rounds of selection they found an aptamer, which bound to FIXa with the K_d value of 0.65 ± 0.2 nM and exhibited 5000-fold higher affinity to FIXa compared with FVIIa, FXa, FXIa and activated protein C [20]. A truncated version of this aptamer (9.3t) maintained high affinity to FIXa (K_d of 0.58 ± 0.1 nM) and totally inhibited FX hydrolysis by the enzyme complex.

The anticoagulation activity of 9.3t was evaluated using activated partial thromboplastin time (aPTT). The aptamer increased clotting time in the dose dependent manner and caused a several fold increase in aPTT. In continuation of the antidote theory Rusconi obtained an RNA-antidote, which caused reversible inhibition of 9.3 t thus creating a drug/antidote pair for the anticoagulation therapy. Using the complementary base pairing principle the second RNA oligonucleotide complementary to the 9.3t aptamer was created. After administration of the antidote

nucleotide the anticoagulation activity of the anti-FIXa aptamer changed in 10 min and this effect persisted for over 5 hours [16]. Almost in 5% of 12 million people receiving heparin therapy heparin-induced thrombocytopenia (HIT) is developed after one year [28] and this is the reason for cessation of the heparin therapy. Patients who need repeated anticoagulant therapy receive hemodialysis, which complicates patient's life.

To prolong the effect of the anti-FIXa aptamer in vivo Rusconi C.P. et al. prepared a cholesterol derivative (Ch-9.3t), which exhibited high affinity ($K_d = 5.3 \pm 1.1$ nM) and anticoagulant activity [27]. Tests on porcine and mouse plasma have shown the same efficacy of the animal models as in the case of human plasma. Using the porcine anticoagulant system the aptamer increased PT and aPTT comparable with its effects on PT and aPTT in human plasma samples. There was significant difference between the modified and initial aptamers: the cholesterol moiety increased half-life of Ch-9.3t to 60–90 min (versus 5–10 min for 5–10 min). The antidote 5-2 C neutralized more than 95% of the aptamer effect within 10 min in animal models [27].

The antithrombotic effect of the aptamer was investigated in mouse thrombosis model, which was induced by administration of ferric chloride to the carotid artery; mice were pretreated with Ch-9.3t or a functionally inactive aptamer with scrambled nucleotide sequence (negative control). All the mice in the negative control group developed an occlusive thrombus in 8.1 ± 0.1 min. In the aptamer-treated group 80% of mice maintained clear normal carotid artery blood flow during 30 min (time required for the occlusive thrombus formation ≥24.4 min) [27]. The effect of the 5-2C antidote was accessed using the model of active bleeding (tail transection). Mice were pretreated with Ch-9.3t or an aptamer with scrambled sequence and the tail was cut 1 h after the treatment. Blood losses were measured for 15 min after tail transection. Animals treated with Ch-9.3t exhibited significantly more blood loss (176 ± 23.7 μl) compared with controls (48 ± 17.8 μl). Administration of the 5-2C immediately after tail transection prevented hemorrhage in the aptamer-treated animals (blood loss was 54.5 ± 13.6 μl) [27].

The biopharmaceutical company Regado Biosciences continues studies on the FIXa aptamer-antidote pair named as REG1, which is under first stage of clinical trials.

2.4. Hepatitis C virus protease NS3 (HCV-NS3)

Hepatitis C virus (HCV) is a major cause of both sporadic and viral hepatites differed from hepatitis A or B [44].

The nonstructural protein 3 (NS3) is a serine protease that exhibits protease and helicase activity and represents a good target for inhibition of HCV. Aptamer selection was performed using a library carrying a 120-nucleotide randomized region and after 6 rounds of selection two aptamers inhibiting protease and helicase activities were obtained [45]. For identification of the aptamer demonstrating affinity to the active site of NS3 subsequent selection was performed using a truncated polypeptide ΔNS3. Using an RNA-library with a 30-randomized region authors performed 9 rounds of selection and identified 45 clones, which bound ΔNS3 [46].

According to their nucleotide sequences aptamers were subdivided into three families. They all contained a conservative region GA(A/U)UGGGAC. These aptamers formed a complex with ΔNS3 with the K_d value of 10 nM, caused 90% inhibition of protease activity of the ΔNS3 peptide and full-sized NS3 bound to a maltose-binding protein (MBP-NS3). In vivo HCV proteins are processed by NS3 and NS4A cofactor. For modeling of physiological conditions the aptamer effect on NS3 activity was tested in the presence of the P41 peptide, which caused a sevenfold increase of MBP-NS3 activity. Under these conditions the aptamer inhibited MBP-NS3 activity by 70% [46].

2.5. Human neutrophil elastase

Human neutrophil elastase (hNE) is involved in various inflammatory diseases, including acute respiratory distress syndrome (ARDS), septic shock, arthritis, and ischemia-reperfusion injury [47]. A covalent inhibitor of hNE, a diphenyl phosphate derivative of valine (valP), was coupled to an RNA library to enhance the binding of the inhibitor with hNE [47]. Ten rounds of selection yielded an RNA-aptamer conjugated to the DNA:valP substrate (RNA 10.11: DNA:valP). The aptamer demonstrated binding to hNE ($K_d = 71$ nM) and enzyme inhibition ($K_i = 5$ nM) in vitro. In contrast to the RNA aptamer 10.11 or the substrate DNA:valP administered separately the aptamer modified with the substrate inhibited hNE ex vivo in the rat model of ARDS [48]. The same group also performed a valyl phosphonate: DNA library selection to find more potent hNE inhibitors. Authors used a single-enantiomer form of the valyl phosphonate, which was compared with a racemic mixture. Inhibitor selection was performed using purified elastase and also secreted elastase in the presence of neutrophils [49]. After 18 rounds of selection the aptamer ED45, which inhibited hNE, was found. The aptamer was truncated to a 42-mer, named NX21909, and tested in a rat model of lung inflammation. A 40 nmol dose of NX2109 inhibited neutrophil infiltration by 53% in the lung of rat in vivo [49].

3. CYTOKINE GROWTH FACTORS

3.1. VEGF

Angiogenesis plays a central role in various physiological and pathological processes. VEGF (vascular

endothelial growth factor) is one of the best characterized growth factors; it is involved into initial steps of angiogenesis and represents one of the most promising targets for anticancer therapy [50].

An increased VEGF level associated with angiogenesis was observed during tumor growth and metastases, premature aging and age-related degeneration of tissues [50–52].

Using the SELEX method Ruckman performed 12 rounds of selection cycles and isolated aptamers to human $VEGF_{165}$ with K_d of 50 pM in a 2'-F-pyrimidine RNA library [19, 20, 53–55]. For increased stability against nucleases two aptamers were additionally modified by the 2'-O-position [55]. These aptamers were characterized by the K_d values of 49 and 130 pM; they were specific to $VEGF_{165}$ and did not bind to related proteins: $VEGF_{121}$ and placenta growth factor $PlGF_{129}$. The aptamers to $VEGF_{165}$ inhibited the binding of $VEGF_{165}$ to its receptors, Flt-1 and KDR (kinase domain receptor). Using ^{125}I-labeled $VEGF_{165}$ inhibition of receptor binding was evaluated: the IC_{50} values for aptamer competition with the Flt-1 receptor and KDR were ranged from 50–300 and 2–60 pM, respectively [55].

Therapeutic potential of the aptamers to VEGF was evaluated by the Miles assay representing simple and rapid means of monitoring the ability of aptamers to inhibit the activity of $VEGF_{165}$ in vivo. It is assessed as vascular wall permeability in animal models. The test was performed using adult guinea pigs and the most effective aptamer inhibited vascular permeability by 58% at 1 μM [55].

Pharmacokinetics of the 2-fluoro-pyrimidine and 2'-O-methyl purine aptamer to VEGF called as NX-1838 has been investigated in monkeys. During intravenous administration of this aptamer as a conjugate with a 40 kDa polyethylene glycol was characterized by half-life of 9.3 h and a clearance rate of 6.2 ml/h. Subcutaneous administration resulted in 80% absorption into the tissues within 8–12 h [25].

Preclinical and clinical trial of NX-1838 also called as Macugen was performed by Eyetech Pharmaceuticals Inc for the treatment of age-related macular degeneration in diabetic patients [11, 12].

The synthetic aptamer NX-1838 was also investigated in the rat model of angiogenesis. These studies confirmed a significant inhibition by 80% of angiogenesis by means of VEGF in the presence of this aptamer. The 1a phase of clinical trials did not reveal any significant complications after a single administration of the drug. In addition 80% of patients demonstrated stable improvement during observation for 3 months after injections, 27% of patients demonstrated a threefold improvement of vision among diabetic patients (ETDRS) [10].

Clinical trials (phase 2) have shown that multiple Macugen administration with or without photodynamic therapy (PDT) did not cause any serious impairments; moreover 87.5% of patients demonstrated stable vision improvement and 25% of patients demonstrated significant improvement evaluated using the ETDRS system (Early Treatment for Diabetic Retinopathy Study).

During the third phase of clinical trials Macugen (under the commercial name Pegaptanib) was used as the only drug every 6 weeks over a period of 48 weeks at a dose of 0.3, 1 and 3 mg intravitreously [56]. All three groups of patients demonstrated significant improvement of vision. The severe loss of visual activity determined as the loss of 30 letters of visual acuity reduced from 22 to 10% in the group receiving 0.3 mg of Macugen. In addition, 33% of patients receiving this dose maintained their visual acuity or gained acuity (versus 23% of control group). No antibodies against Macugen were found. Eytech in cooperation with Pfizer obtained FDA approval for the use of Macugen for the treatment of AMD. These first results demonstrate that aptamers may be effective drug preparations.

It is known that the intensive tumor development is accompanied by neovascularization and therefore an aptamer to VEGF was used for inhibition of tumor vascularization (and tumor growth).

The aptamer isolated and optimized by Ruckman et al. was tested in a mouse model of Wilmis tumor (the most common malignant tumor of the kidneys in children). Tumor was implanted into a mouse kidney and its growth was maintained for one week, and then the aptamer to VEGF (200 μg) or vehicle (phosphate buffered saline) were administered for experimental and control mice respectively, daily for 5 weeks [57]. After decapitation of animals authors observed that tumors weighed 84% less in treated versus control animals. Lung metastases were seen only in 20% of the aptamer-treated animals (versus 60% of animals from the control group). The aptamers tested in the murine nephroblastoma exhibited the decrease in tumor growth by 53% compared with control [57].

3.2. Basic Fibroblast Growth Factor (bFGF)

The increase in bFGF correlates with appearance of various diseases including retinopathy, rheumatoid arthritis, leukemia [58].

Jellinek and co-authors used a 2'-amino-pyrimidine derivative RNA library and performed 11 rounds of aptamer selection [58]. They found an aptamer named as m21A, which exhibited binding to bFGF with K_d of 0.35 nM; a competitive binding study revealed that it competed for bFGF binding with unfractionated heparin and low molecular weight heparin. The inhibitory activity of m21A was also investigated using Chinese hamster ovary (CHO) cells: the aptamer bound to its target with the K_d values of 1–3 nM [59]. The effect of m21A on the endothelial cell motility was also investigated using the migration

of endothelial cells to a denuded area in bovine aortic cells where endogenous bFGF is essential for activity. At concentrations > 50 nM the aptamer inhibited cell migration in a dose dependent manner (as compared with control). The RNA aptamer inhibited bFGF binding to its cell receptor [20].

3.3. Platelet-derived growth factor

Platelet-derived growth factor (PDGF) is a mitogen composed of two homologous (A and/or B) chains linked by three disulfide bonds; this dimeric protein is involved into wound healing and progression of various diseases including atherosclerosis and glomerulonephritis. Many tumor cell lines produce and secrete PDGF [60].

A DNA selection in vitro against human recombinant PDGF-AB was performed and after 12 rounds DNA aptamers characterized by K_d of 50 pM were isolated. Three aptamers effectively inhibited PDGF-BB binding to PDGF α- and β-receptors with the K_i value of 1 nM. The anti-PDGF aptamers also inhibited mitogenic effects of PDGF on cells expressing PDGF β-receptors with K_i of 2.5 nM [61].

One aptamer termed 36t was truncated, 2'O-methyl, 2'-fluoro-modified and capped at the 3'-end (to increase resistance to nucleases) and conjugated to 40 kDa PEG (to increase its lifetime in blood circulation) [62]. The modified aptamer exhibited high affinity binding to the human protein (K_d = 100 pM); it was tested using a rat glomerulonephritis. In this model intravenous administration of this aptamer (2.2 mg/kg) twice a day decreased mitoses by 64% on day 6 and by 78% on day 9. Animals treated with this aptamer were characterized by a decreased monocyte/macrophage index and glomerular matrix overproduction. Control animals received a scrambled sequenced oligonucleotide or PEG for 6 days [63].

3.4. Human interferon γ

Interferon γ (IFN-γ)exhibits various immunoregulatory effects. Although its antiproliferative effect is less pronounced than in IFN-α and IFN-β, IFN-γ is the most potent activator of macrophages and the inducer of expression of MHC class II molecules [58]. In healthy nervous tissue IFN-γ is almost absent, however, during inflammatory processes in the nervous system and in multiple sclerosis it is overproduced. IFN-γ secretion can result in inflammatory and autoimmune diseases. RNA selection using 2-fluoropyrimidine- and 2-aminopyrimidine-RNA or a mixture of these two modifications were screened for aptamers that inhibited receptor binding of IFN-γ [64].

The resultant aptamer, 2'-amino-30 had a K_d value for its complex with receptor of 2.7 nM. In the culture of A549 cells it inhibited receptor binding of IFN-γ with K_i value of 10 nM. This aptamer also inhibited induction of the MHC complex regulated by IFN-γ and ICAM-1 expression with IC_{50} values of 700 and 200 nM, respectively [64].

3.5. Angiopoietin-2

Endothelial receptor tyrosine kinase Tie2 plays an important role in vascular wall stability. Angiopoietin-2 (Ang-2) is a natural antagonist, which is obviously expressed only during active angiogenesis (e.g. tumor growth) [65]. For investigation of Ang-2 by aptamers 11 rounds of RNA selection were performed and RNA molecules exhibiting specific binding to Ang-2 were isolated. One aptamer demonstrated high affinity to Ang-2 (K_d = 3.1 nM); it did not bind to Ang-1 (K_d > 1 μM). This aptamer was truncated to 41-mer (K_d = 2.2 nM) and the truncated aptamer inhibited Ang-2 function in a cell culture and in a rat model, where it significantly inhibited neovascularization by 40% [65].

4. APTAMERS AGAINST INFLUENZA VIRUS PROTEINS

Influenza is one of the most widespread disease in the world. Control of this disease includes active campaigns of vaccination, use of drugs blocking neuraminidase action. The use of aptamers helps to block virus binding to cell receptors. Binding of isolated DNA-aptamers to viral hemagglutinins blocked virus penetration into cells [66, 67].

Extracellular domains of influenza hemagglutinin cause agglutination of blood cells (mainly erythrocytes). Hemagglutinin determines virus binding to cells. Neuraminidase is responsible for : 1) ability of a viral particle to penetrate into the host cell; 2) ability of viral particles to leave host cells after reproduction. Two DNA aptamers were obtained to the hemagglutinin peptide (residues 91–261), which is responsible for binding of an oligosaccharide component of cell receptors. The aptamer A22 exhibited high binding activity and blocked agglutination of chicken red blood cells [66]. The effect of A22 was confirmed by microscopic studies; they revealed preservation of cell structure compared with control preparations, in which damage of cell structure in the presence of influenza virus was observed. The same authors isolated two RNA aptamers to hemagglutinin using an RNA-library containing a 30-nucleotide randomized region. A predicted secondary structure in 73 bases long included nucleotides of the randomized region and also constant sequences of the flanking region. One of the aptamers exhibited binding to hemagglutinin with the K_d value of 2.9 nM. The affinity of this aptamer was 15-fold higher than that of a monoclonal antibody to this protein. Moreover, the RNA aptamer allowed to discriminate hemagglutinins isolated from two various strains [67].

5. APTAMERS TO NUCLEIC ACID BINDING PROTEINS

5.1. Tat-protein

Therapeutic applicability of aptamers has been undertaken during studies of HIV replication. Human immunodeficiency viruses HIV-1 and HIV-2 that belong to a lentivirus class selectively affect T-helpers. A regulatory Tat-protein activates viral replication. The TAR element (of 60 nucleotides long) presented in all predicted viral transcripts is required for functioning of tat-protein. It was proposed to express the 60 nucleotide TAR sequence to capture Tat protein into an RNA-decoy [68, 69]. TAR RNA, HIV transcript, was expressed in CEM SS cells. The RNA decoy inhibited HIV-1 replication over 99% in vitro. Inhibition of viral replication in CD4+ cells proceeded at the high level TAR aptamer expression from the tRNA promoter. Changes in the nucleotide sequence of hairpins or loop in the structure of the TAR aptamer abolished the ability of the TAR aptamer to inhibit HIV replication [68, 70].

The SELEX library consisted of a randomized sequence of 120 nucleotides was used for selection of aptamers to Tat proteins. After 11 rounds of selection a truncated variant of an RNA aptamer named as RNA^{Tat} was obtained; it formed a complex with the Tat protein with K_d of 120 pM. This 37-mer RNA aptamer inhibited HIV-1 in vitro and decreased viral replication by 70% in a cell culture [71].

Other groups also found RNA decoys, which bound Tat protein and inhibited HIV-1 [72, 73].

No significant incompatibility between TAR and Tat interactions of HIV-1 and HIV-2 belonging to various subfamilies were found. However, although Tat-1 could transactivate HIV-2 through TAR-2, Tat-2 did not interact with TAR-1 in HIV-1 [74].

5.2. HIV-1 Rev (revertase)

The viral protein Rev promotes transportation of partially spliced RNA molecules to cytoplasm, where they provide synthesis of usual retroviral products. The RRE (Rev Responsive Element) element composed of about 234 nucleotides forms a complex three-dimensional structure, to which Rev protein binds. Using a tRNA promoter for expression of the RRE element, the major Rev binding site in HIV-1, overexpression of this construct has been estimated in the cells. Expression of the chimeric tRNA-RRE aptamer caused inhibition of viral replication by more than 90% [75]. The aptamers passed phase 1 clinical trials, in which this construct was transduced in vitro to CD34+ cells obtained from bone marrow of a HIV-1 infected subjects followed by subsequent reinfusion into these subjects. Aptamer administration did not cause adverse effects, however, a rather low level of RRE gene expression was observed possibly due to inappropriate conditions for gene transfer [76].

5.3. HIV reverse transcriptase

Reverse transcriptase (RT) was the first target for the development of the SELEX method for HIV therapy. Tuerk and Gold published the pioneer paper on SELEX. They used RT as the target for isolation of RNA ligands, inhibiting HIV replication [1]. After 9 rounds selection from RNA populations randomized at 32 positions authors isolated RNA that specifically bound to HIV RT and inhibited activity of this enzyme [77]. The sructure of the RNA aptamer was subsequently characterized and experiments performed on cells indicated inhibition of HIV-1 replication by 90–99% [78]. In addition, aptamer expressing T-cells completely blocked the spread of HIV in culture [79].

5.4. Transcription factor E2F

Proliferation of myocardial and vascular cells is a central problem in the development of such cardiovascular diseases as hyperplasia, atherosclerosis, malignant tumors [80, 81]. E2F plays a central role in regulation of cell proliferation. This factor exhibits highly specific binding to a double stranded DNA containing eight base pairs TTTCGCGC. The constructed 14-mer DNA aptamer containing a sequences for E2F binding was tested for inhibition of E2F activity [82]. In vascular smooth muscle cells (VSMC) stimulated by E2F the 14-mer oligonucleotide (ODN) inhibited VSMC proliferation and expression of the genes *c-myc* and *cdc2*, controlling cell cycle, and proliferating cell nuclear antigen (PCNA). In vivo the 14-mer ODN was transfected to rats with experimental carotid injury and this markedly suppressed the fibrosis formation compared with nontransfected arterial segments. Furthermore, this inhibition continued up to 8 weeks after a single transfection [81]. Transfer of an E2F decoy can therefore modulate gene expression and inhibit smooth muscle proliferation and vascular lesion formation in vivo. Using the SELEX method other aptamers to E2F were also prepared; they inhibited DNA-binding activity of this protein [82].

These interesting results prompted Dzau's group to test the E2F aptamer in humans in order to determine whether it can limit intimal hyperplasia during intravenous administration [83, 84]. The E2F aptamer was delivered to the infra-inguinal vein by transfection. Cell transfection efficiency was 89%, expression of *c-myc* and PCNA reduced by 73 and 70%, respectively, compared with the control group. After 12 months in the group of patients treated with the E2F aptamers fewer occlusions were registered compared with control.

The E2F- DNA aptamer is now evaluated by Corgenetch Inc., in a phase III study to estimate its efficacy at limiting coronary and peripheral vascular damages. This aptamer is very close to clinical application.

The E2F aptamer was also used for evaluation of long-term protection from neointimal hyperplasia and atherosclerosis [85]. Hypercholesterolemic rabbits were treated with intravenous injections of E2F aptamer or scrambled oligonucleotide. After 6 months (when animals were put on a cholesterol containing diet) the E2F-aptamer treated group of animals was free of plaque whereas animals treated with the scrambled oligonucleotide and also control animals had extensive plaque formation [85].

Finally, SELEX was used to obtain an RNA-aptamer that would bind and inhibit E2F. Insertion of the E2F aptamer into a tRNA expression cassette yielded RNAs exhibited effective inhibition of E2F1 binding to DNA [86]. To test the ability of the E1 RNA-aptamer to block proliferation, human fibroblasts were treated with E1 RNA aptamer and proliferation was then induced. The RNA aptamer inhibited S-phase by 80% compared with control [86]. Thus, natural and in vitro selected aptamer can act as proliferation inhibitors.

5.5 Nuclear factor kappa B (NF-κB)

Transcription factor NF-κB activates genes involved into inflammatory processes and synthesis of cytokines, interferons, MHC proteins, growth factors, and cell adhesion molecules, which play a central role in infarctions and various ischemic pathologies [87]. It is also required for HIV-1 gene expression and regulation of cell tumors. A double-stranded DNA aptamer exhibiting high affinity binding to NF-κB and named as a "natural decoy" was investigated in vivo using a cardiac ischemic/reperfusion model and a significant effect in inhibiting this injury was observed [88]. In a rat model of thrombosis animals transfected with the NF-κB aptamer showed improved recovery of coronary flow (97% versus 61% in control) 3 days after transfection [89]. The aptamer-treated group demonstrated a lower percentage of neutrophil adhesion to endothelial cells (38% versus 81%) and a lower level of interleukin-8 (109 versus 210 ng/mg) as compared with control [89].

A fluorescent-labeled aptamer to NF-κB was investigated in a murine model of nephritis, where it blocked glomerular inflammation and expression of the inflammatory markers IL-1α, IL-1β, IL-6, ICAM-2, VCAM-1 [90].

Using the SELEX method an RNA aptamer was also genetade against the p50 subunit of NF-kB. Fourteen rounds of selection yielded the RNA-aptamer, exhibiting high affinity binding to p50 and inhibition of NF-kB binding to DNA by preventing protein dimerization [91].

CONCLUSIONS

Work with aptamers has important advantages over antibodies:

—in clinical practice aptamers may be applicable in the same fields where antibodies are already used for treatment, but in contrast to antibodies aptamers are non-immunogenic;

—aptamers exhibit the same high affinity to their protein targets as antibodies;

—aptamers can bind and penetrate to a pathological nidus faster than antibodies;

—aptamer antidotes may be developed and they can control activity of the administered aptamer.

The SELEX method originally developed for nucleic acid binding proteins is now actively used for studies of proteins, which lack natural complexes. The method is applicable for manipulations with individual proteins and for work with cell cultures.

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