Innovative Drug Design Using RNA Aptamers for Various Anaemias

a report by

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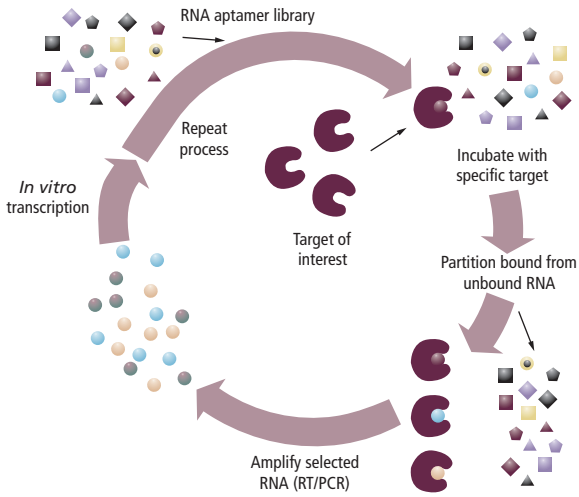
Aptamers are oligonucleotides that bind to molecular targets in a manner conceptually similar to antibodies.[1,2] Through the systematic evolution of ligands by exponential enrichment (SELEX) process, aptamers have been identified against a wide range of therapeutic targets.[3–8] Synthetic ribonucleic acid (RNA) aptamers can be modified to have greatly enhanced plasma stability and longer circulating half-lives, and experience to date suggests that aptamers have low toxicity and immunogenicity *in vivo*. Aptamers can directly inhibit a protein's function by folding into specific 3D structures that provide high-affinity binding to the target protein. To treat various anaemias, including paroxysmal nocturnal haemoglobinuria (PNH) and sickle cell disease (SCD), we have utilised a technology to identify RNA aptamers.

Ribonucleic Acid Aptamers and the Systematic Evolution of Ligands by Exponential Enrichment System

RNA molecules that bind specific ligands are known as aptamers.[9] A selection process to identify and isolate aptamers with specific binding affinities is SELEX.[10] The starting point for this *in vitro* selection is a combinatorial RNA library composed of $10^{14–15}$ single-strand nucleic acids, each containing 20–40 nucleotides of random sequence. The basic approach to *in vitro* selection is outlined in *Figure 1*.[1,2] A starting library of RNA nucleic acids is incubated with the protein target of interest. Molecules that bind to the target protein are partitioned from other sequences in the library. The bound sequences are then amplified repeatedly to generate an aptamer pool enriched in sequences that bind to the target protein. After several rounds of incubation and washing (usually eight to 12), which are typically performed with increasing stringency, the selected RNA ligands are sequenced and evaluated for their affinity to the targeted protein and their ability to inhibit the activity of the targeted protein *in vitro*.

Aptamers are stable under a wide range of buffer conditions and resistant to harsh treatments. Aptamers can be isolated by a simple *in vitro* process for virtually any target, even those that are toxic or have low immunogenicity. Aptamer production takes about eight to 12 weeks with lower costs, whereas antibody production takes about six months. Aptamers can be chemically synthesised, offering a wide variety of targeted modifications such as fluorescent reporters or affinity tags. 'Antidotes', which are short complementary sequences (antisense) to the aptamers, can reverse the properties of aptamers.[6] Aptamer–antidote pairs are safe and highly regulatable drugs. Aptamers can mediate small interfering RNA (siRNA) delivery: cell-type-specific delivery of siRNA with aptamer-siRNA chimaeras.[7] The first aptamer drug (Macugen), which is an anti-vascular endothelial growth factor aptamer for the treatment of age-related macular degeneration, has been approved by the US Food and Drug Administration (FDA),[11] and additional aptamers are in the clinical pipeline.

Figure 1: Overview of the Systematic Evolution of Ligands by Exponential Enrichment Process



Anticomplement Aptamers for the Treatment of Paroxysmal Nocturnal Haemoglobinuria

Paroxysmal nocturnal haemoglobinuria (PNH) is an acquired haematopoietic stem cell disorder.[12] The most common clinical manifestations of PNH include intravascular haemolysis, venous thrombosis, bone marrow failure and occasional transition to a myelodysplastic syndrome or acute leukaemia. The main biochemical feature in PNH is the absence of glycosyl-phosphatidylinositol (GPI)-anchored proteins on the surface of the affected cells, because of incomplete bioassembly of the GPI anchor affixing each protein to the cell surface. The lack of GPI-anchored complement regulatory proteins, including CD55 but especially CD59, results in complement-mediated haemolysis and haemoglobinuria, which is a major clinical manifestation of PNH and an important cause of morbidity and mortality (see *Figures 2A* and *2B*). A humanised monoclonal anti-C5 antibody (eculizumab) that inhibits terminal complement protein activation has recently been approved for PNH in the US and Europe,[13–15] suggesting that blocking complement activation is a new therapeutic option for PNH that could control haemolysis, reduce transfusion dependency and possibly reduce the risk of thrombosis.[16] However, because C5 is critical not only for complement

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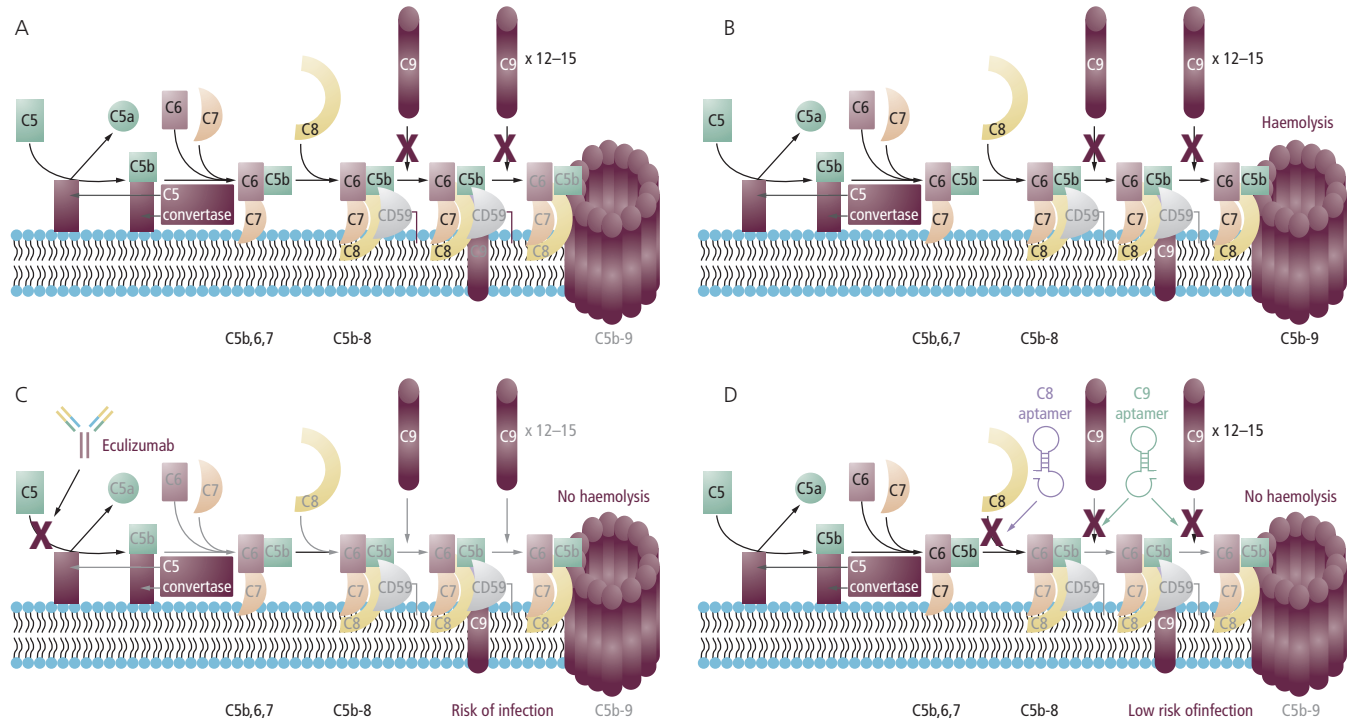
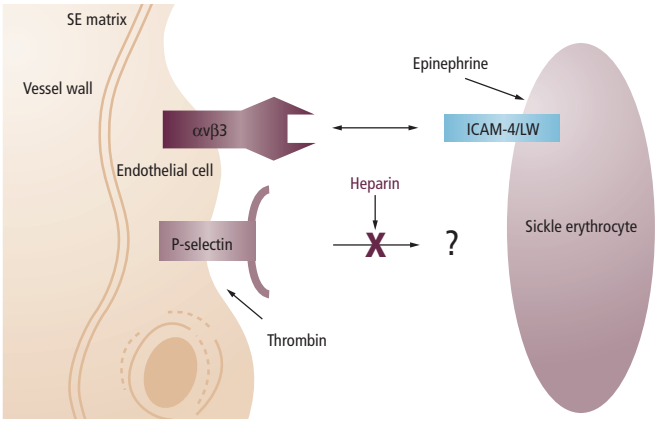
Figure 2: The Complement Pathways



Targeted inhibition. A: Healthy individual: CD59 covers the site for C9 to bind and unfold. B: Paroxysmal nocturnal haemoglobinuria (PNH) patient: CD59 is missing and C9 unfolds, causing initial membrane disruption. After binding additional C9s, the C9 multimer forms a channel on the cell membrane, leading to cell lysis. C: PNH patients with eculizumab (C5 Ab): eculizumab binds to C5, inhibiting its cleavage into C5a and C5b, thereby preventing the formation of the lytic membrane attack complex (MAC) (C5b-9). D: PNH patients with C8 and C9 aptamers: C8 and C9 aptamers bind C8 and C9, respectively, preventing the formation of the lytic MAC.

Figure 3: Adhesive Interactions Between Sickle Erythrocytes and the Endothelium



activation but also for proper regulation of inflammatory processes, prolonged C5 inhibition could have undesired side effects that would limit its utility in PNH (see *Figure 2C*). Terminal complement proteins with more restricted function may represent better targets. Terminal complement proteins C8 and C9 represent potentially better targets, because CD59 inhibits C9 binding to the C5b8 complex. They are critically important in protecting erythrocytes from complement-mediated lysis (see *Figure 2D*). Indeed, inherited deficiencies in complement proteins typically lead to various degrees of illness, including recurrent (neisserial) infection, systemic lupus erythematosus and immune complex diseases, but individuals deficient in C9 are usually healthy. In addition, a patient with PNH and more than 80% type III erythrocytes and a concomitant deficiency in C9 has been asymptomatic (no severe infection and no visible haemolysis) for more than 20 years, indicating that C5b8 complex without C9 might be sufficient to protect from infections but insufficient to haemolyse.[17]

We have therefore begun to identify RNA aptamers that inhibit human complement proteins C8 and C9 by using the SELEX system. One C8 aptamer cloned after four rounds (four-101) and one C9 aptamer cloned after 16 rounds (16-eight) with strong binding (Kd of 15nM and Bmax of 74%, and Kd of 7nM and Bmax of 67%, respectively) and inhibition activity of haemolysis (over 90% inhibition at 250nM, and over 80% inhibition at 125nM, respectively) have been identified.[18–20] These aptamer clones are currently being modified in preparation for future *in vivo* studies using a PNH mouse model. The development of blocking aptamers against terminal complement proteins represents a novel potential therapeutic option for patients with PNH.

Anti-adhesion Aptamers for the Treatment of Sickle-cell Disease

Vaso-occlusive crises are the major clinical feature of SCD, and the adhesion of sickle erythrocytes (sickle red blood cells [SS-RBCs]) to vascular endothelium is important to the generation of vaso-occlusion.[21] SS-RBCs express many adhesion molecules, such as LW (intercellular adhesion molecule [ICAM-4]). Adhesive SS-RBCs may bind to endothelial cell P-selectin, integrins, and extracellular matrix proteins. All of these molecules are potential targets for reagents to prevent or treat the vaso-occlusive crises of SCD (see *Figure 3*). Dr Telen's group has identified an adhesion receptor, ICAM-4, on SS-RBCs that mediates adhesion to endothelium through at least one direct ligand, αVβ3 integrin.[22,23] Importantly, activation of ICAM-4 by the physiological stress mediator epinephrine significantly enhances SS-RBC adhesion. Another important interaction is mediated via thrombin, which causes endothelial retraction with exposure of proadhesive extracellular matrix components and the endothelial expression of P-selectin, involved in erythrocyte, white cell and

platelet–endothelial interactions.[24] To inhibit red cell adhesion for the treatment of SCD, we have begun to target three important adhesion molecules – including αVβ3 integrin, P-selectin and ICAM-4 – using the SELEX technology.

We have synthesised a high-affinity aptamer clone (17.16) that binds to human integrin αVβ3.[25] To measure its anti-adhesion activity, an *in vitro* flow chamber assay was adopted. The anti-adhesion activity of the aptamer clone (17.16) was tested using human umbilical vein endothelial cells (HUVEC) treated with thrombin using a flow chamber assay. Normalised percentage inhibition of aptamer clone (17.16) at 60nM at two dynes/cm^2 was over 70%.[26] We have also synthesised a high-affinity aptamer clone (PF377) that binds to human P-selectin.[27] The anti-adhesion activity of aptamer clone (PF377) was tested using primary HUVEC treated with interleukin (IL)-13, followed by stimulation with histamine immediately prior to a flow chamber assay. Normalised percentage inhibition of aptamer clone (PF377) at 60nM at one dyne/cm^2 was over 90%.[28] Since we confirmed strong anti-adhesion activity of aptamer clones (17.16 and PF377), *in vivo* experiments in mice are currently employing intravital microscopy to measure anti-adhesion activity of aptamer clone 17.16, PF377 and its combination, although ICAM-4-binding aptamers are currently being screened using the SELEX method. The development of combinatorial blocking aptamers against various adhesion molecules, including αVβ3, P-selectin and ICAM-4, represents a novel potential therapeutic option for patients with SCD.

Aptamers that Control/Enhance Erythropoiesis

There are several common clinical situations in which anaemia is the result of hypoproliferation of RBC precursors secondary to erythropoietin (Epo) deficiency. These include anaemias associated with chronic kidney disease, chronic inflammation and cancer. Thus, erythropoiesis is mainly regulated by Epo and stem cell factor (SCF). Agonistic aptamers will be therapeutically beneficial, and we have indeed experienced that dimerised or multimerised aptamers could have agonistic activity. Since we have identified c-kit (SCF-ligand) binding aptamer clones, these clones are currently being tested for their effect on the downstream signaling pathway. It is possible to identify Epo-receptor-binding agonistic aptamers in a similar manner.

Conclusions

Eculizumab is a highly effective therapy for haemolysis in PNH, and seems to be a safer treatment at the moment, with up to 4.5 years of follow-up. However, once a patient with PNH initiates treatment with eculizumab, it may not last long, because PNH is generally a chronic disorder. Therefore, it is not clear whether prolonged C5 inhibition could have undesired side effects in the following decades. Thus, the development of blocking aptamers against terminal complement proteins (C8 and C9) is theoretically safer than the C5 inhibitor and represents a novel potential therapeutic option for patients with PNH.

Table 1: Aptamers versus Antibodies

Aptamers can be modified to have greatly enhanced plasma stability and longer circulating half-lives.
Experience to date suggests that aptamers have low toxicity and immunogenicity *in vivo*.
'Antidotes', which are short complementary sequences (antisense) to aptamers, can reverse the properties of aptamers. Aptamer–antidote pairs are safe and highly regulatable drugs.
Aptamers can mediate small interfering ribonucleic acid (siRNA) delivery: cell type-specific delivery of siRNA with aptamer-siRNA chimaeras.
Aptamers can be isolated by a simple *in vitro* process for virtually any target, even those that are toxic or have low immunogenicity, if the purified target and its screening assay system are available.

SS-RBCs may be a better and more specific target to prevent vaso-occlusion in SCD than vascular endothelium. However, several adhesion molecules in addition to ICAM-4 have been identified, although others (e.g. the ligand for P-selectin) remain unidentified at the moment. Therefore, we propose to identify specific molecular expression on SS-RBCs and inhibit their adhesion to prevent vaso-occlusion in SCD using modified SELEX technology – termed 'complex target' SELEX – selection with activated SS-RBCs as a positive target and non-activated SS-RBCs as a negative target. The concept of complex-target SELEX, whereby targets are not pure proteins but may include whole organisms, intact cells or human plasma, may lead to the identification of aptamers against proteins that can be targeted only in their physiological milieu.[29] A number of functionally exciting RNA aptamers have been selected against a wide range of targets through SELEX to date. Aptamers are attractive alternatives to antibodies for certain therapies (see *Table 1*). In the future, agonistic aptamers will be therapeutically beneficial. ■

Acknowledgements

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