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A New Anticoagulant−Antidote Pair: Control of Thrombin Activity by Aptamers and Porphyrins

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Aptamers are nucleic acids generated by in vitro selection techniques that are able to specifically bind to ligands such as proteins.[1] A series of aptamers targeting blood clotting factors have been described that are highly effective in inhibiting blood coagulation and thus act as anticoagulants.[2] In applications, such as cardiopulmonary bypass surgery, it is crucial to reverse the anticoagulant effect. For this reason, anticoagulant/antidote effector pairs are required, and only one such pair, consisting of heparin as anticoagulant and protamine as antidote, is currently used in daily clinical practice. However, the use of heparin is associated with potential drawbacks including immune reactions.[3] Aptamers that interfere with the blood clotting cascade in turn offer the advantage that antisense molecules can be easily designed that revert the aptamer's action, acting as antidote.[3] Recently, this approach has been developed further by introducing caged thrombin aptamers that can be controlled using light excitation.[4]

The distinct structural features of the thrombin-inhibiting aptamer containing a G-quadruplex open an alternative approach to fine-tune aptamer function. Quadruplex-binding compounds such as the cationic porphyrin TMPyP4 (*meso*-5,10,15,20-tetrakis-(*N*-methyl-4-pyridyl)porphine) have attracted much attention since they bear the potential to interact with G-quadruplexes found in telomere ends of the chromosomes and promoters of proto-oncogenes, thereby often displaying anti-cancer activities.[5] Here, we have investigated if G-quadruplex-binding porphyrins are suited to control the anticoagulant activity of a G-quadruplex-containing aptamer that binds and inhibits human α-thrombin. We demonstrate that a cationic porphyrin, namely, TMPyP4, is able to bind to the aptamer and hence interferes with its active conformation. Consequently, TMPyP4 antagonizes the aptamer-mediated inhibition of blood clotting in a concentration-dependent manner, thus representing a true small molecule antidote of the anticoagulant DNA aptamer.

Interactions of porphyrins with G-quadruplex structures are well characterized.[6] Cationic porphyrins such as TMPyP4 are known to bind to quadruplex structures such as the vertebrate telomeric sequence as well as G-rich promoter elements in proto-oncogenes such as c-myc.[7] Recently, we have engineered hammerhead ribozymes that are regulated by a TMPyP4−quadruplex interaction.[8] Nevertheless, so far, it has not been demonstrated that such interactions could be used to control the activity of functional aptamers containing G-quadruplex elements. In Figure 1A, the crystal structure of human α-thrombin in complex with the quadruplex-forming DNA aptamer inhibitor is shown.[9] Since the aptamer folds into an active G-quadruplex structure and thus allows specific binding to the blood clotting factor thrombin, interaction of G-quadruplex-binding porphyrins might interfere with the capability of the aptamer to bind to thrombin. In order to test this hypothesis, we have first characterized the interaction of TMPyP4

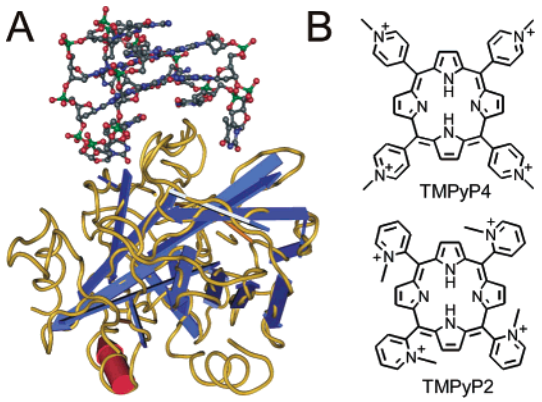

Figure 1. TMPyP4 as antidote of the anticoagulant DNA aptamer. (A) Complex of DNA aptamer and human α-thrombin.[9] The quadruplex-forming aptamer is shown in ball and stick representation, thrombin as ribbon. (B) Cationic porphyrins used in this study.

with the anti-thrombin aptamer by measuring porphyrin fluorescence emission at 650 nm.[10] In addition, TMPyP2 was used as a control since it is known that the *N*-methyl-2-pyridyl analogue binds with much lower affinity to quadruplex structures.[5] In Figure 2A, increasing emission of TMPyP4 is observed upon binding to the DNA aptamer. Half-maximum binding occurs at an aptamer concentration of 570 nM with 500 nM porphyrin used. Fitting of the fluorescence changes revealed a dissociation constant of $k_D = 295 \pm 80$ nM for the TMPyP4−aptamer complex (see Supporting Information). Interaction of TMPyP2 with the DNA aptamer was not observed up to a concentration of 10 μM aptamer. The thrombin aptamer folds into an intramolecular, chair-like antiparallel quadruplex structure with a characteristic circular dichroism spectrum with a minimum at 265 nm and a maximum at 295 nm.[11] In order to elucidate whether binding of TMPyP4 to the aptamer interferes with G-quadruplex formation, we measured CD spectra and thermal denaturation of the DNA aptamer in the presence of TMPyP4. With increasing porphyrin concentrations, disruption of the quadruplex fold was observed by CD (see Figure 2B). Destabilization of the quadruplex was also confirmed by determining the thermal melting point of the four-stranded structure in dependence to TMPyP4 (see Supporting Information Figure S3). This is an interesting finding since other G-quadruplexes are frequently found to be stabilized by cationic porphyrins.[5b,7b]

Next, we have used TMPyP4 to disrupt the anticoagulant protein−DNA interaction. In Figure 3, blood clotting assays are shown. Addition of the DNA aptamer results in prolonged clotting times. Interestingly, TMPyP4 is able to completely antagonize the aptamer-mediated inhibition of blood clotting in a concentration-dependent manner. The increased effective concentrations of TMPyP4 in the clotting assay relative to the binding studies with the aptamer alone likely result from competition of the small

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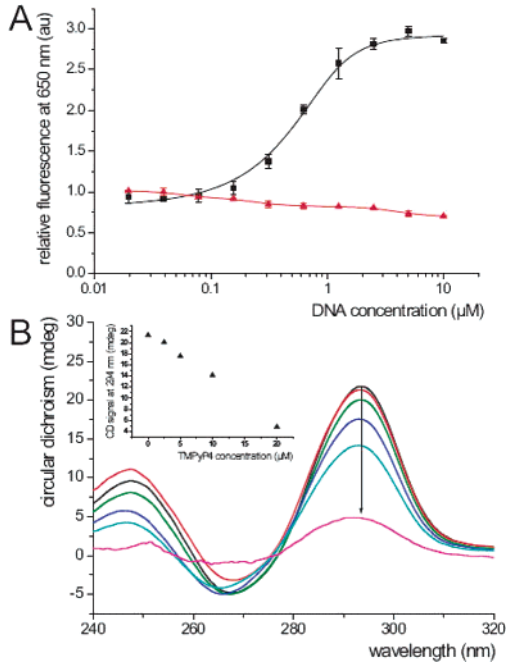

Figure 2. Interaction of cationic porphyrins with the anti-thrombin DNA aptamer. (A) Binding curves were obtained by measuring induction of fluorescence upon interaction with the aptamer, see inset. Black: TMPyP4; red: TMPyP2; porphyrin concentration was 0.5 μM with excitation at 420 nm, emission at 650 nm. (B) CD spectra of TMPyP4−DNA aptamer complexes show that the porphyrin disrupts the G-quadruplex structure. Black line: absence of TMPyP4; red: 0.5 μM; green: 1 μM; blue: 5 μM; cyan: 10 μM; purple: 20 μM TMPyP4; aptamer concentration = 5 μM.

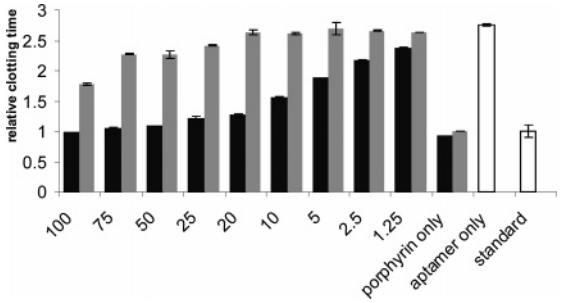

Figure 3. Regulation of blood clotting. Relative clotting times of plasma are shown. Black bars: TMPyP4; gray bars: TMPyP2. Clotting reactions contained 1 μM DNA aptamer except reactions "standard" and "porphyrin only". Numbers indicate porphyrin concentrations (μM). Reactions of "porphyrin only" contained 100 μM of TMPyP4 and TMPyP2. The absolute clotting time in the absence of the aptamer (standard) was 18.9 s, and in the presence it was 53.5 s. For experimental details, see Supporting Information.

molecule with thrombin already bound in the preformed protein−aptamer complex. In addition, unspecific binding of the porphyrin to other components of the blood plasma could contribute to this observation. Importantly, even concentrations of porphyrins as high as 100 μM did not alter the clotting time in the absence of the aptamer; see "porphyrin only" in Figure 3. In accordance with the binding studies, TMPyP2 turned out to be much less efficient in reverting the clotting time.

In conclusion, we have shown that it is possible to antagonize the anticoagulant activity of a DNA aptamer by a small molecule. Since the effect relies on the binding of a ligand to a G-quadruplex structure within the DNA aptamer, the principle might be extendable to other aptamers containing similar structural elements. For example, G-quadruplex aptamers can be generated by constrained, G-rich nucleic acid libraries for SELEX.[12] In principle, this opens the possibility to regulate aptamers that target proteins other than thrombin by small molecules. Besides a variety of studies in cell culture, the porphyrinic compound TMPyP4 has been tested in animal models.[5c] It turned out that the compound was well tolerated and exhibited anti-tumor activity in a mouse xenograft model. With respect to the emerging use of aptamers in animal studies as well as in the clinic,[2b,3,13] it might be possible to use TMPyP4 or derivatives to antagonize the anticoagulant effect of the thrombin aptamer in vivo. Moreover, other compounds known to bind to quadruplex DNA should in principle be suited for controlling G-quadruplex-based aptamer functions as well.

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Supporting Information Available: Details of blood clotting assays, fluorescence, CD, and thermal UV melting measurements, and determination of the dissociation constant are available. This material is available free of charge via the Internet at http://pubs.acs.org.

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