NUCLEIC ACID APTAMERS AGAINST PROTEASES*

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ABSTRACT

Proteases are potential or realized therapeutic targets in a wide variety of pathological conditions. Moreover, proteases are classical subjects for studies of enzymatic and regulatory mechanisms. We here review the literature on nucleic acid aptamers selected with proteases as targets. Designing small molecule protease inhibitors of sufficient specificity has proved a daunting task. Aptamers seem to represent a promising alternative. In our review, we concentrate on biochemical mechanisms of aptamer selection, protein-aptamer recognition, protease inhibition, and advantages of aptamers for pharmacological intervention with pathophysiological functions of proteases. Aptamers can be selected so that they bind their targets highly specifically and with affinities corresponding to K_D values in the nM range. Aptamers can be selected so that they recognize their targets conformation-specifically, for instance with vastly different affinities to zymogen and active enzyme forms. Aptamers can be selected so that they inhibit the enzyme activity of the target proteases, but also so that they inhibit functionally important exosite interactions, for instance cofactor binding. Several protease inhibiting aptamers, mostly directed against blood coagulation, are in clinical trials as antithrombotic drugs. Several of the studies on protease-binding aptamers have been pioneering and trend-setting in the field. The work with protease-binding aptamers also demonstrates many interesting examples of non-standard selection strategies and of new principles for regulating the activity of the inhibitory action of aptamers of general interest to researchers working with nucleic acid aptamers.

INTRODUCTION

Pharmacological intervention with proteolytic enzyme systems - Proteases are enzymes that catalyze the hydrolysis of peptide bonds. Their functions range from extensive degradation of proteins into small peptides or amino acids, as in digestion and extracellular matrix turnover, to limited, regulatory hydrolysis of one or few specific peptide bonds, as in blood coagulation, fibrinolysis, and complement activation. Proteases are most often organized in spatiotemporally highly regulated enzyme systems. They function in complicated networks of cascade-like reactions, in which one protease cleaves a peptide bond in the inactive zymogen precursor of another protease and thereby converts it to its active form. But the functions of the proteases also depend on molecular interactions outside their catalytic active sites, *i.e.*, so-called exosite

interactions. Exosites in the catalytic domains of the proteases are directly involved in recognition of the proteinaceous substrates and the basis for specificity in enzyme-substrate recognition, as the enzymes' active sites *per se* are in general very similar. Exosites can also be areas of the catalytic domain changing conformation after zymogen cleavage and thus necessary for activity. Furthermore, exosites may mediate accumulation of proteases in specific pericellular localizations by binding to extracellular matrix proteins or cell surface receptors (1-5). Many pathological conditions have been linked to dysfunction or dysregulation of proteases and they are therefore often recognized as potential therapeutic targets or prognostic markers. There is a large literature on protease inhibitors as potential drug candidates, as drugs in clinical trials, or as drugs in actual clinical use, including small molecules, peptides, antibodies and nucleic acid aptamers. Especially for small molecule inhibitors targeting the catalytic site of the enzymes, lack of specificity is a recurrent theme and a major challenge. Nucleic acid aptamers against proteases offer a novel type of high specificity, high affinity ligands in the protease field to be used for targeting not only the proteolytic activity but also exosite interactions with other functions. This review will give an overview of protease-binding aptamer described so far with a main focus on the most targeted class of proteases, the serine proteases. We will focus on the biochemical aspects of the interaction between the aptamers and their targets. The *in vivo* work with aptamers on pharmacological intervention with pathological functions of proteases has been excellently covered by several recent reviews (6-8).

Nucleic acid aptamers - Nucleic acid aptamers (RNA or DNA) are selected with an *in vitro* selection strategy called systematic evolution of ligands by exponential enrichment (SELEX) (6-8). The technique combines the ability of RNA or DNA oligonucleotides to fold into a variety of three-dimensional structures depending on their nucleic acid sequence, with the possibility of selecting from very large pools of random sequences (up to 10^{15}), the ones capable of binding to a target of interest, including purified proteins and proteins on the surface of whole cells. The SELEX procedure involves repetitive cycles of selecting binding sequences over non-binding sequences, followed by amplification of the binding pool for a new selection round by PCR for DNA selections, or RT-PCR and RNA transcription for RNA selections. After usually 8 to 15 cycles, 50-100 individual sequences from the final pool can be identified with conventional cloning and sequencing techniques and analyzed for binding to the target. Target binding oligonucleotides are coined nucleic acid aptamers and can often not only bind the target but also influence its functionality. They are remarkable in terms of affinity and specificity, being comparable to antibodies with dissociation constants (K_D) in the picomolar to low nanomolar range and with ability to discriminate between closely related proteins that share common sets of structural domains. To acquire *in vivo* applicable RNA aptamers, selections are routinely performed with modified nucleotides improving their stability in biological fluids significantly. Such RNA aptamers are good starting points for generating agents for analytical and/or therapeutic applications. Aptamers have shown low to no toxicity *in vivo* and unlike antibodies, no immunoreactivity. Additionally, the small size of aptamers (M_r ~10,000 - 15,000) compared to antibodies (M_r ~150,000), together with their polyanionic nature, results in good tissue penetration and rapid blood clearance. These are exactly features that suggest aptamers to be potential agents for molecular imaging. In fact, a radiolabeled aptamer targeting the tumour-associated extracellular matrix (ECM) protein tenascin C was applied to successfully detect tumours in mouse models using PET (9). For

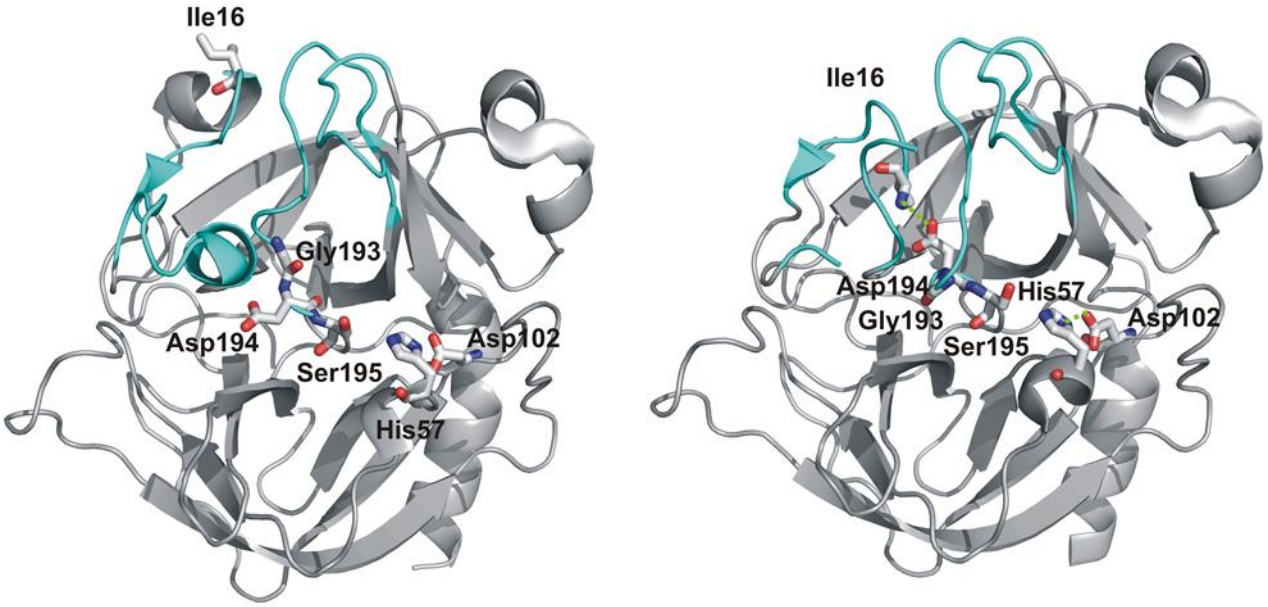

Figure 1. Three-dimensional structures of serine proteases and their zymogen, as exemplified by chymotrypsin and chymotrysinogen. The figure shows ribbon representations of chymotrypsinogen (left) and trypsin (right), constructed by the Pymol program on the basis of the pdb files 1EX3 and 1K21, respectively. The catalytic triad, consisting of His57, Asp102, and Ser195, is indicated. Also shown are the Ile16 residue on the C-terminal side of the activating cleavage; Asp194, organizing the oxyanion hole; and Cys1 and Cys122, connected by a disulfide bond. All residue are indicated as sticks, coloured in CPK. The loops changing conformation upon conversion of chymotrypsinogen to chymotrypsin, *i.e.*, the activation loop (residues 16–21), the autolysis loop (residues 142–152), the oxyanion stabilizing loop (residues 184–194), and the S1-entrance frame (residues 216–223), as shown in green.

other applications, such as in drug development, a decreased clearance rate may sometimes be necessary to increase potency. However, aptamers are produced synthetically and can therefore easily be modified or conjugated to different chemical groups, changing their biodistribution or pharmacokinetics to match the desired application. Although SELEX is a relatively new technology, the first aptamer drug has already received U.S. marketing approval in 2005 and several others are in pre-clinical and clinical trials.

APTAMERS TO SERINE PROTEASES

General properties of serine proteases - The human genome contains about 200 serine protease genes (for a review, see (10)). Serine proteases have been extensively studied and are used as classical examples in enzymology. Their catalytic domains have a common overall fold. The catalytic triad is composed of His57, Asp102, and Ser195[1]. They are synthesized as pro-enzymes or zymogens requiring proteolytic processing in order to achieve full catalytic potential. The zymogen are converted to their active forms by cleavage of the peptide bond between residue 15 and 16. The cleavage results in liberation of a new N-terminus that inserts into a cavity where it forms a salt-bridge to Asp194. The activation is accompanied by stabilization of the activation domain that includes four surface exposed loop-regions referred to as: the activation loop (residues 16–21), the autolysis loop (residues 142–152), the oxyanion stabilizing loop (residues 184–194), and the S1-entrance frame (residues 216–223). These structural changes propagates to the active site cleft resulting in maturation of the catalytic site, including the specificity pocket, the oxyanion hole, and the catalytic triad His57, Asp102 and Ser195 (2) (**Figure 1**). Residues N-terminal to the cleavage site remains bound to the rest of the catalytic

domain by a disulfide bond from Cys1 to Cys122.

The physiological activity of serine proteases is regulated by either of two types of naturally occurring inhibitors, which can react reversibly or irreversibly with the catalytic site cleft of the proteases. The standard mechanism inhibitors, including inhibitors of the Kunitz, Kazal, and Bowman-Birk families, bind reversibly to the active site of the protease. They present a substrate-like peptide bond to the protease, but due to the strained conformation of the loop with the would-be scissile bond, the transition state cannot be stabilized and cleavage does not occur (11). Another class of protease inhibitors is serpins. They also present a substrate-like peptide bond to their target proteases, localized in the surface-exposed, so-called reactive centre loop. The catalytic reaction is initiated but at the acyl-enzyme intermediate stage, the serpin molecule becomes stabilized by insertion of the reactive centre loop into a large central β-sheet. As a consequence, the protease is dragged to the opposite pole of the serpin, the catalytic cycle halted, and a stable, covalent complex formed (for a review, see (12)).

Many serine proteases contain, N-terminal to the catalytic domain, one or more of a variety of other types of domains, including kringles, epidermal growth factor (EGF)-like domains, γ-carboxy glutamic acid (Gla) domains, low density lipoprotein receptor class A (LDLA) repeats, etc. These domains are often involved in other functionally important molecular interactions, like binding to cofactors, extracellular matrix proteins, membrane lipids, or signaling or endocytic cell surface receptors (for a review, see (1)). The domains may thus influence protease function in various ways, either by directly interfering with protease activity or alternatively by mediating other molecular interactions of the proteases.

Being extracellular, serine proteases are easily accessible to nucleic acid aptamers. Many of the aptamer-targeted serine proteases are blood coagulation factors. The reason for the great interest in finding specific inhibitors of blood coagulation factors is that many of the currently available anti-thrombotic drugs are associated with considerable risks of severe side effects in the form of hemorrhages (13). Also, the use of currently available thrombolytic drugs for treatment of stroke, such as tissue-type plasminogen activator, is limited by the increased risk of intracerebral hemorrhage. Strategies that target coagulation factors in the distal end of the coagulation pathway, like heparin treatment, have the capacity to completely block blood coagulation which increases the risk of bleeding. There is therefore great interest in targeting factors at the proximal end of the cascades, as inhibition of those have in several cases been demonstrated to be antithrombotic but without the risk of bleeding side effects.

Thrombin-binding aptamers - Thrombin is a multifunctional serine protease that plays a central role in hemostasis and thrombosis, and among the most investigated targets for anti-coagulant therapy (for a review, see (1,14)). Thrombin catalyzes the conversion of fibrinogen to fibrin and the activation of coagulation factors V, VIII, XI and XIII. Also, thrombin is an initiator of platelet activation and aggregation through cleavage and activation of the platelet thrombin receptor (PAR-1). On the other hand, after binding to thrombomodulin, thrombin alters its substrate specificity and becomes an anti-coagulant enzyme, through the ability to activate protein C, which catalyzes cleavage and inactivation of factor V and VIII. The thrombin-thrombomodulin complex will also protect the fibrin clot by activating thrombin-activatable fibrinolysis inhibitor (TAFI), which removes C-terminal lysine residues from fibrin clots that are otherwise localizing plasminogen to the blood clot. Thus,

thrombomodulin-bound thrombin stops further coagulation and stabilizes the existing clot. The catalytic domain of thrombin has a deep cleft around the catalytic site and two large positively charged anion-binding surface areas, referred to as exosite I and II, located at opposite sides as compared to the active site cleft (**Figure 2**) (15). Exosite I, also called the fibrinogen recognition exosite, is responsible for binding to macromolecular substrates such as fibrinogen, PAR-1, FV and FVIII and cofactors such as thrombomodulin as well as inhibitors such as the serpin inhibitor heparin cofactor II (HCII) and hirudin from leeches. Exosite II, also known as the heparin-binding exosite is responsible for the interaction with sulfated glycosaminoglycans present in heparin. By binding to exosite II, heparin can bridge thrombin and the heparin-binding serpin inhibitor of thrombin, antithrombin (AT), which significantly accelerates the reaction between thrombin and AT. In addition, exosite II is also engaged in the recognition of the substrates FV and FVIII and in the interaction with the platelet surface glycoprotein, GPIbα, involved in platelet activation.

Thrombin was the first published protease target to be applied for nucleic acid aptamer selection. Moreover this first selection opened a whole new research field by highlighting the possibility of selecting aptamers to proteins without a physiological nucleic acid binding function (16). In the study by Bock et al. published in 1992, thrombin-binding single-stranded DNA aptamers with reported binding affinities in the range of 25-200 nM were isolated. The aptamers were found to inhibit thrombin-catalyzed fibrin-clot formation *in vitro* and platelet aggregation *ex vivo* (16,17). The inhibitory activity was retained by a 15-nt short highly conserved consensus sequence GGTTGGTGTGGTTGG. This 15-mer, since then called many names (HD-1, TBA, ODN-1, Arc 183), is today among the most described aptamers in the literature and an often used model aptamer for various studies. It is well established that HD-1 folds into a compact structure containing two G-quartets and binds to exosite I (**Figure 2**). Atomic resolution structural analyses of this aptamer include NMR spectroscopy by two groups (18-20) and an X-ray crystal structure of the aptamer-thrombin complex (21,22). In all structures, the aptamer was folded with two planar guanine quartets in its core. However, differences in the way these central bases are connected were observed between the NMR and crystal structures. This ambiguity was addressed by re-examination of the crystallographic data which resulted in a model that was consistent with both types of data and which places the two TT loops in proximity to the exosite I (23).

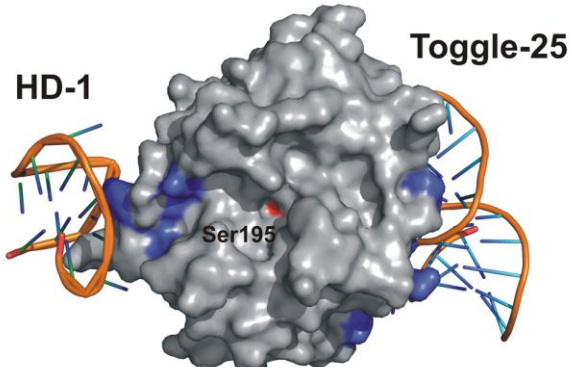

Figure 2. The localization of aptamer binding sites in the three-dimensional structure of the protease domain of thrombin. The figure shows a surface representation of the three-dimensional of the protease domain of thrombin in complex with the two aptamers HD-1 and toggle-25, constructed by the Pymol program at the basis of the pdb files 1hao and 3dd2, respectively. The active site residue S195 is coloured red. Basic residues in exosite I (Arg67, Lys70, Arg73, Arg75, Lys81) and exosite II (Arg93, Arg101, Arg175, Arg233, Lys236, Lys140) are depicted in blue. HD-1 binds exosite 1 and Toggle-25 binds exosite II.

The binding site was also mapped to a part of exosite I by site-directed mutagenesis. Implicated residues were Lys65, His66, Arg70, Tyr71 and Arg73, three of which form electrostatic interactions with HD-1 in the crystal structure (24). The localization of the binding site to exosite I is in agreement with reports of aptamer interference with binding, activation and/or functions related to the exosite I binding substrates fibrinogen, FV,

FVIII and PAR-1 (25-29), as well as with cofactor thrombomodulin binding (25). Aptamer binding also competes with the thrombin inhibitor hirudin, which spans both catalytic site and exosite I (30). Furthermore, exosite I-dependent glycosaminoglycan-mediated inhibition of thrombin by HCII is reduced by HD-I (31). While the aptamer clearly interferes with macromolecular ligand binding, there are mutually contradictory reports as to whether or not HD-1 affects thrombin hydrolysis of small chromogenic peptide substrates (25,32). Though thrombin exosites are inaccessible or immature in prothrombin, HD-1 has been found to bind both prothrombin, thrombin and intermediate forms with similar affinity and to inhibit the activation of prothrombin by the prothrombinase complex resulting from the ability to compete with factor Va in prothrombinase for binding to prothrombin (33).

The *in vivo* anticoagulant potential of the HD-1 aptamer was early on confirmed from pre-clinical tests in animal models arguing for potential utility in certain acute clinical settings (17,34,35). Phase 1 clinical trials were in agreement with the pre-clinical data showing that aptamer infusion resulted in rapid onset of anticoagulatory activity, which rapidly ceased after the infusion was stopped. However, further clinical trials were abandoned due to the high amounts of drug needed to achieve the desired anticoagulant effect. As this limitation was probably a consequence of the affinity, another more potent DNA aptamer (NU172) was developed, which currently is in phase II clinical trials(36) (6).

Other reports have described exosite I-targeting DNA aptamers (37,38) as well as an exosite II-targeting DNA aptamer (39). A truncated variant of the exosite II-binding DNA aptamer, the 29-mer 60-18(29) or HD-22, binds to thrombin with a K_D value of 0.5 nM and like HD-1 has been described in several studies. The localization of the HD-22 binding site is based on protein-aptamer cross-linking studies, competition studies with other exosite I and II oligonucleotide ligands, and reduced affinity to thrombin variants with Arg-Ala mutations in exosite II (39,40). As with heparin, HD-22 was reported to inhibit platelet membrane glycoprotein GPIbα-binding to thrombin (40), and to inhibit GPIbα-mediated acceleration of PAR-1 cleavage (41), thereby reducing thrombin-induced platelet aggregation (40) and thrombin-clotting time (32). In addition, although the primary binding site for binding of fibrinogen to thrombin is exosite I, a subpopulation of fibrinogen having extended contacts in exosite II may also be inhibited moderately by HD-22 (32,39). In agreement with exosite II´s involvement in binding of different ligands, HD-22 also has been found to inhibit thrombin-mediated activation of FV (28) and FVIII (42) and to protect thrombin from heparin-mediated inhibition by AT (43). Whereas HD-1 appears to have no particular preference for the active form over the zymogen form, HD-22 only binds the active form (33).

Petrera et al. (32) used HD-1 and HD-22 to investigate the possibility of an allosteric link between the two thrombin exosites. It was reported that binding of HD-22 to exosite II induces conformational changes in exosite I based on studies with a thrombin variant with a fluorescent probe in exosite I. In addition, HD-22 reduced thrombin binding to fibrin clots and displaced already bound thrombin. HD-1 may in turn be able to partly inhibit binding of HD-22 and other exosite II ligands (32,39). Petrera et al. also observed allosteric inhibitory effects of both aptamers on the catalytic activity of thrombin towards small chromogenic peptide substrates. The anticoagulant effect of exosite I and II aptamers may therefore in part be caused by allosteric effects in addition to shielding the exosites.

The availability of the two thrombin-binding DNA aptamers HD-1 and HD-22 have

allowed the construction of novel bivalent fusion aptamers (43-45). A bivalent aptamer consisting of HD-1 and HD-22 connected by a poly-dA linker (HD1-22) was found to prolong blood clotting time to the same extent as HD-1 alone at a 30-fold lower concentration (43). The construct was subsequently found to have an anticoagulant profile similar to that of the clinically established thrombin inhibitor bivalirudin, moreover with the additional advantage of the possibility of rapid inactivation by complementary oligodeoxynucletides (46).

Besides the thrombin-binding DNA aptamers described above, RNA aptamers binding to thrombin have also been isolated. The first selection of RNA ligands to human thrombin was published in 1994 by Kubik et al. (47). The isolated aptamers had specificity for active thrombin over pro-thrombin. Heparin competed with the binding of the aptamers to thrombin indicating that the binding site most likely includes exosite II. There was no inhibitory effect of a truncated RNA variant (RNA 16.24) on hydrolysis of a small chromogenic peptide substrate by thrombin, and an anticoagulant effect was seen in a clot formation assay. While species-specific RNA aptamers for bovine thrombin have been obtained by Liu et al. (48), White et al. (49) have generated species cross-reactive nuclease-resistant aptamers by "toggling" between porcine and human thrombin as the target during selection ("toggle"-SELEX). Accordingly, the aptamer Toggle-25 prolonged human as well as porcine plasma clotting time and platelet aggregation. The binding site for Toggle-25 was mapped to exosite II by site-directed mutagenesis (50), which was later confirmed by X-ray crystal structure analysis of the thrombin-aptamer complex (**Figure 2**) (51). The effect of Toggle-25 on serpin reactivity with thrombin was described in Jeter et al. (2004) (50). Toggle-25 has also been used together with HD-1 to study possible synergistic effects and the usefulness of aptamer cocktails (52). It appears that HD-1 and Toggle-25 do not compete for binding to thrombin and in three thrombin-sensitive clotting assays as well as in a platelet activation assay, a cocktail of HD-1 and Toggle-25 was found to have potent synergistic anticoagulant effects. Finally, Layzer et al. (53) generated nuclease-resistant RNA aptamers to thrombin, demonstrating anticoagulant activity and clone-dependent binding pattern towards thrombin zymogen and active form. One clone even had sequence similarity with Toggle-25.

Factor VII-binding aptamers - Upon vascular injury, a membrane-bound receptor, tissue factor (TF), becomes exposed to circulating factor VIIa (FVIIa) (for a review, see (1,54)). When FVIIa forms a complex with TF, it achieves its full catalytic potential and activates downstream mediators of the blood coagulation cascade (factor IX (FIX), factor X (FX)), eventually leading to formation of a fibrin clot. FVIIa (M_r ~50,000) consists of, as mentioned from the N-terminus, a Gla domain, two epidermal growth factor-like (EGF) domains, and a catalytic domain. The FVIIa binding site for TF involves all four domains (55). FVIIa is a potential anticoagulant target.

Nuclease-resistant FVII/FVIIa-binding RNA aptamers have been reported in two studies (RNA16.3 (56) and 7S-1 (53)), in both cases with the ability to prolong plasma clotting time. Although limited details are available, the inhibitory activity of RNA 16.3 towards FVIIa was shown to be at least partly due to inhibition of the complex formation between TF and FVIIa, as evident from studies of TF-dependent FX-activation activity in a purified system. In none of the cases it is known whether the aptamers inhibit FVIIa activity in the absence of TF. Interestingly, RNA 16.3 bound the zymogen form and the active form with equal affinity (K_D ~10 nM).

Factor IX-binding aptamers - Factor IX is an M_r ~65,000 serine protease consisting of,

as mentioned from the N-terminus, a Gla domain, two EGF domains, and a serine protease catalytic domain (for a review, see (1)). The zymogen form is activated by the TF-FVIIa complex or by FXIa. Activated FIXa, in complex with its cofactor FVIIIa, in turn activates FX. FIX has been proposed for the development of antithrombotic therapy as anti-factor IXa agents appear to be associated with bleeding risks smaller than those of other antithrombotic agents, like heparin.

Several different FIX-binding aptamers have been reported (53,57,58). Rusconi et al. isolated a 2´-F pyrimidine modified RNA aptamer named 9.3, binding FIX or FIXa with a K_D in the subnanomolar range, more than 1000-fold stronger than other similar proteases (57,59). The aptamer could be truncated (to give 9.3t) and conjugated with either polyethylene glycol (Peg-9.3t) or cholesterol (Ch-9.3t) to increase the circulating half-life *in vivo*, with only a slight reduction in affinity (K_D ~3-5 nM) (57,60). Aptamer 9.3t inhibited FX activation catalyzed by the FIXa-FVIIIa complex (57). Interestingly, it also inhibited the activation of FIX by the TF-FVIIa complex (59). I*n vitro* studies have demonstrated that 9.3t is able to prolong coagulation time (57,59-61). Animal studies made possible by the prolonged *in vivo* half-life of modified variants of 9.3t, validated these *in vitro* findings and demonstrated the ability of 9.3t to induce anticoagulation in pigs and to inhibit thrombosis in mice (60). In many cases, the high specificity of aptamers implies poor cross-reactivity with orthologous proteins from other animal species different from the one from which the protein used for library screening was derived. However, in this respect, 9.3t is different, apparently being able to inhibit FIXa from humans, pigs and mice. Another RNA aptamer, isolated by library screening with bovine FIX, bound bovine FIX with a K_D of ~10 nM but did not measurably bind human FIX (58). The development of the 9.3t aptamer and others have been accompanied by the development of antidotes for rapid reversal of aptamer anticoagulatory effects in the form of complementary oligonucleotides (57,60) or nucleic acid binding polymers (61). A modified version of 9.3t (RB006) and a complementary oligonucleotide antidote (RB007), collectively named the REG1 Anticoagulation System, is currently in clinical trials phase 2B (for a review on this system see (6,36)).

Factor X-binding aptamers - FX consists of, as mentioned from the N-terminus, a Gla domain, two EGF domains, and a serine protease catalytic domain (for a review, see (1)). Activation of the FX zymogen is catalyzed by the TF-FVIIa complex, the FIXa-VIIIa complex, or thrombin. Upon activation, FXa can activate prothrombin to thrombin but the catalytic efficiency is greatly increased by membrane-dependent assembly of FXa with its cofactor, factor Va, into the so-called prothrombinase complex.

Two reports, describe the selection of nuclease-resistant RNA aptamers binding to FX or FXa (53,62). In both cases, the aptamers display a K_D ~1 nM for the active as well as the zymogen form. Both aptamers are able to delay clot formation in human plasma. A truncated and modified version of one of the aptamers, RNA_{11F7t}, was found not to inhibit FXa cleavage of a small peptidylic substrate but to inhibit prothrombin activation by inhibiting the assembly of the FXa-FVa complex, supposedly by an allosteric mechanism. RNA_{11F7t} caused a slight reduction of the rate constant for inhibition of FXa by AT and a strong, 200-fold reduction of the affinity of FXa inactivation to the standard mechanism inhibitor tissue factor pathway inhibitor (TFPI). This aptamer also inhibited activation of FX by the FIXa-VIIIa complex, while no or a minor stimulating effect was observed for activation of FX by the TF-FVIIa complex. The aptamer binding sites were not determined but RNA_{11F7t}-binding was not affected by deletion of the Gla-domain of FXa. The aptamer bound FX

and FXa more than 1000-fold stronger than other, similar proteinases. The concept of antidote-development to aptamers has also been demonstrated with RNA_{11F7t} using nucleic acid-binding polymers (61).

Protein C-binding aptamers - Protein C consists of an N-terminal Gla-domain, followed by two epidermal-growth-factor-like domains, and a C-terminal catalytic serine protease domain (for a review, see (1)). The activation of protein C, as catalyzed by the thrombin-thrombomodulin complex takes place on the surface of endothelial cells where protein C binds to the endothelial cell protein C receptor. The activated form of protein C (APC) binds to the cofactor S and acts as an anticoagulant by degrading FVa and FVIIIa. The macromolecular substrates are recognized by a basic exosite formed by residues in the 37-, 60-, and 70-80-loops of APC. The activity of APC is controlled by the heparin-dependent serpin, protein C inhibitor (PCI). Recombinant APC is used for treatment of severe sepsis but a life threatening side effect is major bleedings. Therefore, a specific and fast-acting inhibitor could be useful for managing these unwanted side effects (for a review, see (63)). In addition, blockade of activated protein C anticoagulant activity might be an adjuvant approach for treatment of hemophilia (64).

RNA as well as DNA aptamers binding to APC have been isolated (65,66). Gal et al. (65,67) reported the selection of an RNA aptamer, APC-167, binding to human APC with a K_D-value of ~110 nM. The aptamer inhibited hydrolysis of a small chromogenic peptide substrate (K_i ~83 nM) in a non-competitive manner. No measurable affinity was observed for thrombin, FXa, NS3 protease, or bovine serum albumin. APC-167 was truncated from 167 to 99 nucleotides (APC-99) without loss of affinity (APC-99, K_D ~120 nM, K_i ~137 nM). Further truncation resulted in significant loss of affinity and inhibitory activity. Using biochemical methods, the minimum binding motif of APC-99 was identified as a 56-nt two stem-loop structure in which certain wobble base pairs of the stem and several phosphates were found to play an important role.

Recently, a single-stranded DNA aptamer, HS02, with subnanomolar affinity for APC (K_D of ~0.43 nM) was also isolated (66,67). HS02 binds with comparable affinities to APC, free or in complex with cofactor protein S. The aptamer inhibits the catalytic activity of activated protein C measured both as hydrolysis of a small fluorogenic peptide substrate and hydrolysis of the macromolecular substrates FVa and FVIIIa. The inhibitory effect was further established in a plasma clotting assay where HS02 was found to inhibit APC anticoagulant activity. There was no measurable affinity for other similar serine proteases. Interestingly, the affinity of HS02 for binding to APC is 2-3 orders of magnitude higher compared to the zymogen form. HS02 was truncated down to a size of 44 bases, which encompass a predicted stem-loop structure together with a CT bulge, without any significant loss of affinity. The basic exosite on the 36-, 60-, and 70-80-loops in APC was found to be the most likely binding site for HS02, based on several observations. First of all, HS02 only partly inhibits the hydrolysis of a small fluorogenic peptide substrate suggesting an indirect, allosteric inhibitory mechanism. Secondly, APC-mediated degradation of the physiological substrates FVa and FVIIIa was nearly completely blocked by HS02, although the presence of protein S markedly decreased the inhibitory effect. Finally, heparin, which binds to the basic exosite competed with binding of the aptamer to activated protein C. Interestingly, HS02, like heparin, stimulated the rate of inactivation of APC by PCI but the degree of stimulation was dependent on the size of HS02.

Aptamers binding to urokinase-type plasminogen activator - Urokinase-type plasminogen activator (uPA) catalyzes the conversion of the zymogen plasminogen to

the active protease plasmin, by hydrolysis of a single peptide bond in plasminogen. Plasmin in turn catalyzes the degradation of extracellular matrix proteins including fibrin. uPA (M_r ~50,000) consists of, as mentioned from the N-terminus, an EGF domain, a kringle domain, and a C-terminal catalytic serine protease domain. The EGF domain can bind to the cell surface-anchored uPA receptor (uPAR), thereby concentrating plasminogen activation activity at cell surfaces. The form of uPA initially secreted from cells is the zymogen form pro-uPA. The primary inhibitor of uPA is the serpin plasminogen activator inhibitor-1 (PAI-1) (for a review, see (68)). uPAR-bound uPA-PAI-1 complex may become endocytosed and targeted for lysosomal degradation by endocytosis receptors of the low density lipoprotein receptor (for reviews, see (12,69)). uPA participates in many events in tissue remodeling in the normal organisms, but is also known to be a prognostic marker in cancer (for a review, see (70)) and to mediate cancer metastasis (for a review, see (68)), making it a potential target for anti-cancer therapy.

A selection of DNA aptamers for binding to pro-uPA was described by Skrypina et al. (71). Sequencing after 12 selection rounds revealed a group of similar sequences but no further information was reported.

Dupont et al. (72) screened a library of 2´-F-pyrimidine modified RNA aptamers with human uPA and isolated several aptamers which were able to block the binding of uPA to uPAR with IC_{50} values in the low nanomolar range, but none of the aptamers affected the ability of uPA to catalyze the conversion of plasminogen to plasmin. The aptamers did not cross-react with mouse uPA. As judged from experiments with truncated forms of uPA, the aptamers seemed to have a binding site encompassing the EGF domain, in good agreement with the fact that this domain is the one binding to uPAR. Accordingly, the aptamers inhibited the accumulation of uPA-catalyzed plasminogen activation at cell surfaces. Previously generated inhibitors of the uPA–uPAR interaction include peptides, which bind uPAR and sterically interfere with the binding of uPA to the receptor. However, although they are antagonists of uPA-uPAR binding, such peptides may still activate the adhesive and cell signaling functions of uPAR. In contrast, the aptamers are pure uPAR antagonists.

Neutrophil elastase-binding aptamers - Neutrophil elastase (NE) consists of only a serine protease catalytic domain. The active site preferentially accommodates small hydrophobic residues. NE is one of several leukocyte proteases associated with inflammatory processes (for a review, see (73)). Neutrophils are the first cells to be recruited to inflammatory sites in response to invading pathogens. Here, NE is both secreted to the extracellular space and stored in the phagocytic compartments and facilitating degradation of pathogenic bacteria. However, NE also plays a role in tissue repair after injury. Due to the ability to degrade host proteins such as fibronectin and elastin, its activity must be tightly regulated to avoid injury of the host tissue. The primary inhibitor is the serpin α_1-proteinase inhibitor (α_1-PI), also known as α_1-antitrypsin. NE is associated with a number of pathological states and diseases, including emphysema, cystic fibrosis, idiopathic pulmonary fibrosis, acute respiratory distress syndrome (ARDS), ischemia-reperfusion injury, and rheumatoid arthritis.

Several sets of aptamers binding to NE have been isolated, including a set of nuclease resistant RNA aptamers (74) and a set of DNA aptamers (75,76), in both cases with K_D values in the low nanomolar range. Both sets of aptamers did not display measurable binding affinity to related proteins like thrombin, uPA, or porcine pancreatic elastase. The RNA and DNA aptamers displayed no or only weak inhibitory activity towards NE

hydrolysis of small artificial chromogenic substrates. However, one of the DNA aptamers were found to be able to modulate the reaction of human NE with the inhibitors secretory leukoprotease inhibitor (SLPI) and α_1-proteinase inhibitor (α_1-PI) (76). In an interesting construct made on the basis of these selections, a NE-binding DNA aptamer was covalently tethered to a tetrapeptide, which on its own weakly inhibited the enzymatic activity (K_i ~1 mM) of human NE (75). The conjugate was able to completely inhibit human NE, with a K_i of ~28 nM.

In another attempt to develop inhibitory aptamers towards human NE, a "blended SELEX" strategy was applied. By this strategy, the 5´ constant region of RNA (77) or DNA (78) libraries were hybridized to a complementary DNA oligonucleotide carrying a small-molecule covalent inhibitor of neutrophil elastase. From the RNA library, sequences were obtained, which could confer the small-molecule covalent inhibitor with a two orders of magnitude increased second order rate constant. The constructs selected from the DNA library had an even higher potency, and in an *in vitro* elastinolysis assay, several constructs were more efficient inhibitors than the natural protease inhibitor α_1-PI. High specificity was confirmed using another serine protease, cathepsin G, which has substrate specificity similar to that of human NE. In an isolated rat lung model of ARDS, the RNA construct was found to protect from human neutrophil-mediated damage (77). As elastase activity should be strictly confined to inflammatory sites, a 99mTc labeled DNA-based construct was used to image inflammation in an *in vivo* rat inflammation model (79). The DNA construct was also shown to inhibit vascular leakage and neutrophil infiltration in this model (80).

Prostate-specific antigen-binding aptamers - Prostate-specific antigen (PSA) is an M_r ~34,000 serine protease consisting of only a serine protease catalytic domain. It is a member of the tissue kallikrein family. It is primarily produced by prostate epithelium. Transcription of the PSA gene is stimulated by androgens. The zymogen form of PSA can be activated by glandular kallikrein. PSA that enters the circulation is rapidly inhibited by protease inhibitors, mainly the serpin α_1-antichymotrypsin. PSA is normally present in low quantities in the blood of men with healthy prostates but is often elevated in cases of prostate cancer. A high serum level of PSA is for this reason a commonly used, although presently not undisputed, marker for prostate cancer. The interest in measurements of serum PSA for early detection of prostate cancer has been a motive for selecting PSA-binding aptamers. Selective measurements of zymogen PSA, active PSA, or inhibitor-PSA complexes in serum could potentially be used for diagnosis of early-stage prostate cancer. The method currently available is a standard one for assaying total PSA in serum (for a review, see (81)).

On this basis, Jeong et al. (82) selected RNA aptamers specific for human active PSA by applying counter-selection against the zymogen form. They isolated aptamers with binding activity towards active PSA with a low nM K_D, whereas no or very little binding was observed for the zymogen form. Savory et al. (83) isolated DNA aptamers binding to human PSA purified from human semen, using a combination of standard SELEX and *in silico* maturation. Applications of these PSA binding aptamers in assays for measuring PSA concentrations have not been reported. Neither is it known whether they inhibit the enzyme activity of PSA.

Hepatitis C virus NS3-binding aptamers - Hepatitis C virus non-structural protein 3 (HCV NS3) is one of 10 proteins encoded by the HCV RNA genome. Initially after translation, it is part of one large polyprotein precursor, which is subsequently processed into individual proteins. NS3 is a dual-function protein consisting of an N-terminal trypsin-like serine protease catalytic domain linked with a C-terminal helicase consisting

of 3 independently folded domains (84,85). The serine protease is responsible for polyprotein processing at four different sites and the catalytic activity depends on non-covalent complex formation with its 54-residue cofactor NS4A, itself a viral protein. The helicase has the ability to move along nucleic acid polymers while displacing complementary strands at the expense of ATP hydrolysis. The exact role in the viral lifecycle is unknown, but both helicase and protease activities are necessary for viral replication and NS3 is therefore a potential target for anti-HCV drugs.

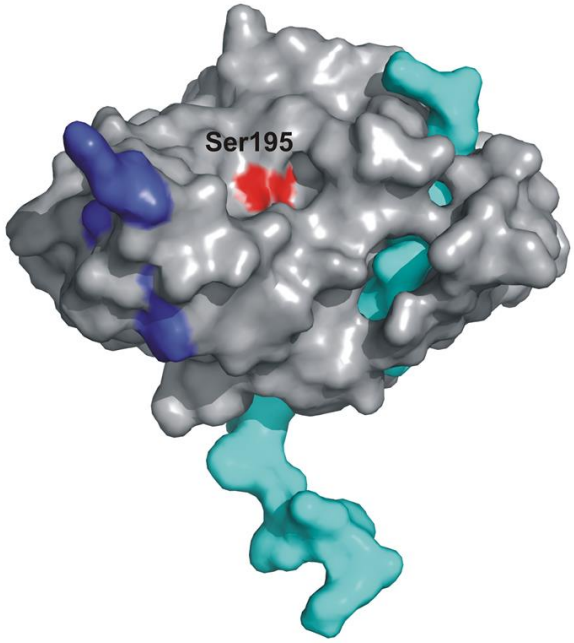

Figure 3. The localization of an aptamer binding site in the three-dimensional structure of the protease domain of HCV NS3. The figure shows a surface representation of the three-dimensional structure of the protease domain, constructed by the Pymol program at the basis of the pdb file 1a1r. The active site Ser195 is coloured red. The basic residues in the binding site for RNA aptamer G9-I (Arg186, Arg240, and Lys244), are coloured blue. Residues Gly21 - Ala36 of the cofactor NS4A are shown in cyan.

With the aim of targeting the protease activity of NS3, several RNA aptamer selections have been conducted. After limited success with selection using the native protein (86,87), an aptamer G9-I, binding to NS3 with a high affinity (K_D ~10 nM) was derived from a screening using the isolated protease catalytic domain (88). It could reduce NS3 proteolytic activity for a peptidylic substrate by 70% and 90% in the presence or absence of the cofactor NS4A respectively. The mechanism of inhibition was found to be non-competitive, with a K_i of ~100 nM. A model for the binding interaction of G9-I with NS3 was proposed from mutagenesis and nucleotide analog interference mapping, highlighting the importance of the conserved region and suggesting a role of the constant regions in tertiary interactions important for aptamer structure stabilization (89). A 52 nucleotides truncated variant, with properties close to those of the full-length aptamer was produced (89,90). By alanine scanning mutagenesis, the binding site for G9-I was localized to one of three clusters of basic residues on the surface of NS3 protease (91) (**Figure 3**). One of the clusters corresponds to the fibrinogen-binding exosite of thrombin and the proposed APC aptamer binding site of HS02 in APC. Interestingly, however a different cluster encompassing R130, R161 and K165 was identified. It has been speculated that this area near the catalytic site is important for NS3 substrate recognition.

Probably as a consequence of the non-specific affinity of the NS3 helicase for RNA, several aptamers targeting the protease domain of NS3 were found to also inhibit the NS3 helicase activity or helicase substrate binding, although with low affinity (87,88,92). In an attempt to improve such dual inhibitory behaviour, protease-directed aptamers were extended with poly-U sequences, for which the NS3 helicase has a preference (90,92). The extensions were found to both improve helicase and protease inhibitory activity. To specifically target the helicase activity, selections were conducted with an isolated helicase module, in the presence of poly-U to increase stringency (93). The selection yielded several aptamers, one named aptamer-5, with potent helicase inhibitory activity, without effect on protease activity. Coupling

the helicase inhibitory aptamer-5 with protease inhibitory aptamers by a poly-U linker resulted in an aptamer constructs with subnanomolar affinity to native NS3 and superior dual inhibitory activity (94). Interestingly, the NS3 binding aptamer G9-II alone and in a bivalent construct with aptamer-5 were shown to inhibit HCV replication in cell cultures (94,95).

Subtilisin-binding aptamers - Subtilisin-like serine proteases are in general mostly of bacterial origin and well represented by various species of *Bacillus* (for a review, see (96)). It contains a catalytic triad with a charge-relay system involving an Asp, a His and a Ser residue. They are structurally unrelated to the chymotrypsin-clan of serine proteases, and compared to this family a classic example of convergent evolution of this structural feature. Subtilisin-like serine proteases have a preference for cleaving after aromatic and hydrophobic residues and are generally secreted extracellularly by bacteria for the purpose of scavenging nutrients. The proteases are also widely used in a variety of industrial products such as detergents and cosmetics.

Takeno et al. (97) isolated an RNA aptamer, RNA-1, for subtilisin BPN´ from *Bacillus amyloliquefaciens*. The aptamer was reported to be a competitive inhibitor of subtilisin cleavage of a small peptide substrate (K_i ~2.5 µM). Furthermore, RNA-1 was demonstrated to have specificity for subtilisin BPN´ over trypsin and chymotrypsin. RNA-1 is the first RNA aptamer selected towards an extracellular bacterial enzyme.

APTAMERS TO OTHER PROTEASES

Cathepsin E-binding aptamers - Cathepsin E is an M_r ~42,000 aspartic protease belonging to the pepsin superfamily and consists of two homologous domains both involved in the formation of the active site (98,99). As is the case for cathepsin E´s close relative, cathepsin D, the active site preferentially accommodates small hydrophobic amino acids. Cathepsin E is mainly present in intracellular, non lysosomal vesicles of cells of the immune system. However, its exact function is yet to be elucidated. For functional studies on cathepsin E, the most widely used inhibitor is the broad-spectrum aspartic protease inhibitor pepstatin A. Specific potent inhibitors of cathepsin E would facilitate the understanding of physiological and pathofysiological functions of this protease.

Generation of cathepsin E-binding aptamers has been described in two studies (99,100). Naimuddin et al. [82] performed 3 rounds of selection for aptamers with affinity to cathepsin E, using a single-stranded DNA library. The resulting pool of aptamers was then linked directly to a peptidylic cathepsin E substrate with a biotin moiety. Upon incubation with cathepsin E, DNA sequences preventing cleavage of the peptide substrate were isolated with streptavidin beads. The most promising among the isolated aptamers, SFR-6-3, had a K_D of ~15 nM for binding to cathepsin E, and specifically (over cathepsin D) inhibited its proteolytic activity towards a fluorogenic substrate with an IC_{50} of ~30 nM. In comparison, the most widely used inhibitor of cathepsin E, pepstatin A, had an IC_{50} of ~1 nM, but does not discriminate between cathepsin E and D. In a follow-up study (100), computational approaches were applied to direct the development of aptamers with higher inhibitory activity.

Aptamers binding to *β*-secretase 1 - *β*-secretase amyloid cleaving enzyme (BACE1) is a transmembrane aspartate protease belonging to the pepsin subfamily with an extracellular protease domain, a transmembrane sequence, and a short cytoplasmic tail, consisting of 24 amino acids. Physiological ligands to the cytoplasmic domain have been identified, but the biological roles of the interactions are largely elusive. BACE 1 participates in the processing of *β*-amyloid precursor protein to

β-amyloid peptide that aggregates and deposits in the brain of Alzheimer patients (101).

Rentmeister et al. (102) decided to select RNA aptamers binding to the cytoplasmic domain, to be used as tools for elucidating the biological function of this domain and its significance in Alzheimer´s disease. An initial selection procedure resulted in an aptamer clone with a relatively low affinity for the target (K_D ~4 µM). Affinity maturation was performed by error prone PCR leading to the isolation of several aptamers binding with considerable higher affinity. One aptamer, S10, bound to the cytoplasmic domain with a K_D-value of 360 nM and was shown to inhibit binding of Golgi-localized γ-ear-containing ARF-binding protein, a known ligand to the cytoplasmic domain.

Aptamers binding to botulinum neurotoxin - Botulinum neurotoxins (BoNT) from *Clostridium botulinum* are the most toxic molecules known to mankind (103). The neurotoxin is released as a single polypeptide (M_r ~150,000) from different sized progenitor toxins, and nicked to generate two disulfide linked fragments. The heavy chain (Hc, Mr ~100,000) is responsible for translocation into the cytosol, while the light chain (Lc, Mr ~50,000) is a zinc-dependent endopeptidase with specific activity towards proteins involved in neurotransmitter release. BoNT-mediated inhibition of acetylcholine release at the nerve-muscle junction makes BoNTs extremely toxic, and in combination with the easy production methods, BoNTs have become potential biowarfare agents. For this reason, there is a great interest in development of agents targeting this protease for therapy and sensors.

Single stranded DNA aptamers binding to type A BoNT with K_D values in the low nanomolar range were isolated in two studies (104,105). One of the aptamers was subsequently used to develop an electrochemical sensor for BoNT based on target-induced conformational changes of the aptamer (106). A group of 2´-F-pyrimidine-modified RNA aptamers binding to type A BoNT, also with K_D values in the low nanomolar range, were isolated with only the protease domain as a target (107). All the aptamers showed non-competitive inhibition. The nature of the interaction between the aptamers and BoNT was explored with molecular docking, which suggested that all bind near the Zn atom in the active site.

Aptamers binding to prostate-specific membrane antigen - Prostate-specific membrane antigen (PSMA; not to be confounded with prostate-specfic antigen, PSA) also known as glutamate carboxypeptidase II, is a glutamate carboxypeptidase and a type II membrane glycoprotein. PSMA is expressed by prostate cells. The expression is strongly upregulated in prostate tumor cells, making it an excellent target for carrying imaging and therapeutic agents to prostate cancer cells (108).

Lupold et al. (109) isolated two PSMA-specific RNA aptamers, xPSM-A9 and xPSM-A10, using the extracellular portion of PSMA as a bait in the selection procedure. The aptamers were found to inhibit the proteolytic activity of PSMA, with K_i values of 2.1 and 11.9 nM, respectively. Interestingly, the two aptamers display different inhibitory mechanisms with one being a competitive inhibitor and the other a non-competitive inhibitor. xPSM-A10 could be truncated by 15 nucleotides from the 3'-end without significant loss of inhibitory potency (K_i of 20.5 nM). The truncated version of xPSM-A10 (xPSM-A10-3) was fluorescently labeled with rhodamine-red-X and its ability to specifically recognizing PSMA-expressing cultured prostate cancer cells was verified by fluorescence microscopy.

PSMA-binding aptamers have been used for delivery of toxins (110), siRNA (111-113) and toxin-loaded nanoparticles (114) for

therapy and various nanoparticles and dyes for imaging (115,116).

GENERAL FEATURES CONCERNING PROTEASE-BINDING APTAMERS

General features of selection strategies used for isolating protease-binding aptamers - The studies of protease-binding aptamers include a number of interesting observations and highlight recurrent themes concerning aptamer selections in general. The typical library screening setups have consisted in the use of a nucleic acid library (single stranded DNA or RNA, in later years preferably containing modified nucleotides) with a random region around 40 nucleotides and a diversity in the range 10^{13}-10^{15}. The preferred method for separation of free and target-bound nucleic acid is nitrocellulose filter. The reported standard affinity-based aptamer selections typically resulted in a limited number of aptamer clones or families which displayed a uniform profile in terms of target binding site and functional effects. This fact is probably caused by a combination of several factors, such as the cloning and sequencing being performed after many selection rounds, the limited number of clones investigated, and the presence of, presumably, an only limited number of dominating aptamer binding sites on the targets.

The existence of dominating aptamer binding sites was suggested on the basis of several studies. The dominant site in thrombin is presumed to be the most electropositive site, the heparin-binding exosite II. Accordingly, aptamer selections utilizing nitrocellulose filter partitioning have resulted in aptamers binding to this area. It has been speculated that aptamers binding to exosite I, as described in other studies, were isolated only when using concavalin A-Sepharose-immobilization of thrombin which may mask exosite II (39). In the case of NS3, the helicase domain is a nucleic acid hot spot and aptamers, which potently inhibited the protease function, were not identified until selections were performed with the protease domain alone (88). The results of screening of aptamer libraries with a mixture of targets (53) also illustrates the existence of dominant sites. After a few initial screening rounds, the majority of the aptamers obtained were directed towards the most abundant protein in the mixture, thrombin. However, by subsequently deselecting for thrombin-binding aptamers or selecting for other individual proteins alone, aptamers for other targets began to dominate. The latter example shows that the final pool obtained after standard affinity-based selections may contain many more interesting aptamer sequences than those found after analysis of a small number of the most abundant clones.

Another type of attempt to direct the outcome of selections were presented by Jeong et al. (82), who used counter-selection against the zymogen form of PSA to isolate aptamers specific for the active form. It is also possible to select for aptamers with specific functions. Thus, Smith et al. (77) and Charlton et al. (78) screened libraries of aptamers coupled to a small molecule inhibitor of neutrophil elastase to isolate molecules inhibiting this protease with high affinity and high specificity ("blended SELEX"). Here, the direct blocking of the active site is the function of the small molecule inhibitor moiety, while the nucleic acid moiety confers the specificity and the affinity, presumably by binding to sites adjacent to the active site. Naimuddin et al. (99) selected for cathepsin E-inhibitory aptamers by screening for aptamers able to inhibit the cleavage of a small peptide substrate. A number of other studies also highlight how the choice of selection conditions may have an impact on the final outcome. Including K^+ in the selection buffer may promote the selection of G-quartet containing aptamers (16,74). Selections at low pH resulted in isolation of C-rich i-motif containing aptamers (99). Aptamers selected at low salt or ambient temperature had reduced target-affinity at physiological

conditions (56,77). Therefore, the intended application of the aptamers should be considered carefully when setting up the selection experiment.

Reported protease aptamers bind their targets with K_D-values in the high pM to low μM range, the majority having low nM affinities. Specificity, in terms of affinity to proteins related to the target, has usually been studied with aptamer concentrations up to the low μM range.

After an initial successful screening of a library for a protease-binding aptamer, a typical step has been attempts to truncate the aptamer to a size compatible with chemical synthesis. Protease aptamers currently or previously in clinical trials, consisted of 15 (HD-1 for thrombin), 29 (NU172 for thrombin) and 31 nucleotides (RB006 for FIXa). Most protease aptamer studies therefore report truncation analyses on the basis of a combination of secondary structure predictions, confirmed or not experimentally, footprinting, and RNA mutational analyses. The degree of truncation possible without loss of affinity and/or inhibitory activity however appears highly variable. For example, the APC aptamer APC-167 could only be reduced from 167 to 99 nucleotides (65), the FVII aptamer 16.3 from 80 to 56 nucleotides (56), and the uPA aptamer from 79 to 49 nucleotides (72). In a few cases, reduction in size was even accompanied by an increased affinity as exemplified by the thrombin-binding RNA aptamers Toggle25, which could be reduced from 96 to 25 nucleotides with a change in K_D from 3 to 0.5 nM for human thrombin, and RNA 16.24, which could be reduced from 79 to 24 nucleotides with a change in K_D from 37 to 9 nM, However, even in the absence of an affinity loss, size reduction may modulate the functionality and binding specificity. In the case of Muller et al. (2009), HS02 variants of different sizes with similar affinities for APC had size-dependent functional effects with a certain intermediate size being most optimal in complex matrices (66). Binding also appears not always to be 1:1 between aptamer and the target. A simple 1:1 binding mode could not convincingly be concluded from functional assays with FX aptamer RNA_{11F7t}, data being more consistent with the binding of 2 moles of RNA per mole of FX (62). Interestingly, this report also mentioned a lower limit estimate of 20 μM for the equilibrium dissociation constant for dimer formation of the aptamer. The ability to oligomerize was also observed for an elastase aptamer (74).

Mechanisms of inhibition of the enzyme activity of proteases by aptamers - Protease-binding aptamers often inhibit the enzymatic activity of their target. Four different mechanisms for inhibition of enzyme activity have been observed.

By the first mechanism, the aptamers sterically block the access of the substrate to the active site by binding to the catalytic domain. In some cases, the aptamers inhibited the turn-over of even small synthetic peptidylic substrates competitively. This type of inhibitory mechanism was suggested for aptamers to cathepsin E (99), aptamers to subtilisin (97), and aptamers to prostate specific membrane antigen (109), but the majority of proteases cleave macromolecular substrates which often rely on enzyme-substrate recognition outside the active site. Blocking these sites by binding of aptamers would therefore be expected to influence the proteolytic processing of macromolecular substrates. A number of aptamers seem to inhibit proteolytic activity by this mechanism. These aptamers include the thrombin-binding aptamer HD-1, which was reported to inhibit the proteolytic activity of thrombin by competing with its substrates for binding to exosite I, whereas no significant effect on hydrolysis of a small chromogenic substrate was observed (25).

By a second mechanism, the aptamers induce conformational changes in the protease

resulting in distortion of the active site or alternatively of exosites involved in the binding of substrate. This type of inhibitory mechanism has been inferred from studies where the binding epitope of the aptamer has been localized to an area outside the active site or outside the affected exosites of the protease. In most such cases, the aptamers exhibit non-competitive mode of inhibition of turn-over of small peptidylic substrates. Thus the NS3-binding aptamer G9-I was found to have an epitope distant from the active site but still to be an inhibitor of the hydrolysis of a small peptide substrate by NS3 (88,91). Other aptamers towards proteases reported to be non-competitive inhibitors include a BoNT-binding aptamer (107), a PMSA-binding aptamer (109), and an APC-binding aptamer (66), but in these cases, detailed characterizations of the aptamer binding sites were not reported.

By a third inhibitory mechanism, aptamers act by competing with binding of cofactors to proteases. Thus, it was suggested that aptamers to FX and FVII sterically interfere with association of these coagulation factors to their respective cofactors, *i.e.*, FVa and TF (56,62).

By a fourth inhibitory mechanism, the aptamers target the zymogen proteases and inhibit their proteolytic activation. This has been illustrated by the thrombin-binding aptamer HD-1, which inhibited prothrombin activation catalyzed by the TF-FVIIa complex (33). Furthermore, the FIX-binding aptamer 9.3 inhibited TF-FVIIa catalyzed FIX activation. Interestingly the activation of FIX by FXIa was not affected by 9.3 suggesting that FIX is recognized differently by its activating proteases (59).

Inhibition of molecular interaction other than the enzyme activity - Aptamers inhibiting non-proteolytic functions of proteases are also described in the literature. Rentmeister et al. (102) isolated aptamers binding to the cytoplasmic tail of BACE I inhibiting the binding of Golgi-localized γ-ear-containing ARF-binding protein. In another study by Dupont et al. (72), aptamers binding to the N-terminal domain of uPA was found to inhibit the binding of uPA to the cell surface receptor uPAR. Furthermore, an aptamer binding to and inhibiting the helicase of HCV NS3 was reported by Nishikawa et al. (93).

Advantages of using aptamers as inhibitors of proteolytic enzyme systems - Small molecule inhibitors are the type of inhibitors most frequently worked with in attempts to develop protease inhibitors. Such molecules are designed to block the catalytic site and prevent access of the natural substrates. Although much success has been achieved with this approach, in some cases, issues primarily related to the lack of specificity towards a single protease have led to disappointing results in clinical trials in other cases. This was experienced with synthetic organochemical inhibitors of matrix metallo-proteases directed against the active sites which displayed systemic side effects due to lack of specificity (for a review, see (117)).

One great advantage of aptamers is their high specificity towards the target against which they are selected. The specificity of aptamer is comparable to the specificity displayed by monoclonal antibodies. This has been exemplified in the above described selections of aptamers against proteases. As mentioned, compared with similar proteases aptamers are in general specific for their targets until at least the low micromolar range, which is the typical upper limit for testing specificity. Aptamers can be conformation-specific as exemplified by thrombin aptamer HD-22 and a PSA aptamer recognizing the active form but not the zymogen form of their targets (39,82). They can also have species-specificity as found for bovine FIX aptamers preventing bovine but not measurably human thrombin-catalyzed fibrinogen conversion (58). Likewise, uPA-uPAR blocking uPA aptamers were able to inhibit the interaction

in a human but not a mouse setting (72). For therapeutic applications species cross-reactivity is desired to enable animal testing. Cross-reactivity was found for several aptamers including FIX aptamer 9.3, thrombin aptamer Toggle-25 (49,57) as well as elastase aptamers (77,78).

As a side effect, anticoagulant therapy can provoke the development of acute hemorrhage (13). Antidotes to nucleic acid aptamers can be constructed from oligonucleotides with a sequence complementary to that of the aptamer. Hybridization of such oligonucleotides will thus disturb the aptamer three-dimensional folding and thus its target recognition. The principle was elegantly demonstrated with the bivalent anticoagulant thrombin-binding aptamer HD-1-22 (46) and FIXa binding aptamer (57,60). The antidote principle using nucleic acid binding polymers was illustrated with FIXa- and FXa-binding aptamers (61). Thus aptamers are inhibitors of choice when a balanced regulation is important, such as in the case of therapeutics against coagulation proteases. This possibility is unique for aptamers compared to other inhibitor classes such as small molecule inhibitors, peptides or monoclonal antibodies.

Another advantage of aptamers is the ease by which their pharmacokinetics can be changed. In general, 'naked' RNA or DNA aptamers have short half-lives *in vivo* of a few minutes because of degration. A short half-life can be advantageous in some cases where a rapid onset of action followed by a rapid reversal is wanted. In the case of thrombin the possibility of a rapid reversal will minimize the risk of hemorrhage. In clinical situations, where systemic anticoagulation poses a great risk, regional anticoagulation has been demonstrated to be feasible with for example thrombin aptamers with very short half-lives (17). In other cases, aptamers need to remain in circulation for longer time periods. In such cases, increasing resistance towards nuclease digestion by using modified nucleotides and conjugating aptamers with moieties such as polyethylene glycol or cholesterol has been shown to dramatically increase the half-life of aptamers *in vivo*. Such modifications have been carried out for most aptamers tested in *in vivo* studies (6).

It is possible to couple two aptamers against the same target, but with different epitopes, using various types of linkers. In this way, it is possible to construct bivalent inhibitors with an improved affinity. Moreover, such constructs may be bifunctional, inhibiting more than one function. Thus, a bivalent aptamer against HCV NS3 inhibited the protease as well as the helicase activity (94). The bivalent adduct between the two thrombin-binding DNA aptamers HD-1 and HD-22 have targeted exosite I and exosite II (43-45).

An obvious disadvantage when considering the use of aptamers as drugs is the fact that they cannot be made orally available.

Other applications of protease-binding aptamers - But there are great perspectives in other potential applications of protease-binding aptamers, which they share with aptamers directed against other targets. The applications of aptamers described in the literature also include their use as biosensors, delivery vehicles, therapeutic, and imaging agents.range from biosensors to delivery, therapeutic, diagnostic, and imaging agents.

Concerning the use of biosensors for analytic purpose, it is relevant to mention that aptamers often display conformation-specific affinity to their targets and thus are potentially useful for quantitative analyses of different conformational (and therefore functional) forms of their targets in biological samples.

With respect to the potential use of aptamers for *in vivo* imaging directly, Dougan et al. (29) investigated the thrombin imaging potential of HD-1 and HD-22. *In vitro* experiments suggested the ability of HD-22 but not HD-1 to form a ternary complex with

thrombin and fibrin but no specific *in vivo* association was obtained probably as a consequence of rapid aptamer clearance from the circulation together with slow mass transfer found in thrombuses. A 99mTc labeled elastase aptamer construct was investigated for the ability to image inflammation in a rat Arthus reaction model (79). The aptamer conjugate displayed better peak target-to-background signal compared with a clinically used antibody, but two-three fold lower signal intensity.

The ability to act in delivery of cargo for therapeutic or imaging applications has been illustrated with the aptamer for the prostate cancer-associated membrane protein PSMA.

PERSPECTIVES

As will appear from the above text, studies of protease-binding nucleic acid aptamers have played a pioneering and trend-setting role in the aptamer field. In spite of the amount of work done, there is still considerable un-characterised aspects of aptamer-protease interactions, which need to be elucidated by further X-ray crystal structure analysis of aptamer-protease complexes. Also, small angle X-ray scattering, analytical ultracentrifugation, and isothermal titration calorimetry are tools which have so far been under-employed but which may give important information about aptamer-protease interactions. In the future, we see a potential for the use of aptamers being able to give new information about basic enzymatic, regulatory, or inhibitory mechanism of proteases, as well as being useful tools for the elucidation of the biological functions of proteases.

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LIST OF ABBREVIATIONS

α_1-PI, α_1-proteinase inhibitor; APC, activated protein C; ARDS, acute respiratory distress syndrome; AT, antithrombin; BoNT, botulinum neurotoxin; FVa, blood coagulation factor V; FVIIa, blood coagulation factor VII; FVIIIa, blood coagulation factor VIII; FIXa, blood coagulation factor IX; FXa, blood coagulation factor X; Gla, γ-carboxy-glutamic acid; HCII, heparin cofactor II; HCV NS3, hepatitis C virus non-structural protein 3; LDLA repeats, low density lipoprotein receptor class A repeats; PAI-1, plasminogen activator inhibitor-1; PAR-1, protease-activated receptor-1; PCI, protein C inhibitor; PSA, prostate-specific antigen; PSMA, prostate-specific membrane antigen; SELEX, systematic evolution of ligands by exponential enrichment; TF, tissue factor; uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor.

FOOTNOTES

[1]Numbering of amino acids in the catalytic domain of serine proteases is according to the chymotrypsin template numbering.

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