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X-Aptamers: A Bead-Based Selection Method for Random Incorporation of Druglike Moieties onto Next-Generation Aptamers for Enhanced Binding

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Supporting Information

ABSTRACT: By combining pseudorandom bead-based aptamer libraries with conjugation chemistry, we have created next-generation aptamers, X-aptamers (XAs). Several X-ligands can be added in a directed or random fashion to the aptamers to further enhance their binding affinities for the target proteins. Here we describe the addition of a drug (*N*-acetyl-2,3-dehydro-2-deoxyneuraminic acid), demonstrated to bind to CD44-HABD, to a complete monothioate backbone-substituted aptamer to increase its binding affinity for the target protein by up to 23-fold, while increasing the drug's level of binding 1-million fold.

Aptamers are single-stranded RNA and DNA oligonucleotides that can bind a specific molecular target and exhibit high binding affinity. Aptamers are thus emerging as viable alternatives to small molecules and antibodies for many applications in research, diagnostics, imaging, and therapeutics.[1,2]

A diverse range of modifications have been reported[1,3−5] for either enhancing aptamers' nuclease resistance or expanding their chemical functionalities. Here we present the first example of X-aptamers that were endowed with both properties via addition of druglike molecules to 5-positions of certain uridines on a complete monothiophosphate backbone-substituted oligonucleotide aptamer. By combining our one-bead, one-sequence thioaptamer selection method[6,7] with the incorporation of pseudorandomly placed bases containing chemical linkers, we can append additional X-ligands onto aptamers or thioaptamers to create a next-generation, X-aptamer library, and the best binding X-aptamers can be selected from this large pool of sequences. In a previous report,[8] we described thioaptamers substituted with monothiophosphates on the 5′-side of dA that bind to the hyaluronic acid binding domain of CD44 (CD44-HABD) (K_D = 187−295 nM). On the basis of the primary sequence of several of these thioaptamers and observed variations, we synthesized a pseudorandom, one-bead, one-sequence bead library of aptamers using an automated four-column, split-pool synthesizer.[9] This synthesis produced a library with >1 million (4^{10}) unique X-aptamer sequences (Figure 1) in which X = 5-[*N*-(2-aminoethyl)-3-(*E*)-acrylimido)]-2′-deoxyuridine (amino-dU) or, later, a conjugated druglike appendage. The bead-based library consists of a 5′-primer region, a 30-nucleotide pseudorandom sequence (nine split-pool steps), and a 3′-primer region that is covalently linked by a noncleavable hexaethylene glycol linker to a 65 μm polystyrene bead (ChemGenes). The unique sequence on a given bead may contain 0−12 X-positions, but three or four Xs is most likely. To select highly nuclease resistant X-aptamers, the library was prepared with a fully monothiophosphate (permonothioated) backbone. This original library served as the base library from which a variety of additional X-aptamer libraries were derived by conjugation with NHS ester forms of druglike molecules. To select small molecule ligands as binding affinity enhancers that could be attached to the X-aptamer base library, we carried out *in silico* screening using AMBER9[10] and DOCK6.4[11] (see the Supporting Information for details). ADDA (*N*-acetyl-2,3-dehydro-2-deoxyneuraminic acid) was selected as the lead compound (Figure S3B of the Supporting Information), and several other compounds are under investigation. ADDA binds to CD44-HABD with an equilibrium dissociation constant of 2.22 ± 0.82 mM (Supporting Information). The docking experiments suggested that ADDA binds into part of the hyaluronic acid binding pocket, which is believed also to be the binding site of the originally selected

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Col. 1:  5′-GAGATTCATCACGCGCATAGTC CCAA-GGCC-TGC-AAG-GGA-ACC-AAG-GAC-AC-AG CGACTATGCGATGATGTCTTC-3′
Col. 2:  5′-GAGATTCATCACGCGCATAGTC XGCA-GATC-CAG-TAG-GTA-XCC-ATA-TCC-AA-TA CGACTATGCGATGATGTCTTC-3′
Col. 3:  5′-GAGATTCATCACGCGCATAGTC TTGG-GACG-XGX-TAA-ACG-AAG-GGG-ACG-GT-GA CGACTATGCGATGATGTCTTC-3′
Col. 4:  5′-GAGATTCATCACGCGCATAGTC XXAA-GAXA-CAX-AAX-XGA-AXG-XAA-XAC-AX-XG CGACTATGCGATGATGTCTTC-3′
Example: 5′-GAGATTCATCACGCGCATAGTC CCAA GATC CAX TAG ACG ACC XAA TCC AX GA CGACTATGCGATGATGTCTTC-3′
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Figure 1. Four sequences used in the split-pool, complete monothioate XA library synthesis and one example of a resulting bead sequence. X = 5-[*N*-(2-aminoethyl)-3-(*E*)-acrylimido)]-2′-deoxyuridine created by incorporation of "Amino Modifier C2 dT" (Glen Research). Dashes represent the presence of a split-pool synthesis step. The example sequence would result from a bead that followed the column path (from 3′ to 5′) 3-4-2-4-1-3-2-4-2-1, depicted as shaded parts, during the split-pool method.

thioaptamers (Figure 2A and Figure S3 of the Supporting Information).

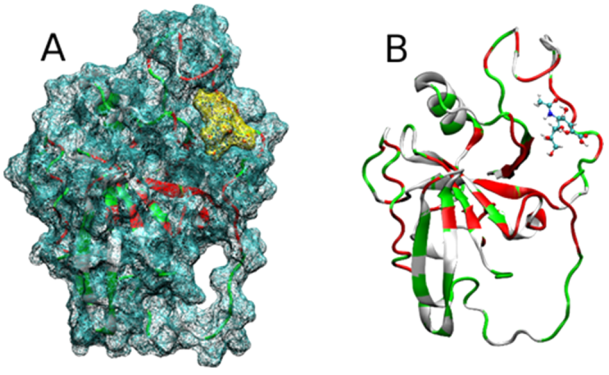

Figure 2. (A) Predicted docking of ADDA to CD44-HABD. (B) Chemical shift changes observed in a $^{1}H-^{15}N$ HSQC experiment mapped onto the structure of CD44-HABD.[12] Large changes are colored red, small changes white, and unassigned residues green.

The ^{15}N HSQC NMR experiments (Figure 2B and Figure S4A of the Supporting Information) also support these calculations, as shown by large changes in amide proton chemical shifts[13] surrounding the purported binding pocket.

ADDA was conjugated to the amino-dU of the original XA library by using amine−carboxy coupling. A bead-based selection method was conducted to find the XAs specific for CD44-HABD (see the Supporting Information for details). The selected positive X-aptamer beads were used for the "one-bead, one-polymerase chain reaction" amplification process.[6,7] The X-positions in the sequence were determined on the basis of the original library design, using the adjacent bases as "bar-coding". Table S1 (Supporting Information) lists the XA sequences obtained.

Nitrocellulose-nylon filter binding assays were used to determine equilibrium dissociation constants (K_D) of the selected XAs with CD44-HABD that are listed in Table S2 (Supporting Information). Saturation binding curves were generated by curve fits assuming a single binding site.

Although our previously reported[8] thioaptamer had a K_D value of 191 ± 25 nM, the sequence was monothioated adjacent to only the dAs, with nuclease-susceptible phosphate backbones at all remaining positions. When the same sequence was permonothioated to enhance nuclease resistance, it bound to CD44-HABD with a similar affinity [K_D = 230 ± 47 nM (Table 1)]. However, the selected permonothioated amino X-aptamers and ADDA-modified X-aptamers exhibited K_D values around 60−80 nM (Table S2 of the Supporting Information), an improvement of approximately 3−4-fold from those of the unmodified permonothioate thioaptamers.

Secondary structure predictions performed using MFold[14] suggested that all selected XA sequences can form hairpin loop structures in which the random regions form loops and the primers form stem regions (Figure S1 of the Supporting Information). On the basis of these predicted structures, we identified several binding motifs and smaller constructs of various stem−loop regions. The equilibrium binding constants of these small XA constructs (Table 1) were also determined by filter binding assays.

Remarkably, coupling ADDA with smaller stem−loop constructs from the best X-aptamer sequences, motifs 2 and 4 (ADDA adduct), yields an ∼2 nM affinity for CD44-HABD, which is an increase in binding affinity of ∼115-fold between the full-length permonothioated parent sequence and the final ADDA-conjugated XA (phosphoform) and ∼23-fold between

Table 1. X-Aptamer Sequences and Equilibrium Dissociation Constants for Binding to the CD44 Hyaluronic Acid Binding Domain

		Dissociation constant (nM)			
Parent[a]	Full-length sequence, partially monothioated	191 ± 25[b]			
Parent[a]	Full-length sequence permonothioated	230 ± 47			
		Phospho X-aptamers		Thiophospho X-aptamers	
	Motif Sequence[c]	X = amino-dU	X = ADDA-dU[d]	X= amino-dU	X = ADDA-dU[d]
Motif 1[e]	5′- AAGGGAACCAAGGACACTAC-3′	10.3 ± 1.3		15.0 ± 2.0	
Motif 2	5′-CXGXTAGGGAACCAAGACGA-3′	43.1 ± 9.5	2.0 ± 0.6	48.0 ± 18.0	15.5 ± 3.2
Motif 3	5′- GCCTGCAAGACGXCCATAGACAC-3′	27.6 ± 3.5	19.5 ± 3.2	81.2 ± 30.9	64.8 ± 13.7
Motif 4	5′- GATCTGCAAXGTAACCATAGACA-3′	6.8 ± 1.8	2.1 ± 0.2	35.4 ± 7.4	13.6 ± 3.0
Motif 5	5′- AGAXACAGTAAACGXCCATAGACAC-3′	13.8 ± 4.1	3.9 ± 1.0	18.0 ± 3.7	10.1 ± 2.6

[a]The sequence of the full-length parent aptamer is shown in the first row of Figure 1 and corresponds to TA1 from ref 3. [b]From ref 3. [c]Alternating shading indicates split-pool sequence sections in Figure 1 for these aligned sequences. [d]ADDA-dU is the ADDA adduct with 5-(aminoethyl-3-acrylimido)deoxyuridine. [e]Motif 1, which contains no X, was included to compare the phosphoaptamer and the thioaptamer forms.

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the full-length sequence complete monothioates and motif 5 (thiophosphoform). Moreover, in every case in Table 1, the ADDA-conjugated XA showed increased affinity compared to that of the unconjugated XA. In the best case, ADDA conjugation increased affinity ~22-fold. ADDA binds more weakly (~2 mM) to CD44-HABD. By conjugation to an aptamer, the binding affinity of ADDA-modified XAs has improved the affinity 1 million-fold (2 nM).

By introducing a protein binding small drug molecule, ADDA, into the 5-position of dU residues at random positions of the aptamers and/or replacing one of the nonbridging phosphate oxygen atoms with sulfur atoms, we are able to select an X-aptamer with a <10 nM affinity for CD44-HABD through a noniterative bead-based selection from large combinatorial libraries of X-aptamers. Our bead-based method is compatible with both monothiophosphate- and dithiophosphate-modified thioaptamers, and even complete monothiophosphate modification of the backbone, as reported here, which is not possible with traditional SELEX methods.[15,16] In addition, only one or two rounds of aptamer selection are required in contrast to the 10−15 rounds necessary in traditional SELEX.

As expected, the effect of ADDA as a binding affinity enhancer is dependent of the location within the aptamer. The process to find the optimal position of the ligand was part of our X-aptamer selection because ADDA was attached to the aptamers at various positions. By simultaneously selecting the optimal sequence of the aptamer scaffold and orientation and position of the small drug presented by the optimal scaffold, we enhance affinities. The incorporation of ADDA expands not only the XA's chemical diversity but also the surface area of binding; thus, the XA can also offer enhanced specificity.

While this work describes conjugation with one specific drug at a time, multiple drug hits can be randomly attached as well, to provide enhanced combinations of binding moieties. More than one ligand can be attached by pausing the DNA synthesis for the addition of a ligand and subsequent DNA synthesis and coupling reactions. By using two or more chemical linkers in one root library, multiple drugs can be selectively incorporated. Our methodology can be applied to most target proteins with a variety of small molecules to create highly chemically modified X-aptamers that have the combined characteristics of drug molecules, proteins, and nucleic acids.

■ ASSOCIATED CONTENT

Supporting Information

Additional experimental details, Molecular Dynamics (MD) protocols, and supplemental figures and tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

D.E.V., A.S., V.T., and D.G.G. designed the bead library. J.E. synthesized the bead library. M.-A.E.-R. and C.N.C. designed and conducted docking and MD experiments. W.H. conjugated drugs to the library. W.H. and R.H.D. designed and conducted bead selection experiments. W.H., X.L., and G.L.R.L. performed binding assays. All authors contributed to writing the manuscript.

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Notes

The authors declare the following competing financial interest(s): D.G.G. and the University of Texas Health Science Center at Houston have research-related financial interests in AptaMed Inc. and AM Biotechnologies LLC (Houston, TX). R.H.D. and J.E. are employees of AM Biotechnologies, which has a commercial interest in this technology.

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