Nucleic Acid Aptamers for Cardiovascular Therapeutics

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Abstract Nucleic acid aptamers can fold into a multitude three-dimensional structures allowing them to bind to almost any small molecule, nucleic acid or protein target with high specificity and affinity. Aptamers can be generated in a controlled and entirely in vitro process known as SELEX. Advances in aptamer technology have made possible the application of this therapeutic modality to many therapeutic areas including cardiovascular indications. To that end, aptamers have been generated to coagulants, adhesion, and angiogenic targets leading to the pre-clinical and clinical development of numerous aptamer drugs.

1 Nucleic Acid Aptamers

An aptamer is a structured nucleic acid molecule that binds tightly to a specific molecular target. The term "aptamer" is derived from the Latin word "aptus" that means "to fit." Aptamers fold into unique, stable tertiary structures that allow for molecular recognition through van der Waals, hydrogen bonding, and electrostatic interactions. From a mode of action point of view, aptamers can be thought of as nucleic acid or "chemical" antibodies. They bind with high affinity, K_D = pM–nM, and have the ability to disrupt protein–protein interactions. Aptamers are typically 15–40 nucleotides in length or 5–15 KDa and can be produced by a readily scalable

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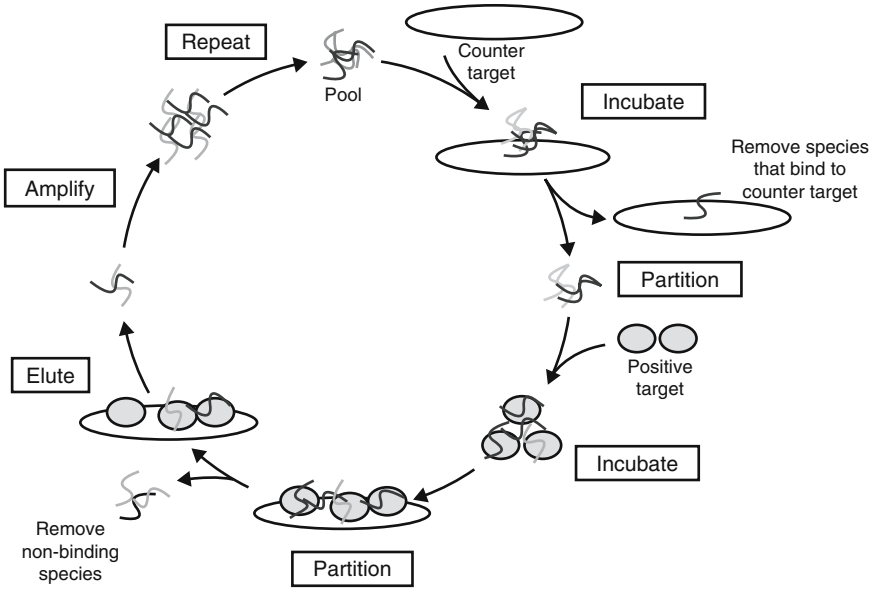

Fig. 1 General SELEX scheme. Pool RNA is mixed with the counter target (ex: selection matrix or counter-SELEX target). Molecules of the pool that do not bind to the counter target are partitioned from the bound molecules. These molecules are then incubated with the positive selection target. Target:RNA complexes are partitioned from non-binding molecules and bound molecules are eluted and then amplified. Multiple cycles of SELEX result in RNA aptamers that have high affinity and specificity toward the target

chemical manufacturing process. Aptamers are discovered through the application of the SELEX (Systematic Evolution of Ligands through EXponential enrichment) process (Fig. 1) (Tuerk and Gold 1990). The initial SELEX experiment was designed to study the key determinants for the binding interaction between T4 DNA polymerase and the RBS of its mRNA, yet Tuerk and Gold proposed that this process would allow for the ability to select nucleic acid-based binders to any molecule and these nucleic acid-based binders could modulate protein function. Since the introduction of the SELEX process in 1990, researchers have identified high affinity aptamers targeting a broad cross-section of protein families including cytokines, proteases, kinases, cell-surface receptors and cell adhesion molecules (Fig. 2).

2 Therapeutic Potential of Aptamers

In addition to target validation and research applications, aptamer technology has progressed to the point that aptamers can be developed as therapeutic agents. Although natural RNAs/DNAs are subject to nuclease degradation when dosed into animals, chemical modifications can be introduced to protect aptamers from serum endonucleases and exonucleases. For instance, endonucleases can be blocked by

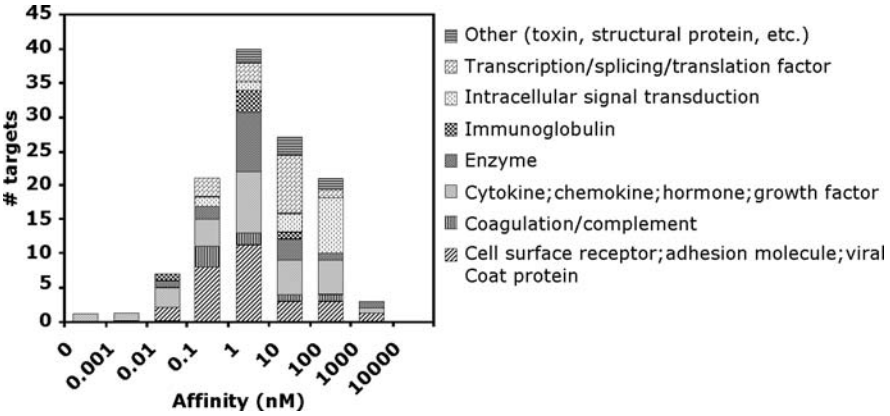

Fig. 2 Affinities for aptamers for different target types. Affinity versus number of aptamers selected plotted for a broad cross-section of targeted protein families

modifications to the 2′-hydroxyl position (Green et al. 1995). Nucleic acid-derived pools with chemical modifications at the 2′ position on the ribose can be used in the SELEX process to modulate the nuclease stability of output aptamers. These modified bases are chemically stable, nuclease resistant, and can be efficiently incorporated into the SELEX process through the use of T7 polymerase mutants (Burmeister et al. 2006). Exonucleases can be blocked by modification of the 5′- and 3′-ends of an aptamer by using a 3′-3′-linked thymidine cap for the 3′ end (Dougan et al. 2000) or by attaching a high molecular weight polyethylene glycol (PEG) group to either end. High molecular weight PEG has the added benefit of dramatically decreasing the clearance via the kidney of aptamer (Healy et al. 2004) and allowing increases in pharmacokinetic half-life by more than ten-fold (Ostendorf et al. 2001; Eyetech Study Group 2003).

Aside from their great specificity, affinity, efficacy and ability to modulate pharmacokinetic properties, aptamers have other competitive advantages over protein biologics. For instance, unlike antibodies, aptamers are produced by an entirely in vitro process. This allows for a more rapid generation of initial therapeutic leads and gives researchers more control over determining the specificity and affinity of said leads. Aptamers have also demonstrated little or no toxicity or immunogenicity and because of their superior solubility can be administered at high doses by subcutaneous injection as well as by intravenous injection (Cload et al. 2006).

3 Anticoagulant and Antithrombotic Aptamers

The primary control of bleeding is via the biochemical cascade that results in the formation of the hemostatic plug through fibrin polymerization and platelet activation. In the healthy blood vessel this cascade is critical to control bleeding. In the

atherosclerotic or inflamed blood vessel, these events can result in thrombosis with associated life-threatening myocardial infarction, stroke or other ischemic events that effect tissue and organ survival. The development of safe and effective agents to inhibit thrombosis has focused on specific enzymatic or cellular components of the clotting pathways. Factor Xa and thrombin have been and continue to be the primary approach to effective control of coagulation. Other approaches have been directed to Factor IXa and Factor VIIa/Tissue Factor. Cell mediated pathways have also been targeted. Inhibitors of the selectin molecule, P-selectin, have been shown to reduce both tissue factor mediated thrombosis and prothrombotic cell accumulation on the damaged vascular surface. Inhibitors of von Willebrand Factor (VWF) inhibitors have also been shown to reduce the initial platelet accumulation. Inhibitory aptamers have been developed to all of these targets. Multiple aptamers have entered into clinical development.

Thrombin specific inhibitory aptamers were first reported by Toole and colleagues (Bock et al. 1995). They reported on a single-stranded DNA aptamer containing a highly conserved 14–17 base region with binding affinities for thrombin of 25–200 nM. One aptamer, with binding specificity for alpha-thrombin (GGTTGGTGTGGTTGG), inhibited fibrin formation at nM concentrations. A role for the anion binding exosite in aptamer binding was suggested by the fact that hirudin competed with the aptamer for binding. The specific binding site of the aptamer was identified as Lys-21 and Lys-65 on the B chain located within or in proximity to the anion binding site, suggesting that the aptamer competes with fibrinogen and platelet thrombin receptor binding and inhibits thrombin-catalyzed cleavage of PAR-1 in a dose-dependent manner (Paborsky et al. 1993; Boncler et al. 2001). The anticoagulant activity of the aptamer was reported in two studies. The aptamer inhibited thrombin induced platelet aggregation with an IC50 of 70–80 nM and, in an ex vivo whole artery angioplasty model, the aptamer inhibited fibrinopeptide A production and platelet adhesion at both low and high shear conditions. Clot bound thrombin was also inhibited by approximately 80% (Li et al. 1994). Non-human primate studies in cynomolgus monkeys demonstrated a rapid prolongation of the prothrombin time during infusion and a rapid reversal once infusion was stopped. The half-life was estimated at 108 ± 14 s. Thrombin-induced platelet aggregation was also inhibited, while collagen aggregation was unchanged. The aptamer was evaluated in a sheep model of extracorporeal hemofiltration. The prothrombin time was doubled in the circuit during treatment. The authors concluded that the rapid onset and the short half-life could make such an aptamer a useful anticoagulant for extracorporeal circuits with some distinct advantages over current anticoagulant therapy (Griffin et al. 1993). As of 2007, the only thrombin inhibitory aptamer to reach a phase I clinical trials is ARC183. The administration of ARC183 resulted in a rapid onset of anticoagulation and demonstrated a stable, dose-associated anticoagulation. The effects of ARC183 rapidly reversed after drug infusion was stopped. The efficacy of ARC183 was limited by the amount of drug needed to achieve the desired anticoagulation. This resulted in an unacceptable dosing profile for use in coronary artery bypass graft surgery (CABG). A second generation molecule with greater potency is currently under

development (Hutabarat et al. 2007). This molecule has a very high affinity (K_D ~0.1 nM) for thrombin and, when administered by IV bolus + infusion to pigs and monkeys, it achieves a significant anticoagulation effect. In these studies, an ACT ≥400 s and a rapid reversal of the activity was noted within 25 min of stopping infusion. In a pig bypass pump model, this second generation molecule was administered at doses sufficient to maintain ACTs ≥400 s throughout a 3-h bypass procedure. It prevented clot formation while clots formed in the circuits of the saline control animals (Wagner-Whyte et al. 2007).

A primary stimulus to physiologic and pathologic clot formation is the Tissue factor/Factor VIIa complex. A specific stable RNA aptamer inhibitor of Factor VII/VIIa has been developed to inhibit this pathway. The aptamer was shown to inhibit tissue factor activation of Xa by VIIa with a prolonged half-life of approximately 15 h (Rusconi et al. 2000)

The Factor IXa inhibitory apatmer RB006 (Rusconi et al. 2004a,b; Nimjes et al. 2006) has resulted in a significant advancement in the development of anticoagulant aptamers. This aptamer did not use the strategy of unstable constructs to promote rapid inactivation by endo- and exonucleases that was part of the thrombin inhibition strategy. The high affinity and specific Factor IXa inhibitory RNA-based aptamer RB006 has been formulated to have a prolonged duration of action, using both chemical stabilization against nucleases and conjugation with 40-kD PEG to improve pharmacokinetic parameters. The mechanism of action for RB006 results from the inhibition of factor IXa's role in the generation of factor Xa. Its better stability and PK characteristics result in a more durable and controlled systemic anticoagulant and antithrombotic activity and a much slower reversal of Factor IXa inhibition compared to the thrombin apatmers previously discussed. In order to accelerate reversal, a modified-RNA oligonucleotide antidote complementary to RB006 and designated RB007 was developed. RB007 retains the rapid clearance of the less stable aptamers resulting in both neutralization and clearance of the RB006/RB007 complex. The results of the Phase I clinical trial was recently reported by Dyke et al. (2006). A predictable dose–response as measured by activated partial thromboplastin time was observed with escalating doses. Volunteers treated with the antidote RB007 had a rapid (1–5 min) and sustained return to baseline.

4 Antiadhesion Aptamers

Platelets and leukocytes have a central role in the thrombotic process. At the same time, the antithrombotic efficacies of inhibitors of platelet-leukocyte adhesion have been reported (Furie and Furie 2007; Hennan et al. 2006). P-selectin and von Willebrand Factor are two important mediators of platelet/leukocyte and platelet adhesion that have been targets for anti-adhesion apatmer development. Anti-P-selectin aptamers with an affinity of 16–710 pM (10^5–10^6 higher than the native minimal carbohydrate ligand sialyl Lewis X) were reported by Jenison et al. (1998). The aptamers were found to bind with subnanomolar affinity to P-selectin expressed

on the surface of thrombin-activated platelets and blocked the binding of activated platelets to neutrophils. In vitro studies on sickle cell disease were recently reported, with one of these aptamer clones (PF377), for its ability to prevent cellular adhesion of SS-RBCs to endothelial cells. The aptamer binds P-selectin with a Kd of 6 nM and a Bmax of 87%. The aptamer at 60 nM had anti-adhesion activity similar to heparin and an inhibitory antibody to P-selectin in inhibiting sickle rbc adhesion (Nishimura et al. 2007). No in vivo data have been reported.

von Willebrand Factor (VWF) multimers adhere to exposed collagen via the vWF A3 domain and, under conditions of high shear force common to diseased arteries with luminal atherosclerotic lesions, platelets in transit through these vessels become immobilized through interactions between the adherent VWF A1 domain and the platelet glycoprotein Ib (GPIb) receptors. This interaction stimulates platelet activation, recruitment and thrombus formation. Aptamers to VWF that inhibit these interactions have been developed. A high affinity VWF aptamer that can inhibit VWF mediated platelet aggregation and can be reversed by an antidote similar to that described above for Factor IXa has been reported (Oney et al. 2007). A more complete characterization of a second VWF aptamer with specificity for the A1 domain, has been completed (Lagassé et al. 2007a; Rottman et al. 2007). The aptamer has high affinity for human VWF (K_D = 1.4 nM, or ~0.02 μg ml^{-1}) and minimal binding to other closely related A1 domain-containing proteins CD49c, ICAM, collagen type VI and Factor B, as well as complement factors C3 and C5, prothrombin, thrombin, Factor Xa, fibrinogen and HSA. Assessment by ELISA for inhibition of VWF activity demonstrated that the aptamer was a potent inhibitor of VWF activity. The IC_{90} value for inhibition of VWF activity in human plasma was 3.72 μg ml^{-1} (~283 nM). Human platelet aggregation was also assessed with the PFA-100®. The IC_{90} and maximal prolongation of closure times (≥300 s) values were 1.15 μg ml^{-1} (0.088 μM). Despite potent inhibition of VWF-mediated platelet aggregation, concentrations up to 130 μg ml^{-1} or 10 μM had no effect on the VWF-independent platelet aggregation induced by platelet agonists such as epinephrine, arachidonic acid, ADP, collagen and thrombin. The aptamer demonstrated a significant anti-thrombotic activity when it was administered to 15 monkeys by a bolus + continuous iv infusion at doses of 100 μg kg^{-1} bolus + a 1 μg kg^{-1} min^{-1} infusion up to a 600 μg kg^{-1} bolus + a 3.7 μg kg^{-1} min^{-1} infusion. In this study, it was compared to saline and the GPIIb/IIIa inhibitor abciximab (250 μg kg^{-1} bolus + a 0.125 μg kg^{-1} min^{-1} infusion. Bleeding times were minimally elevated over saline except at the highest dose of aptamer tested. This aptamer has also been evaluated in a Phase I clinical trial (Gilbert et al. 2007). This was a randomized, double-blind, placebo-controlled study in 47 healthy volunteers at doses of 0.05–1.0 mg kg^{-1}. PD effects were measured by an ELISA for free VWF A1 binding sites and by a platelet function analyzer. The concentration-time profile appeared monophasic. C_{max} and AUC were dose-proportional. The mean apparent elimination half-life ($t_{1/2\beta}$) was ~2 h and mean residence time (MRT) was ~3 h. The mean apparent volumes of distribution (V_z and V_{ss}) were ~1/2 the blood volume, suggesting that distribution is in the central compartment. The mean clearance (CL) ranged from ~10 to 21% of GFR, suggesting that renal filtration may not be a major

mechanism of clearance. Inhibition of VWF A1 binding activity was achieved with an EC_{90} value of 2.0 μg ml^{-1} (151 nM) and of platelet function with an EC_{90} value of 2.6 μg ml^{-1} (196 nM). The aptamer was generally well tolerated and no bleeding was observed. Adverse events tended to be minor and not dose related.

5 Antiangiogenic Aptamers and Potential for Treatment of Atherosclerosis

Inhibition of angiogenesis has been one of the early target areas of interest for the development of therapeutic aptamers. In fact, the first approved aptamer (Macugen, penaptamib sodium) inhibits vascular endothelial cell growth factor (VEGF) and the abnormal vasculogenesis associated with wet age-related macular degeneration (AMD) (Ng and Adamis 2006). An aptamer inhibiting the angiogenic factor platelet derived growth factor (PDGF-B) and an antiangiopoietin-2 (Ang-2) aptamer are also under development for treatment of AMD. Antiangiogenic aptamers have also been a focus for the treatment of cancer by reducing vascular growth, and thus the nutrient supply necessary for tumor growth and vascular channels that carry metastatic cells. This effort has included studies of aptamers inhibiting PDGF-B, Ang-2, and VEGF (Sennino et al. 2007; Sarraf-Yazdi et al. 2007; Huang et al. 2001). Angiogenesis and the inhibition of angiogenesis by aptamers may also have an important therapeutic benefit in the treatment of atherosclerosis. The outer layer of blood vessels, the adventitia, is characterized by a vascular network known as the vasa vasorum. The normal function of this network is the supply of nutrient flow to the blood vessel. However, in the developing atherosclerotic lesion, there is significant vascular proliferation in the vasa vasorum, and this proliferation can extend into the media (smooth muscle rich portion of the vessel wall) and the atherosclerotic lesion. Like the abnormal, leaky vessels observed in AMD and within tumors, these vessels are also leaky. In addition, it is thought that they may also be the source of infiltrating inflammatory cells that can both increase the lesion growth as well as further destabilize the lesion resulting in intralesion hemorrhage and thrombosis (Doyle and Caplice 2007; Kahlon et al. 1992; Michel et al. 2007; Chyu and Shah 2007; Petrovan et al. 2007; Kolodgie et al. 2007). The source of the angiogenic stimulus may come from one of the key cells known to be involved in the development of the proliferative atherosclerotic lesion, the platelet. Angiogenic growth factors have been reported to be stored and released from platelet granules (Italiano et al. 2008). Thus, localization of platelets in an area of lesion formation could also be expected to release factors that would also promote local angiogenesis. It would be expected that aptamers inhibiting one or more of these angiogenic factors could have therapeutic benefit in the treatment of lesions, especially the highly vascular unstable lesion or early lesions created after dilation by stent placement. PDGF inhibitory aptamers have been evaluated their effect on smooth muscle proliferation following balloon injury of the rat carotid artery, a model of post angioplasty re-occlusion. Treatment was shown to be effective during a 2-week period.

However, once treatment was stopped, the beneficial effect was lost. This suggested to the authors that a longer treatment would be required for full therapeutic benefit (Leppanen et al. 2000). No other studies with antiangiogenic aptamers in atherosclerosis have been reported.

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