Turning aptamers into anticoagulants

Michael Famulok

An RNA-based anticoagulant and antidote are effective at controlling blood coagulation in animals.

Blood coagulation involves a delicate balancing act. It must occur immediately in response to trauma to avoid blood loss, yet it would be fatal if it occurred in an uncontrolled fashion. Accordingly, drugs that modulate blood coagulation should allow exquisite control of this vital process. But existing anticoagulant drugs are relatively crude, with excessive bleeding a common side effect. In this issue, a study by Rusconi *et al.* suggests that an RNA-based anticoagulant aptamer and its RNA antidote represents a safer approach to anticoagulant therapy[1].

Blood clotting is accomplished by activation of a cascade of proteolytic zymogens that ensures a rapid and amplified reaction to injury. Abnormal clotting arising either from disease or during cardiopulmonary surgery is a major cause of disability and death. Anticoagulant therapy for such patients is widespread, emphasizing the need for a new generation of anticoagulant drugs that can be rapidly neutralized by antidotes.

Rusconi *et al.* originally obtained their anticoagulant aptamer by screening a large combinatorial library of RNA sequences for nucleic acid ligands, or aptamers, that specifically bound to one member of the human zymogen activation cascade, the coagulation factor IXa. In plasma assays, the aptamer acted as a potent factor IXa inhibitor with strong anticoagulant activity[2].

Aptamers are short single-stranded oligonucleotides that fold into distinct three-dimensional structures that recognize target molecules. Binding of an aptamer to its target can be prevented by disrupting the aptamer's structure, for example, through addition of a complementary oligonucleotide that hybridizes to the aptamer. Thus, the design of an aptamer-antidote pair is intuitive: each aptamer sequence inherently carries the prescription for its antidote. Indeed, an antidote oligonucleotide complementary to one half of the anti-factor IXa aptamer efficiently neutralized the aptamer's anticoagulant activity in plasma assays *in vitro*[2].

In the present study, Rusconi *et al.* evaluated the activity of this aptamer-antidote pair in two clinically relevant animal models: a porcine anticoagulation model and a murine thrombosis model. Moving from *in vitro* studies to animals is not trivial. Two severe problems are rapid clearance of aptamers from circulating blood and blood nucleases that rapidly hydrolyze both aptamers and antidotes before they reach their target in the bloodstream. A solution to the latter problem was achieved previously with 2′-fluoro-2′-deoxy pyrimidine–modified aptamers[2], which are nuclease-resistant[3]. The aptamer's plasma residence time was increased by attachment of a cholesterol group[4] (**Fig. 1**). Not only did the cholesterol-modified aptamer exhibit a significantly extended blood half-life compared with the nonderivatized version, it also increased the clotting time in pigs. The antidote neutralized this effect quickly, and Rusconi *et al.* provide evidence that this was due to the expected association with the aptamer rather than to some incidental clotting-related activity.

Most important, the antidote's neutralization activity rapidly levelled off, suggesting that blood clotting could be inhibited repeatedly after antidote administration by reinjecting the aptamer. In this respect, the aptamer-antidote pair appears to be advantageous compared with the most common anticoagulant-antidote pair used in clinical

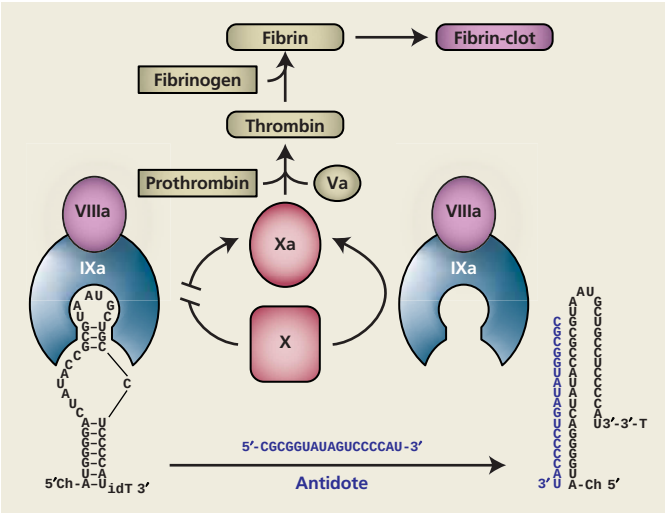

Figure 1 Mechanism of the aptamer-antidote pair. The aptamer is stabilized by a 3′-3′-linked deoxythymidine at the 3′-end and by 2′-fluoro-2′-deoxy modifications at every pyrimidine residue. It carries a cholesterol-modification at the 5′-end to increase the plasma residence time. The aptamer binds to activated factor IX and prevents the proteolytic cleavage of factor X (left). In the presence of the antidote (blue sequence), the aptamer is released from factor IXa (right). Together with activated factor VIII (VIIIa), factor IXa catalyzes the cleavage of factor X to yield activated factor X (Xa), which is required for the blood clotting cascade: with factor Va, Xa cleaves prothrombin to yield thrombin, which catalyzes cleavage of fibrinogen to yield fibrin. Cross-linked fibrin forms the clot.

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practice, namely heparin and its neutralizing polypeptide protamine.

Blood clotting is mediated by two pathways: an extrinsic pathway, activated as a consequence of trauma, and an intrinsic pathway, activated by rupture of the endothelial lining of the blood vessels. Both cascades converge on a common pathway that finally induces cross-linking of the protein fibrin, which forms the clot[5] (**Fig. 1**). Rusconi *et al.* used a murine arterial injury model to show that the aptamer also prevented occlusive thrombus formation compared with a nonfunctional mutant aptamer. Using a second mouse model, they investigated whether the aptamer-antidote pair could control bleeding caused by surgical trauma following treatment with high doses of the aptamer. Indeed, application of the antidote immediately after surgical injury significantly reduced blood loss and restored the procoagulant activity of factor IX, indicating that the antidote inactivates the aptamer *in vivo* extremely quickly.

These promising results further demonstrate the potential of aptamer technology in drug development. Despite the urgent need for novel selective inhibitors of activated zymogens in the coagulation cascade, the search for such compounds, let alone combinations of drug-antidote pairs, has been very difficult. The advantage of Rusconi *et al.*'s approach is the ease with which such aptamer-antidote pairs can be identified and their impressive performance *in vivo*.

The future of aptamer anticoagulants depends in part on how they will compete economically with the heparin-protamine system, the current standard of clinical practice. Large quantities of the aptamer and antidote will be required for widespread applications. The synthesis of a chemically highly modified aptamer, like the anti-factor IXa aptamer, in kilogram quantities is probably possible[6,7], but may still be an expensive alternative to heparin/protamine. The promise of this aptamer-antidote pair and of several other therapeutic aptamers in clinical development[8] should encourage investment in the development of methods for large-scale syntheses of functional nucleic acids. Certainly, economic issues will not be a priority for those patients who do not tolerate classical treatment. However, this economic issue is perhaps best treated according to the old Latin proverb: "*Prius antidotum quam venenum*" ("Don't take the antidote before you are poisoned"), which can be interpreted to mean "let's not worry about it until we have to."

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Fluorescent proteins in a new light

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A new photoswitchable fluorescent protein will facilitate protein tracking in cells.

Painting a complete picture of a protein's movement in a cell often can prove difficult using the small palette of fluorescent proteins currently available[1]. To be ideal, such protein paints should be photoconvertible, high-contrast and monomeric. In this issue, Chudakov *et al.*[2] describe the development of just such a protein, photoswitchable cyan fluorescent protein (PS-CFP), which changes from a cyan to a green fluorescent form upon irradiation at 405 nm.

PS-CFP was generated by mutagenesis of aceGFP, a green fluorescent variant of a colorless *Aequorea coerulescens* protein. As PS-CFP is monomeric, it can likely be fused to other proteins without altering their behavior. Because of the high contrast between optically labeled and unlabeled proteins, photoswitched proteins can be tracked in space and time. The authors demonstrate the utility of PS-CFP for visualizing filopodial transport and the constitutive endocytosis of a human dopamine transporter (hDAT), uncovering a direct exchange of cargo proteins between physically interacting endosomes.

The recent realization that the spatiotemporal regulation of signal transduction is unexpectedly dynamic has provoked interest in the diffusion and transport of proteins involved in signal transduction pathways. The mobility of fluorescently labeled molecules has conventionally been assessed using a photobleaching technique called fluorescence recovery after photobleaching (FRAP)[3]. This method, which allows the acquisition of a series of images after photobleaching a small region of the cell, is useful for semi-quantitative descriptions of the diffusion of fluorescently labeled molecules (**Fig. 1a**). However, because it involves full photobleaching with relatively long and intense illumination in a specific region of the cell, FRAP has been useful for measuring the relatively slow movements of proteins within membrane structures with a diffusion coefficient below 1 $\mu m^2/s$.

In contrast, tracking faster protein movements requires optical marking techniques that employ photoactivation (**Fig. 1b**) or photoconversion (**Fig. 1c**). Fluorescently tagged proteins can be optically labeled by partial photoactivation or photoconversion using unique color markers that require faster, less intense light exposure, thus permitting measurements of fast protein movements with a diffusion coefficient greater than 1 $\mu m^2/s$.

Several methods of optical marking have been developed based upon photo-induced alteration of the excitation or emission spectra of certain fluorescent proteins, such as wild-type *A. victoria* GFP[4,5] and DsRed[6]. However, these methods are limited by the dimness and instability of the photoconverted product and by the unavailability of simple, efficient and specific illumination for marking.

In the past few years, three fluorescent protein variants have been generated that overcome some of these limitations and allow for efficient, selective optical labeling: photoactivatable GFP (PA-GFP)[7], Kaede[8] and kindling fluorescent protein 1 (KFP1)[9]. To highlight the advanced features of PS-CFP, I shall compare it to PA-GFP and Kaede.

PA-GFP is a derivative of *A. victoria* GFP, whose performance has been proven over a decade of research with GFP fusion proteins. *A. victoria* GFP has a bimodal absorption or excitation spectrum with two peak maxima at 395 and 475 nm, which correspond to the protonated and the deprotonated states of the chromophore, respectively. When

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