Aptamer-based modulation of blood coagulation

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Summary
Nucleic acid based aptamers are single-stranded oligonucleotide ligands isolated from random libraries by an in-vitro selection procedure. Through the formation of unique three-dimensional structures, aptamers are able to selectively interact with a variety of target molecules and are therefore also promising candidates for the development of anticoagulant drugs. While thrombin represents the most prominent enzymatic target in this field, also aptamers directed against other coagulation proteins and proteases have been identified with some currently being tested in clinical trials.

In this review, we summarize recent developments in the design and evaluation of aptamers for anticoagulant therapy and research.

The advantages of aptamers are manifold, the most prevalent being their chemical synthetic access.

Moreover, aptamers have also been used for diagnostic purposes, target validation, targeting of tumours and metastases, and as molecular probes for the screening of small molecules that compete with an aptamer for protein interaction.

In this review we focus on recent developments in the application of aptamers in the field of haemostasis.

Keywords
Aptamer, SELEX, coagulation, photo-control

Schlüsselwörter
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Zusammenfassung

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HD1, HD22
The first aptamer that was described to bind to and inhibit a coagulation factor is the thrombin aptamer HD1. This 15-base DNA oligonucleotide, identified in 1992, inhibits the procoagulant functions of thrombin such as conversion of fibrinogen to fibrin and platelet aggregation (1). Shown by structure analysis, it forms a stable G-quadr-

During the last decades, nucleic acids matured from mere blueprints of genetic information towards advanced regulatory elements. Among them, aptamers have been developed as synthetic and short nucleic acids interacting with target proteins. While recent studies revealed that nature already developed aptamer domains for efficient regulation of bacterial gene expression, reflected by so-called riboswitches, the generation of nucleic acid libraries and their use for in vitro selection purposes commenced during the late 1980’s. Since then, many aptamers have been developed and their exploitation as alternative therapeutics has gained enormous interest. In contrast, only one aptamer has been yet approved for medical uses but a handful of next generation aptamers are currently in different phases of clinical trials.

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Aptamers that bind to and inhibit thrombin via recognition of exosite I (a), exosite II (b), or both exosites (c).

d) Anticoagulant activity of thrombin-targeting aptamers in comparison to argatroban and bivalirudin. The indicated molecules were added to normal human plasma at equimolar levels and partial thromboplastin times (aPTT) were measured.

A comparison between the aptamers HD1 and HD22 displays the fact that slight modifications within the aptamer sequence can completely alter the binding affinity and direct an aptamer to a distinct epitope of a protein. HD1 has been subjected to clinical trials as an anticoagulant during bypass surgery intervention. However, due to low efficacy, development has been stopped.

Instead, a next generation DNA aptamer named NU172, that also recognizes thrombin’s exosite I and inhibits coagulation, has been developed and corresponding clinical phase 1 trials have been completed.

**HD1-22**

Others and we followed a distinct strategy to generate potent blood clotting inhibitors. Inspired by the protein Bothrojaracin (6, 7), a snake venom ingredient, we designed a bivalent aptamer that simultaneously binds to both exosites of thrombin (8). This behaviour has been also demonstrated for Bothrojaracin and leads to superior coagulation inhibiting properties. The corresponding molecule named HD1-22 joins the distinct thrombin aptamers HD1 and HD22 via a polynucleotidic linkage (Fig. 1c). This molecule is the most potent “aptameric” thrombin inhibitor to date (9).

Simultaneously, others also employed this concept using a poly-T linker or PEG-like spacer phosphoramidites to connect the aptamer domains. When compared to the precursor aptamers, these bivalent aptamers reveal significantly enhanced anticoagulant activities, as tested in various whole blood or plasma-based coagulation assays (Fig. 1d). In addition, it has been shown that HD1-22 inhibits the activation of prothrombin more efficiently than HD1 (9, 10, 11).

Besides its potential as an anticoagulant, the bivalent aptamer HD1-22 also repre-
sents an important diagnostic tool. Plasma levels of active thrombin represent a prom-
isating biomarker reflecting a patient’s indi-
vidual haemostatic status. Due to its very
low off rates, HD1-22 recently enabled the
development of a thrombin detection sys-
��. At this, the active site of thrombin is
protected from inactivation ex vivo by the
reversible inhibitor argatroban. The in-
hibitor can be removed by washing after
thrombin has been separated from plasma
in microtiter modules coated with HD1-22
aptamers. Since binding of HD1-22 leaves
the active site of thrombin functional and
accessiblen, small peptide substrates can be
subsequently used to quantify the amount
of thrombin captured in the wells (12).

**TOG25**

In addition to the thrombin binding DNA
aptamers, also RNA aptamers that inhibit
thrombin or other factors of the coagu-
lution cascade have been developed (13).
Among them, a RNA aptamer, named
TOG25, bearing 2'-deoxy-2'-fluoropyri-
midine nucleotides was described to in-
hibit plasma clot formation and platelet ac-
tivation (14). Crystal structure analysis re-
vealed TOG25 to recognize the exosite II
with a succession of adenine-arginine
stacking interactions (15).

**Aptamer stability**

Several attempts have been described to en-
hance aptamer stability and in-vivo half-
life. While addition of PEG-moieties seems
to be beneficial regarding the attenuation
of renal clearance of aptamers, and cer-
tainly represents the method of choice yet,
backbone modifications and the addition
of CAP-like structures enhance nuclease
resistance. These measures were taken into
account for the development of aptamers
targeting activated factor IX (FIXa) and
von Willebrand factor (VWF). Both mol-
ecules are currently tested as antithrom-
botics in clinical trials (Aptamers as anti-
thrombotics).

Alternatively, oligonucleotides contain-
ing locked nucleic acids (LNA) are reported
to possess increased thermal stability but
also enhanced stability towards nuclease
digestion. LNA nucleosides are ribonucleo-
tide analogues containing a cyclic methyl-
eene ether linkage between the 2'-O and
4'-C of the ribose ring. This ring closure re-
sults in a locked 3'-endo conformation that
coordinates the LNA nucleotide monomers
for high affinity hybridization (16). LNA
residues were introduced in the HD1 ap-
tamer to benefit from these advantages but
resulted in decreased anticoagulant activ-
ity. In general, changes in the sugar back-
bone of nucleotides directly involved in the
binding to the target seem to result in de-
creased affinity (17).

**Specificity**

Exemplified by thrombin exosites’ recog-
nizing aptamers, protein sub-domain spe-
cificity allows access to highly selective ap-
tamers. In this regard, selective inhibition
of the anticoagulant activities of activated
protein C (APC) was recently shown by a
DNA aptamer. Herein, the selected ap-
tamer, namely HS02, recognizes the basic
exosite of APC and inhibits its anticoagu-
lation activity and, thus, represents a poten-
tial antidote to counteract on the action of
recombinant APC (Xigris®) in case of
major bleeding during treatment of severe
sepsis. Advantageously, the anti-apoptotic
and cytoprotective functions of Xigris re-
main unaffected in the presence of the ap-
tamer (18).

**Control of aptamer activity**

The efforts described point out the great in-
terest in selecting aptamers for regulation
of the coagulation cascade. A number of
anticoagulant drugs have improved the clini-
cal outcomes. However, their use, es-
pecially during surgery, is limited by the
lack of a specific antidote for clinical situa-
tions in which rapid reversal of the anti-
coagulant activity is required. In order to
that, strategies for controlling the activity
of aptamers and, thus, their cognate target
molecules were brought into centre stage.

One simple and smart strategy to con-
trol the activity of an aptamer is the addi-
tion of a complementary oligonucleotide
molecule. This principle has first been
demonstrated to control the activity of an
aptamer targeting FIXa (Aptamers as anti-
thrombotics) and subsequently ex-
tended to the bivalent aptamer HD1-22
(Fig. 2a) and an aptamer targeting VWF
(9, 19). Since this concept has been proven
to be successful in vivo, clinical trials have
been initiated to develop correspondingly
controlled anticoagulation regimens. Be-
sides this (aptamer-)sequence-specific
strategy, it has also been shown that several
polymer-based molecules are able to in-
hbit the activity of aptamers in a more gen-
eral manner (20).

An alternative approach to gain control
over aptamer function is the addition of
photolabile groups at strategic positions
within the aptamer. These positions might
be either essential for folding of the active
aptamer conformation or represent sites
that are directly in contact with the target
molecule. This technique, referred to as
“caging”, enables the irreversible activation
or inactivation of aptamers by UV-light ex-
posure (21).

Different photolabile caging groups,
such as o-nitrophenylethyl (NPE) and o-
nitrophenylpropyl (NPP), have been used for
regulating aptamers with light (Fig. 2b,
Fig. 2c). These groups can be introduced at
different sites of nucleobases and sub-
sequently incorporated into aptamer se-
quences by solid phase synthesis and phos-
phoramidite chemistry. In this way, several
caged variants of the aptamer HD1 have
been synthesized and investigated with re-
spect to the efficiency of light mediated
control (22).

Applying this strategy, not only an on-
switch HD1-variant could be developed by
introducing photo-labile groups at position
T4 or G6 (Fig. 2c), but also variants that can
be switched off by irradiation with UV light.
The latter bears intramolecular antisense-do-
mains at either the 3’ or 5’-terminus (Fig.
2b). Base pairing capabilities were tempor-
arily blocked by introduction of photolabile
groups (23). Upon irradiation, the Watson-
Crick-base-pairing properties were reacti-
vated and, thus, the active G-quadruplex
structure of HD1 irreversibly disturbed.

Ultimately, this concept has been driven as
far as to modulate individual aptamer
domains of the bivalent aptamer HD1-22.
This has been achieved by caging the G-quadruplex domains of HD1 or HD22 in a reciprocal manner. In this way, HD1-22 has been generated to be light-dependent with regard to either inhibit thrombins’ exosites I or II activity. Hence, it has been shown that modulation of individual aptamer domain’s activity and, thus, function of protein sub-domains by light is possible (24). While this concept can be employed to generate safer anticoagulant drugs, which can be switched off upon light irradiation, it also has been recently extended to other than thrombin aptamer-target pairs to allow for spatiotemporal control of protein function (25).
Aptamers as antithrombotics

Aptamers inhibiting the prothrombotic activities of coagulation factors are interesting candidates for the development of new anticoagulation strategies. At present, aptamers inhibiting activated factor IX, the platelet binding site of VWF, and thrombin are tested in clinical trials (13). The REG1 Anticoagulation System™ (Regado Biosciences, Durham, NC, USA) consists of the nuclease-resistant RNA-aptamer RB006 (pegivacogin), inhibiting activated factor IX (FIXa), and RB007 (anivamsen), a corresponding complementary antidote molecule (26). To prolong the circulating half-life of RB006, the molecule has been modified with a PEG-moiety at its 5’-end (26). After successful completion of phase 1A-C- and phase 2A-clinical trials (28–32), the primary results of a randomized phase 2A trial (RADAR) have been recently announced (33).

Clinical studies

The RADAR study was enrolled to evaluate the safety and efficacy of REG1 in comparison to heparin in subjects with acute coronary syndrome scheduled for percutaneous coronary intervention (PCI) within 24 hours. The objective of the RADAR study was to verify that 1 mg/kg b.w. of pegivacogin results in near complete inhibition of FIXa and, in addition, to determine the dose of anivamsen needed to sufficiently reverse pegivacogin-mediated anticoagulant activity. Regarding the latter aspect, dosing of anivamsen was adjusted to reverse 25%, 50%, 75%, or 100% of pegivacogin anticoagulant activity after completion of PCI (34).

While the lowest-dose anivamsen arm (25% reversal) was stopped early on in the trial due to an increased number of bleeding events, rates of bleeding were lower in the 75% and 100% reversal arms when compared to conventional treatment with heparin. Furthermore, albeit based on only a small number of events, the incidence of ischemic events appeared to be lower within the group of patients treated with REG1. Adverse events were reported to have been rare during the trial but included three allergic reactions that followed the administration of pegivacogin with one patient needing hemodynamic support. Nevertheless, phase 3 clinical trials on the REG1 Anticoagulation System are currently under design (33).

The clinical study program on the von Willebrand factor recognizing aptamer ARC1779 includes a phase 1 clinical trial, enrolled to evaluate its pharmacokinetics, pharmacodynamics, and safety (35). ARC1779 is a nuclease-resistant, DNA/RNA-chimera conjugated to a PEG molecule that targets the A1 domain of human von Willebrand factor and thereby inhibits the binding of VWF to the platelet GPIb receptor.

The phase 1 clinical trial revealed that ARC1779 inhibited VWF-activity and platelet function in a dose- and concentration-dependent manner. Although nucleic-acid based aptamers are generally thought to induce only minimal immune responses, even when applied intravenously, ARC 1779 was initially accom-
panied by one hypersensitivity reaction. According to that, the administration scheme was changed to lower concentration of the injected drug solution and reduced rate of delivery. After modification of the administration scheme, ARC1779 was well tolerated with occurring adverse events being minor and unrelated to dose. Also no bleeding events were observed.

In addition to its general potential as an anti-(athero)thrombotic drug, ARC1779 has been shown to prevent desmopressin-induced platelet drops in patients with type 2b von Willebrand disease (36). Furthermore, administration of ARC1779 adjuvantic to plasma exchange therapy improved the outcome in cases of thrombotic thrombocytopenic purpura (37, 38).

In contrast to RB006 and ARC1779, the thrombin-targeting DNA-aptamer NU172 is intended as a fast-acting anticoagulant demonstrating a short circulating half-life. Thus, NU172, that comprises 26 deoxynucleotides, is not conjugated to PEG and therefore shows a short duration of action in vivo. In phase 1A and 1B clinical trials including healthy volunteers, NU172 mediated dose-dependent increases in activated clotting times (ACT). After termination of continuous infusion, the ACT showed rapid return to baseline. No serious adverse events were reported (39). A phase 2 trial of NU172 in patients undergoing coronary artery bypass graft surgery is proclaimed (NCT00808964).

Conclusion

Aptamers have been developed towards versatile tools for studying and controlling the coagulation process. Moreover, the availability of efficient approaches to regulating aptamer and, thus, target protein activity renders them as unique molecular probes and potential therapeutics. At present, the most prevalent targets within this field (▶ Fig. 3) are

- thrombin (still),
- factor Xa, and
- von Willebrand factor.

This is underlined by corresponding aptamers that are currently investigated in clinical trials and hopefully one of them will get approved for medical use, either as therapeutical or diagnostic tool. This would be of eminent importance for the aptamer field.

References


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