The application of a modified nucleotide in aptamer selection: novel thrombin aptamers containing 5-(1-pentynyl)-2'-deoxyuridine

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ABSTRACT

Combinatorial libraries of nucleic acids are developing into novel sources for lead compounds in drug development. In order to diversify the pool of ss DNA sequences, we have used a modified nucleotide, 5-(1-pentynyl)-2'-deoxyuridine, in place of thymidine in a random nucleic acid library and screened this library against human thrombin. Previously, we described this screening method to identify a novel structural inhibitor (an aptamer) of the coagulation protease thrombin (Bock, L. *et al.* (1992) *Nature* 355 564 – 566). Using the modified nucleic acid library, we have now isolated a second pool of thrombin inhibitors with strikingly different sequence composition compared to the selection using natural bases. This second class of aptamers is dependent on the presence of the modified nucleotide for protein binding and clotting inhibition. Our method represents a potential strategy to enhance the diversity of libraries for *in vitro* selection, and thereby increasing the utility of this technique in the identification of molecules with novel biochemical properties.

INTRODUCTION

Chemical synthesis of random sequence oligonucleotides enables the construction of a diverse pool, in practice usually greater than 10^{13} different molecules, each theoretically capable of adopting a unique three dimensional structure. Capitalizing on this structural diversity, one can combine affinity chromatography and the polymerase chain reaction (PCR) to isolate nucleic acid molecules, termed aptamers, which specifically interact with a target attached to the affinity chromatography matrix. Using this approach, aptamers interacting with small molecule ligands such as Cibacron Blue and with nucleic acid binding proteins have been described^{1–7}. In addition, we recently described the isolation of a single stranded DNA (ss DNA) aptamer which specifically binds to the coagulation factor thrombin and is capable of inhibiting the enzymatic activity of this protease, even though this protein's natural function does not involve interaction with oligonucleotides⁸. The thrombin aptamer contains a unique

consensus sequence that adopts a distinct tertiary structure stabilized by G quartet formation, and is capable of inhibiting thrombin activity at nanomolar concentrations^{9,10}.

To date random library screening involving nucleic acids has utilized only naturally occurring building blocks. The four bases routinely found within either RNA or DNA do not display abundant information content when compared to amino acids. Cationic and anionic character, hydrophobic character (aliphatic and aromatic) and uncharged polar hydrogen bonding describe a limited number side chain interactions which take place in proteins. Theoretically, it is possible to transfer some elements of this diversity to oligonucleotides using modified nucleotides. In the case of aptamer selection, novel contacts offered by the modified base may alter the binding site location, the structural motif employed, the biochemical behavior the aptamer (e.g. enzyme inhibition properties) or the affinity level between the aptamer and the target.

Here, we describe *in vitro* selection studies involving thymidine substitution by a modified nucleotide, 5-(1-pentynyl)-2'-deoxyuridine (5-pentynyl-dU). We demonstrate that the incorporation of this hydrophobic group into the random oligonucleotide library significantly alters the outcome of the selection process against human thrombin. The isolated aptamers display enrichment for molecules containing extensive substitution with this modified nucleotide. Binding and anticoagulant activity of the isolated aptamers are discussed.

MATERIALS AND METHODS

Enzymes, nucleotides, oligonucleotides and reagents

Agarose linked avidin was purchased from Vector laboratories; Nick and NAP 10 chromatography columns were purchased from Pharmacia; dNTPs and pGEM3z were purchased from Promega; Vent DNA polymerase, Vent DNA polymerase buffer, T4 DNA ligase and restriction endonucleases were purchased from New England Biolabs; human α -thrombin, γ -thrombin, prothrombin and Factor X were from Haemetologic Technologies Inc. (Essex Junction, VT); human albumin, chymotrypsin and trypsin were from Sigma; Sequenase were purchased from U.S. Biochemical.

All other enzymes and reagents were purchased from

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commercial sources. Oligonucleotides were prepared using standard phosphoramidite chemistry on an Applied Biosystems DNA synthesizer.

Preparation of 5-(1-pentynyl)-2'-deoxyuridine triphosphate and 5'-dimethoxytrityl-5-(1-pentynyl)-2'-deoxyuridine cyanoethyl (bis-diisopropyl) phosphoramidate. 5-(1-pentynyl)-2'-deoxyuridine was prepared using Pd/Cu mediated coupling of 1-iodo-pentyne and 5-iodo-2'-deoxyuridine via the method of Hobbs¹¹. 5-(1-pentynyl)-2'-deoxyuridine triphosphate was synthesized and purified via the method of Kohler et. al.¹². The protected phosphoramidite was prepared using the method of Koster¹³.

Preparation of and in vitro selection with 5-(1-pentynyl)-2'-deoxyuridine containing single stranded DNA. The selection method was conducted as follows: a 60 nucleotide oligomer of the sequence 5'-TAGAATACTCAAGCTTCGACG-(N)₂₀-AGTTTGGATCCCCGGGTAC-3' was prepared via automated synthesis using standard phosphoramidite chemistry. After deprotection in NH₄OH, followed by desalting using a NAP10 column (Pharmacia), the oligonucleotide was gel purified, extracted and recovered. The synthetic fragment was amplified using large scale PCR (5 mL combined total volume) in 200 μ L reactions containing: 10 μ M primer 5'-TAGAATACTCAAGCTTCGACG-3'; 10 μ M primer 5'-biotin-GTACCCGGGGATCCAAACT-3'; 250 μ M dGTP, 250 μ M dATP, 250 μ M dCTP and 250 μ M pentynyl dUTP; 10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% Triton X-100 in 20 mM Tris pH 8.8. The reaction was brought to 100°C for 3 minutes and 10U of Vent Polymerase was added. This was cycled 18 times through 100°C, 1 min.; 56°C, 1 min.; 75°C, 2 min. using an extension of 5 sec/cycle at this temperature. A small aliquot of this primary amplification was then removed and amplified an additional 18 cycles in the presence of 60 μ Ci of α -³²P-dNTP (dCTP, dGTP, dATP). The replacement of thymidine by pentynyl dU in this and in all subsequent amplifications results in DNA containing primarily pentynyl dU in the initial round and only this modified pyrimidine in the locations originally thymidine within the random and 3' constant region of the synthetic oligonucleotide. The resulting 60 nt ds DNA product was applied to a Nick column equilibrated in 100 mM NaCl/100 mM Tris-HCl pH 7.5 and purification was performed as per the manufacturers instructions. ss 60 nt DNA was then isolated via the use of avidin agarose; ss 60 nt DNA was eluted from the matrix using 0.25 N NaOH, neutralized with glacial acetic acid to pH 6 and the buffer exchanged via a Nick column equilibrated in 10 mM Tris-acetate pH 7.4. The eluent was then brought to 20 mM Tris-acetate pH 7.4 containing 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂ and 1 mM CaCl₂. Selection was conducted using Concavalin A-immobilized thrombin and specific recovery of the protein/DNA complexes was accomplished by α -methymannoside elution as in ref 8.

Cloning and sequencing of pentynyl deoxyuridine thrombin aptamers. Round six ss DNA aptamer pool was amplified using thymidine triphosphate in place of pentynyl deoxyuridine triphosphate as described above in the absence of α -³²P-dNTP. The resulting double stranded DNA was purified via a Nick column equilibrated in 10 mM Tris-HCl pH 7.5/0.1mM EDTA. The selected ds DNA was digested with HinDIII and BamHI, gel purified and cloned into pGEM3z. Miniprep DNA was

prepared via the lysozyme boiling method and clones screened for single inserts using restriction enzyme digestion with HinDIII and EcoRI¹⁴. Single insert clones (>85% of those screened) were sequenced using Sequenase.

Determination of relative binding affinities of pentynyl dU thrombin aptamers via nitrocellulose filter binding. Radiolabelled ss 60 mer for each clone was prepared from miniprep plasmid DNA using standard amplification conditions described above with two minor modifications. First, the concentration of each dNTP was reduced to 100 mM and second, the amplification reactions were performed at increased α -³²P-dNTP levels (150 μ Ci each dATP, dGTP & dCTP). Limiting quantities of each ss DNA clone were incubated with human thrombin (62.5 nM through 8000 nM), and applied to prewetted nitrocellulose filters. Each filter was subsequently washed with 3 mL of wash buffer (140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂ in 20 mM Tris-Ac pH 7.4). The ³²P-ss DNA associated with the nitrocellulose filter was quantitated via Cerenkov counting.

Analysis of protein/ssDNA aptamer interaction specificity using electrophoretic mobility shift. 5'-³²P-labelled pentynyl dU aptamer was prepared using γ -³²P-ATP (Amersham) and T4 polynucleotide kinase (US Biochemical) using standard procedures¹⁵. The ³²P aptamer was heated to 95°C in selection buffer (140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂ in 20 mM Tris-Ac pH 7.4) for 5 minutes and cooled to room temperature. This ss DNA was then added to the respective protein equilibrated at 4°C (final concentration: 500 nM in selection buffer). This was allowed to stand on ice for 90 minutes. The sample was then brought to 10% glycerol and electrophoresed at 4°C using 20 mM NaCl/50 mM HEPES pH 7.1 in a 4% (39:1) native acrylamide gel.

Inhibition of thrombin catalyzed fibrin formation by thrombin pentynyl dU aptamers. Inhibition was assayed using purified human thrombin (Haemetologic Technologies Inc.) and human fibrinogen (Sigma). DNA was incubated with thrombin at 37°C for 1 minute and fibrinogen pre-equilibrated at 37°C was added. The final concentration of thrombin and fibrinogen in the mixture was 13 nM and 2 mg/mL respectively. Oligonucleotides concentrations are at or greater than their respective K_d as determined via nitrocellulose filter binding when determined. Clotting times were measured using an automated fibrometer as described⁸.

RESULTS

In vitro selection using a modified nucleotide combinatorial library

A number of modified pyrimidine heterocycles containing functional groups are readily synthesized and found to be polymerase substrates when prepared as their ribo- or deoxyribonucleoside triphosphates¹⁶⁻¹⁹. When incorporated into a PCR based method, it is critical that a modified nucleotide exhibits standard base pairing and template properties to ensure faithful replication in PCR. We have used pentynyl deoxyuridine (Figure 1), a nucleotide fulfilling both of the criteria stated above, in aptamer selection towards human thrombin. This nucleotide analog introduces a potential hydrophobic interaction site, via

the alkynyl substituent, which is largely unavailable with natural nucleotides. In addition, 5-alkynyl pyrimidines have been observed to display enhanced thermodynamic and biological stability which may also influence the selection outcome beyond novel protein/DNA contact^{20,21}.

A random oligonucleotide pool was chemically synthesized containing 20 random bases flanked by two constant regions for use in PCR. This template was amplified in the presence of 5-pentynyl-dUTP to construct an initial pool which was completely substituted with the modified base at thymidine position in the random and 3' constant regions of the original synthetic fragment. The ss DNA was isolated and the selection conducted using immobilized thrombin⁸. After six rounds of selection and amplification, a modified base containing aptamer pool towards thrombin was isolated (see Table 1).

Sequence composition of pentynyl dU thrombin aptamers

To determine the sequences of the DNA, the selected pool was converted back to thymidine substituted dsDNA via PCR and cloned into pGEM3Z. The individual sequences are shown in

Figure 1. Structure of modified nucleotide triphosphate used in aptamer selection. Synthetic DNA was amplified in the presence of **1** to generate the starting substituted random pool. All subsequent PCR amplifications were conducted using **1** in place of TTP. Automated chemical synthesis employed the protected phosphoramidite **2** during the preparation of synthetic fragments described in this study.

Table 1. Selection of pentynyl dU ss DNA aptamers towards human thrombin.

Cycle	Percent bound DNA
1	0.05
2	0.2
3	0.9
4	3
5	7
6	13
7	13

Selection was performed as in ref. 8, including the use of negative selection with Concanavalin A/agarose (Con A/agarose). A 60 nucleotide oligomer of the sequence 5'-TAGAATACTCAAGCTTCGACG(N)₂₀AGTTTGGATCCCCGGGTAC-3' substituted in the random region and 3' constant region with pentynyl deoxyuridine in place of thymidine was used in these studies. Percent bound represents that fraction of input ³²P ss DNA specifically eluted from the Con A/agarose with α -methyl mannoside treatment.

Table 2 where X represents the pentynyl dU position in the aptamer. These clones show limited sequence homology when compared to our sequences identified in studies with natural base containing DNA; of the twenty seven sequences analyzed, only one fit the GT-rich pattern previously observed (clone 21). The original consensus sequence has been shown to fold into a compact G quartet structure and it is possible that this alkynyl pyrimidine grossly interferes with formation of this type of quartet^{9,10}. One clone (clone 5) was isolated multiple times; this sequence contains a strong bias towards the modified base.

Pentynyl dU aptamer affinity

Nitrocellulose filter binding with purified human thrombin was then performed to determine the K_d of the initial pentynyl-dU random pool and the sixth round pentynyl-dU aptamers²². The dissociation constant of the initial pool was greater than 10 μ M (data not shown) whereas the selected pool was 400 nM (Figure 2). Three clones representing a small sample of the selected pool sequences each with different extents of pentynyl-dU substitution, were chosen for further study (clone 3, clone 5 & clone 7). Their protein dependent dose response curves are shown in Figure 2. From these data dissociation constants of clone 3, 5 and 7 were estimated to be 800, 400 and 1000 nM respectively. Clones exhibiting a high pentynyl dU content represent a significant proportion of the sequence data base. Clone 5, a high pentynyl dU content clone isolated multiple times, exhibits comparable

Table 2. Round six thrombin pentynyl dU aptamer variable region sequences.

Clone no.	Aptamer insert sequence
5	---G-XAXAXA-GXAXAGXAX-XGGC
18	X-AC-XAXCAXGXAXAXXACCC
4	--XAG-XAXGXAXX-AXGXG-X-AG
1	---AG-GAXAXAG-AXAXGAXXCGG
19	AX-AGAG-XAXAX--XGCGXGXCX
32	XAXAGAG-XAXA-GXAXGX-G-CX
3	CA--GAXACAGGC-CAX---GXGC
28	--XGGAXA--GCA-CAXX--GGXGXA
13	GACGACXCCAG-XXXA---GGXCG
33	AGXACAXGCAGGX-A-----GXXCAC
21	A-CGGAXG--G-X-C-X---GGCXGGACA
10	GXAC-AX-----X-CA---GGCXGCCXGCC
8	X-ACCAXCCCG-X-----GGACGXAAC
14	AAC-GAAG--GG--CACGCCGCGCXG
7	---GACXAAA---CGCAXXGXGCCCC
31	---GAACAAA---CGCAXXGX-CCCC
2	-----XCA---GXAXXAGGCCCCXCGAA
34	--GCCCAACA---CGCAXXGXGCCCC
20	--CACCAA-A---CGCAXXGCAXXCC
23	G-----A---CGCACCGXACCCCGX
22	GCXX---XXX-----GCAXXAAACGC-GA
27	--CAGXXXA---CGXGCCACXGXAC
29	GXGXAGXXXA---CGXCCCACCC
35	GAG---XXCA---CGXGCGAXGXGAC
26	CX-CCCAXAA-----XGCCXAGCCC

Clone sequences are grouped via homology analysis using the MEGALIGN sequence alignment program of LASERGENE (DNASTAR). Round six thrombin-associated aptamers were amplified in the presence of thymidine triphosphate to convert the selected pool to natural nucleotide containing ds DNA, and the 60 bp fragment was then cloned into pGEM3Z. Individual clones were then sequenced with Sequenase (US Biochemical). X denotes pentynyl dU as determined via sequencing.

Figure 2. Thrombin/pentynyl dU dissociation constant evaluation. Nitrocellulose filter binding was employed to evaluate the interaction between radiolabelled aptamer and thrombin. Percent ^{32}P ss DNA bound to nitrocellulose in a thrombin dependent format is plotted versus thrombin concentration. In this evaluation system, K_d is estimated at the fifty percent binding point.

affinity to the selected pool. The dissociation constant equivalence suggests that the biochemical character of the selected pool is dominated by this class of sequences. Clones 3 and 7, both lower in pentynyl dU composition, show decreased affinity indirectly supporting a correlation between binding strength and pentynyl dU substitution. Importantly, removal of pentynyl dU from the sequence via thymidine substitution abolishes binding indicating that the modified nucleotide plays an essential role in the interaction between the aptamer and thrombin (Figure 2; clone 5 substitution with thymidine).

Protein specificity of selected aptamers

The protein specificity of this pool was analyzed using an electrophoretic mobility shift assay²³. Radiolabelled clone 5 aptamer was prepared, incubated with various concentrations of human thrombin and then assayed via native polyacrylamide gel electrophoresis; a protein dependent bandshift was observed. The observed dissociation constant using this method is equivalent to the K_d determined via nitrocellulose filter binding (data not shown). Furthermore, this band shift is specific to thrombin. We did not observe a mobility shift when examining a series of related proteins (trypsin, chymotrypsin, coagulation factor X, prothrombin, γ -thrombin or albumin) (Figure 3). The loss of binding with prothrombin (the precursor of the mature protease) and γ -thrombin (a proteolytic derivative inactive in fibrinogen processing) implicates the anion binding exosite as the aptamer binding site. In prothrombin the exosite is still partially masked and in γ -thrombin it is destroyed^{24,25}. Previous work using lysine protection and site-directed mutagenesis has independently established that natural base-containing thrombin aptamers bind in this exosite^{26,27}. Our findings suggest that this second class of aptamers is also directed towards this site, implying this region of thrombin is the dominant aptamer determinant. Furthermore, our studies suggest multiple motifs of sequence specific recognition of this site are possible which vary depending on the novel substitution incorporated in the selection process.

Figure 3. Protein specific binding of pentynyl-dU containing aptamers to human thrombin. An electrophoretic mobility shift assay was used to assay protein/ssDNA aptamer interaction. Lane 1, no protein; lane 2, thrombin; lane 3, prothrombin; lane 4, γ -thrombin; lane 5, human albumin; lane 6, Factor X; lane 7, chymotrypsin; lane 8 trypsin.

Enzyme inhibition properties of pentynyl dU aptamers

Our previous studies have shown a correlation between protein-DNA binding and inhibition of enzymatic activity⁸. Oligonucleotides of the 20 base variable region of two clones (3 & 5) were next prepared by chemical synthesis and analyzed for their ability to inhibit thrombin enzymatic activity in a clotting assay. These two 20 base oligomers and a mixed sequence control were then analyzed for clotting inhibition activity (Table 3). Surprisingly, all three molecules were inactive. To investigate the importance of the primer region for activity the entire 60 base sequences containing the random region flanked by the two constant regions of clones 3 and 5 were prepared. These oligonucleotides contained pentynyl-dU in the 3' constant region and T in the 5' constant region, analogous to the PCR product. In addition, a clone 5 analog was also prepared replacing all pentynyl-dU residues with thymidine. Each was then analyzed for its ability to inhibit thrombin (Table 3). Clotting inhibition is observed with the 60 nt clone 5, and furthermore this activity is dependent on the presence of pentynyl-dU (Table 3 entry 8). We conclude from these results that one or both of the constant regions of the oligomer play a significant role for thrombin inhibition. In addition, we have independently verified, via this coagulation assay, that thrombin-aptamer interaction is dependent on the pentynyl-dU derivative (as judged by the clone 5 thymidine substitution result).

Secondary structure analysis of pentynyl dU aptamers

To investigate the potential structural motif of the pentynyl dU aptamers, we constructed a phylogenetic secondary structure using the sequence alignment data of Figure 2. From the three sequence groups, we observed potential hairpin structures; the general structure is conserved between the three groups. Primer binding regions are envisioned to participate in base-pairing that varies by group (explaining the primer requirements for anticoagulant action). The proposed hairpin would be significantly stabilized by the introduction of pentynyl dU and destabilized by its removal in accord with the data described^{20,21}. The group III hairpins successfully inhibit thrombin in the fibrin formation assay; these oligonucleotides are predicted to adopt the most extended duplex structure when compared to group I or group

Table 3. Clotting inhibition by modified base containing oligonucleotides

	Concentration	Time(sec)
(1) Clone 5 GXAXAXAGXAXAGXAXXGGC 20 nucleotide variable region	(1 μ M)	24
(2) Clone 3 CAGAGXACAGGCCAXGXGCA 20 nucleotide variable region	(1 μ M)	24
(3) Clone 5 GCXGXAGAXAXXAGXAXAGX 20 nucleotide variable region scrambled	(1 μ M)	24
(4) GGTTGGTGTGGTTGG	(20 nM)	43
(5) Clone 5-60 nucleotide fragment with pentynyl-dU	(4 μ M)	104.5
(6) Clone 3-60 nucleotide fragment	(4 μ M)	37.5
(7) Clone 5/T-substituted-60 nucleotide fragment	(4 μ M)	25.1
(8) Control		24.3

X=pentynyl-dU

Inhibition was assayed using purified human thrombin (Haematologic Technologies Inc.) and human fibrinogen (Sigma). DNA was incubated with thrombin at 37°C for 1 minute and fibrinogen pre-equilibrated at 37°C was added. The final concentrations of thrombin and fibrinogen in the mixture were 13 nM and 5.9 mM respectively. Oligonucleotide concentrations are as listed above and equal to or greater than their respective K_d as determined via nitrocellulose filter binding. Clotting times were measured using an automated fibrometer.

II. The correlation between duplex length and inhibition activity suggests that extensive secondary structure is required in order to effectively interfere with fibrinogen processing by the active site.

DISCUSSION

We have shown that modified nucleotides can be successfully incorporated into *in vitro* selection, and that by doing so they can dramatically alter the outcome. Sequences of a selected pool generated using a modified pyrimidine base differ significantly from those obtained with natural deoxynucleotides when selected against a common target. In the natural base experiments, a defined short consensus sequence was observed which was subsequently shown to be the binding determinant for this class of aptamer. With the of pentynyl dU pool on the other hand, our sequence analysis of has not established a consensus oligonucleotide which is responsible for the observed thrombin binding activity. Instead our data suggests the involvement of a conserved secondary or tertiary structure(s) which is dependent on pentynyl-dU for its formation and interaction with thrombin. These structures clearly involve the use of defined primer sequence. This phenomenon has been observed with a variety of targets ranging from small molecules to proteins, and appears to correlate with the size of the variable region incorporated into the selection strategy^{1,28-31}. For example, in studies by Yarus and coworkers using a comparable length variable RNA, aptamer recognition of arginine was found to exhibit a similar primer requirement to that described here³⁰. The 3' constant region sequence used in the recovery and amplification process during *in vitro* selection was identified as a critical feature for arginine/RNA interaction.

The dissociation constant of the full length pentynyl dU aptamers binding towards thrombin is comparable to that observed in the original full length natural ss DNA aptamers. In our original studies, the consensus sequence observed within the variable region showed enhanced affinity for thrombin when prepared separately from the flanking sequences. Size reduction with this class of aptamers clearly effected protein/nucleic acid contact or alternatively eliminated a nonbinding competing DNA structure(s). In either case, flanking sequence removal translated

Figure 4. Proposed hairpin secondary structures of pentynyl dU thrombin aptamers. Aligned sequences from figure 2 are grouped into similar predicted secondary structures composed of three distinct classes. Distinguishing features of these groups are: sequence composition of the hairpin loops, duplex length and the level of primer involvement in duplex formation. Conserved nucleotides found in all members of a particular group are shown. Boxed regions indicate areas of conserved base pairing where box size is proportional to overall duplex length; shaded areas indicate constant sequence for primer binding in PCR. Solid lines connecting these regions indicate contiguous duplex except in group III where the curved connection depicts a conserved bulged loop.

into increased affinity between thrombin and the oligonucleotide. The modified base aptamers on the other hand lack a distinguishing consensus sequence and are biochemically inactive when the variable regions are synthesized separately. We feel it is unlikely that these nucleotides have perturbed or limited the structural motifs of this class of oligonucleotides to generate this behavior. Pentynyl-like oligonucleotides have been synthesized previously, and are functional in hybridization and exhibit normal nuclease sensitivity^{20,32}. Given this different structural requirement, it is difficult to compare the binding affinity between this new class of aptamers and those isolated earlier.

As mentioned earlier, the site of interaction between thrombin and the two classes of ss DNA aptamers is the anion binding exosite^{26,27}. This site contains two dominant structural features: (a) high cationic charge density and (b) an extensive cluster of surface hydrophobic residues³³. This combination of hydrophobicity and cationic character make it the ideal interaction site for both classes of ss DNA aptamers. A second class of thrombin inhibitors, the hirudins, also interact with this site^{34,35}. These leech derived peptides bind very tightly to thrombin ($K_d < 10^{-12}$ M) by interacting with the exosite via extensive hydrophobic and ionic contacts³³. This interaction is significantly weakened when the exosite is modified, behavior identical to both classes of oligonucleotide inhibitors^{36,37}.

A second important feature of this exosite is its distance from the enzyme's active site. Fibrinogen cleavage is a coordinated process involving substrate recognition in the exosite and peptide bond hydrolysis at the active site. Enzyme inhibition can result from interference at either of these steps. It is unlikely that the pentynyl dU and natural base oligonucleotides are positioned identically within the exosite given the different sequence and functional group requirements of these aptamers. In addition, it appears that clotting inhibition is not a universal property of the pentynyl dU class of aptamers. The group III sequences (figure 4) with the largest potential hairpin structure are the only group that exhibits significant clotting inhibition. This data indicates that effective pentynyl dU aptamer inhibition correlates with the degree of secondary structure. Aptamer repositioning within the exosite may have disrupted the original substrate/inhibitor overlap requiring a more extensive structure in order to interfere with fibrinogen binding and processing (a property fulfilled only by aptamers of group III).

In conclusion modified nucleotide aptamers, like their natural counterparts, are able to retain enzyme inhibition properties, and more importantly this activity is dependent on the modified base. We believe these initial studies provide insight into the type of changes that can occur when modified bases are employed. Further studies using other classes of modified nucleotides will be required in order to develop a more complete understanding of their potential within the aptamer selection process.

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