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Selection of 2′-Fluoro-Modified Aptamers with Optimized Properties

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Supporting Information

ABSTRACT: RNA or single-stranded DNA aptamers with 2′-F pyrimidines have been pursued to increase resistance to nucleases, and while it seems likely that these and other modifications, including the modification of purines, could be used to optimize additional properties, this has been much less explored because such aptamers are challenging to discover. Using a thermostable DNA polymerase, SFM4-3, which was previously evolved to accept nucleotides with 2′-modifications, we now report the selection of 2′-F purine aptamers that bind human neutrophil elastase (HNE). Two aptamers were identified, 2fHNE-1 and 2fHNE-2, that bind HNE with reasonable affinity. Interestingly, the 2′-F substituents facilitate the selection of specific interactions with HNE and overcome nonspecific electrostatic interactions that can otherwise dominate. The data demonstrate that inclusion of only a few 2′-F substituents can optimize properties far beyond simple nuclease resistance and that SFM4-3 should prove valuable for the further exploration and production of aptamers with properties optimized for various applications.

Aptamers, single-stranded RNA or DNA (ssDNA) oligonucleotides that recognize a specific target with high affinity and high specificity, are emerging as an important class of biomolecules with potential applications ranging from affinity reagents, molecular imaging, and diagnostics to potentially eventually even therapeutics.[1−3] Nonetheless, the nature of the natural nucleotides limits their potential properties and functions and thus their potential applications. For example, it may be challenging to evolve aptamers specific for positively charged targets, as nonspecific electrostatic interactions with their highly negatively charged backbones may dominate recognition. An approach to circumventing these limitations is to include modified nucleotides bearing alternate functionality, including on the phosphate,[4] sugar,[5] or nucleobase moiety.[6,7] Aptamers with 2′ sugar substituents have attracted the most interest because of their potential to stabilize nucleotides against nucleolytic degradation.[8,9] Such modifications are also expected to impart the aptamers with other properties, but this possibility has received little attention.

As with other nucleotide modifications, the challenge with developing aptamers with 2′-substituents lies in their poor recognition by the DNA polymerases required for the amplification step of the selection procedure. Thus, the 2′-modified oligonucleotides are first "reverse-transcribed" into their fully natural DNA counterparts, amplified, and then "transcribed" back into their modified form for selection.[10,11] While these transcription and reverse-transcription reactions are more tolerant of nucleotide modification, at least in part because they only need to tolerate modification in the template of synthesized strands, many modifications remain inaccessible. For example, while aptamers with 2′-F-modified pyrimidines may be transcribed and reverse-transcribed and thus have been used for aptamer selection,[11] the analogous purine modifications are not well tolerated. To enable a greater exploration of the range of physicochemical properties available with 2′ modifications, we recently reported the directed evolution of variants of the Stoffel fragment of Taq DNA polymerase that efficiently amplify oligonucleotides that are partially modified with 2′-substituents, including 2′-F-modified purines, with the most efficient being SFM4-3.[12]

To begin to explore the use of SFM4-3, we initiated selections for 2′-F-modified purine aptamers that bind human neutrophil elastase (HNE) (Figure 1A and the Supporting Information), a serine protease associated with numerous inflammatory diseases.[13,14] With an isoelectric point of 9.5, HNE is positively charged at neutral pH, and natural anti-HNE aptamers have been reported previously.[15] Libraries of ∼10^{13} 68-mer DNA fragments with a central 30-mer random region flanked by primer binding sequences and biotinylated at their 5′ termini were first converted into fRdY (2′-F-modified purines (fR), deoxypyrimidines (dY)) DNA by asymmetric emulsion PCR using SFM4-3 with the primer for reverse strand synthesis also biotinylated (Figure 1A). Streptavidin partitioning was then used to generate a DNA-free fRdY single-stranded library, which was incubated with immobilized HNE. After incubation and washing, fRdY ssDNA was eluted with hot formamide. The recovered oligonucleotides were then subjected to SFM4-3-mediated amplification with natural dNTPs, followed by a second amplification with 2′-F purine triphosphates. The increased efficiency of the first amplification was found to be required to produce sufficient template concentration for the second, less efficient amplification. The process of selection and amplification was then repeated with the selection stringency progressively increased each round by employing longer wash times, increasing ionic strength of the wash buffer, and adding increasing amounts of yeast-tRNA to compete with nonspecific fRdY binding.

After six rounds of selection, a significant reduction in the qPCR-measured C_t value of the eluted fraction prompted us to analyze the libraries by PAGE, which interestingly revealed two bands (Figures S1 and S2): the expected 68-mer and a longer 75−90-mer, which likely resulted from template switching during amplification.[16,17] Five additional rounds of selection

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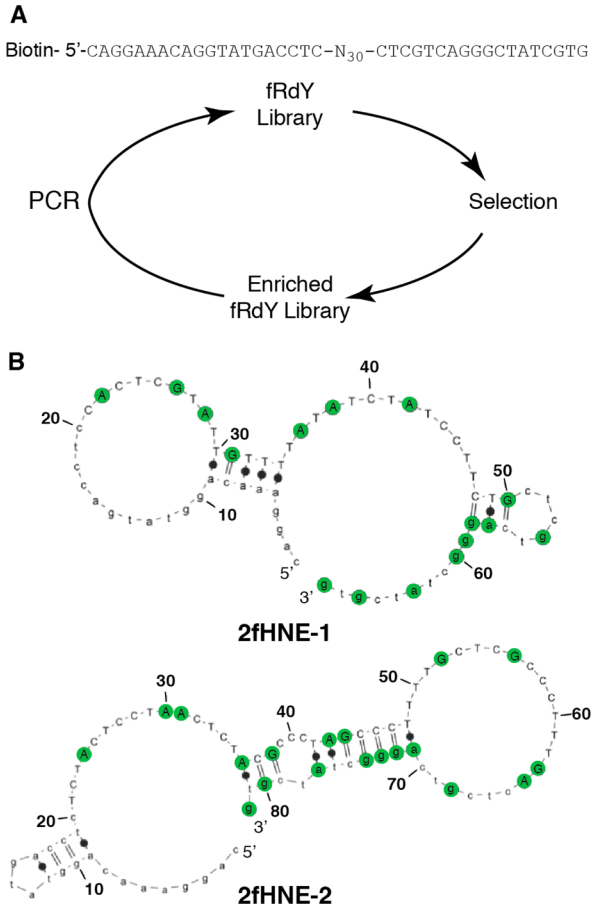

Figure 1. (A) Library design and selection scheme for anti-HNE fRdY aptamers (see the text and Supporting Information for details). (B) Predicted secondary structures of 2fHNE-1 and 2fHNE-2 (mFold[18]). Nucleotides from the constant primer regions are shown in lower-case letters, and 2′-F-modified nucleotides are highlighted in green.

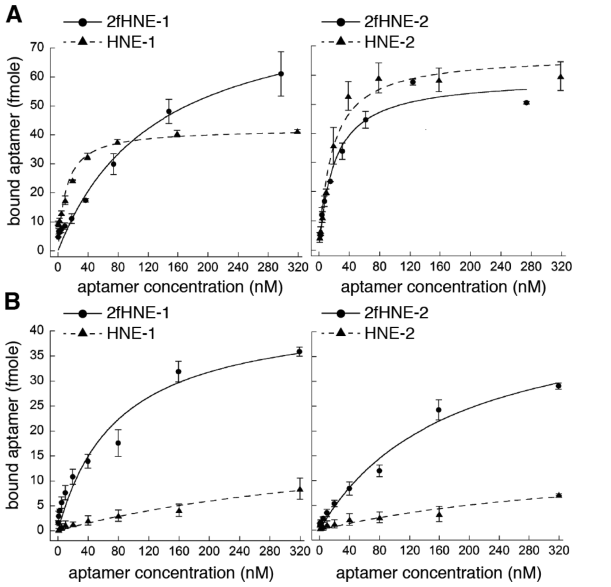

Figure 2. (A) Binding of 2fHNE-1, 2fHNE-2, and their natural counterparts to HNE under low salt and low yeast tRNA concentrations. (B) Binding of 2fHNE-1, 2fHNE-2, and their natural counterparts to HNE under high salt and high yeast tRNA concentrations. Data are averages and standard deviations of three independent determinations (see the Supporting Information for details).

were performed with only the oligonucleotides of the expected length (purified by PAGE after each round) or with the entire library, in which case the slower-migrating band increased and dominated the selected libraries (Figure S2). Individual library members were identified via conversion to their natural counterparts by SFM4-3 and sequencing, and of the 42 members of the faster-migrating population sequenced, the sequence 2fHNE-1 was identified seven times (Figure 1B). Upon sequencing 65 members of the slower-migrating population, 10 contained the consensus sequence GCCCT, including 2fHNE-2 (Figure 1B), which drew our attention because it may form an activity-promoting stem−loop structure with the AGGGC sequence of the 3′ constant primer-binding region.

To characterize the affinity of 2fHNE-1 and 2fHNE-2 for HNE, we employed a fluorescence-based microwell plate binding assay (see the Supporting Information). In addition, to elucidate the effects of 2′-F modifications, we also produced their fully natural counterparts, HNE-1 and HNE-2, respectively, via asymmetric PCR with SFM4-3 and natural triphosphates. Finally, for comparison, we synthesized the previously reported, fully natural HNE aptamer DNA-I.[15] We first examined the binding in the presence of 150 mM NaCl and 25 ng/μL yeast tRNA (Figure 2A). Under these conditions, 2fHNE-1 and 2fHNE-2 bound to HNE with K_d values of 130 and 20 nM, respectively. The slightly higher level of HNE-1 required for saturation may result from a low level of nonspecific binding. DNA-I bound HNE with a K_d of 106 nM, which is larger than the reported value of 17 nM, likely as a result of the presence of the competitor tRNA (Figure S3). Surprisingly, HNE-1 and HNE-2 also bound HNE, and in fact, with K_d values of 11 and 17 nM, did so with greater affinity than their 2′-F counterparts.

HNE is positively charged, and thus, to examine the effects of nonspecific electrostatic interactions, we examined the binding of each oligonucleotide in the presence of 1 M NaCl and 200 ng/μL yeast tRNA (Figure 2B). Under these conditions, 2fHNE-1 and 2fHNE-2 clearly retained affinity, with K_d values of 78 and 172 nM, respectively. However, DNA-I showed undetectable binding (K_d > 1 μM) (Figure S3). Most importantly HNE-1 and HNE-2 also showed undetectable binding under these conditions (K_d > 1 μM). Thus, the recognition of HNE by DNA-I, HNE-1, and HNE-2 appears to be mediated largely by nonspecific electrostatic interactions, while recognition by the 2′-F modified aptamers is mediated by more specific interactions.

To further confirm that the presence of the 2′-F substituents results in a different mode of binding, we performed binding competition experiments using Alexa-488-labeled oligonucleotides (Figure 3 and the Supporting Information). As a control, we first demonstrated that binding of fluorophore-labeled HNE-1 or HNE-2 to plates coated with HNE was eliminated with the addition of an excess of their unlabeled counterpart. In contrast, the binding of 2fHNE-1 was slightly increased and that of 2fHNE-2 only marginally reduced by the addition of excess HNE-1 and HNE-2, respectively. The relative changes in binding of 2fHNE-1 and 2fHNE-2 with their unlabeled counterparts are consistent with the changes in K_d observed with increased salt and yeast tRNA. These data demonstrate that the presence of the 2′-F substituents within otherwise

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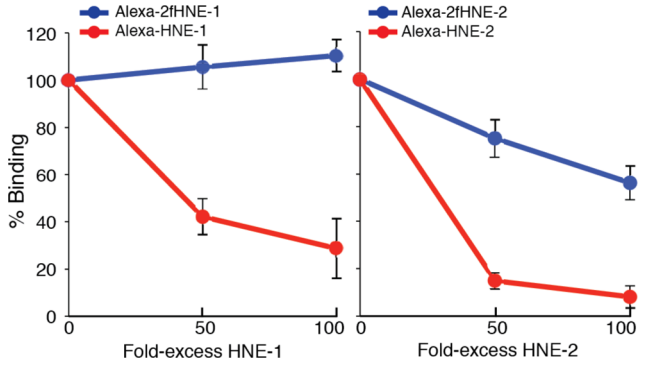

Figure 3. Competitive binding of Alexa-488-labeled oligonucleotides (10 nM) with excess unlabeled HNE-1 or -2. Binding in the absence of any competition is taken as 100%, and relative percent binding is plotted. Data shown are averages and standard deviations of three independent determinations.

identical oligonucleotides results in the recognition of different epitopes of HNE.

To elucidate how the 2′-F purine substituents might mediate the altered and more specific recognition of HNE, we utilized their NMR activity. The secondary structure of 2fHNE-1 was first predicted using mFold,[18] which identified a core stem–loop that contained four 2′-F modified purines (two 2′-F-modified A and two 2′-F-modified G nucleotides). To examine the behavior of this potential core structure of the aptamer, we chemically synthesized the fragment mf2fHNE-1, corresponding to nucleotides 5–35 (Figure 4A). At low temperature, only two broad peaks were observed in the ^{19}F NMR spectrum of m2fHNE-1 (Figure 4B), suggesting the presence of aggregation and/or conformational heterogeneity.[19−21] With increasing temperature, the upfield-most peak split into two and an additional, downfield-shifted, broad peak appeared. By 40 °C all four peaks were sharp. We then synthesized the corresponding oligonucleotides bearing only one of the 2′-F-modified purines (with the other three replaced by their natural counterparts). At low temperature, no peaks were observed for any of the singly modified oligonucleotides, and between 32 and 35 °C, single broad peaks appeared, two of which remained broad even at 40 °C (Figure 4B). While the chemical shifts observed for each singly modified variant are similar to one of the peaks observed with m2fHNE-1, allowing for a likely peak assignment, at no temperature do they match precisely. Thus, the data reveal that the precise structure of the putative core of 2fHNE-1 requires all four 2′-F substituents and that the structure is likely more aggregated in their absence.

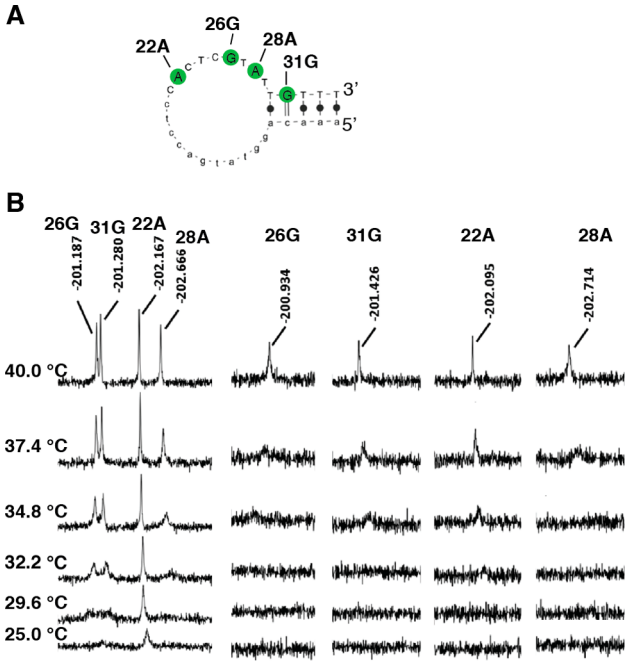

Figure 4. (A) Predicted structure of m2fHNE-1, with nucleotides from the constant primer regions shown in lower case and 2′-F-modified nucleotides highlighted in green and labeled. (B) Proton-decoupled ^{19}F NMR spectra of m2fHNE-1 and each singly 2′-F-labeled variant. Signals in m2fHNE-1 are assigned on the basis of the chemical shifts of the singly modified variants.

There has been much interest in the development of 2′-modified aptamers, but their identification has historically been a laborious procedure, involving three different polymerases, and generally only pyrimidine modification was possible. The directed evolution of SFM4-3 now allows for the use of a simplified procedure, requiring only a single polymerase, and the inclusion of modified pyrimidines and/or purines. This advance has allowed us to select for aptamers containing 2′-F-modified purines in order to demonstrate that the modifications significantly increase aptamer selectivity, likely by stabilizing specific conformations that recognize their target. It has long been known that 2′-F substituents favor the 3′-endo sugar pucker, which disfavors duplex formation with DNA.[22] Perhaps in the absence of 2′ modification, other structures with greater inter- or intramolecular duplex formation that lead to aggregation are favored. Thus, the optimized properties of the selected aptamers may result from duplex avoidance that allows for the selection of more soluble species that more specifically recognize their target. Regardless, it is clear that 2fHNE-1 and 2fHNE-2 more specifically recognize HNE than the previously reported DNA-I aptamer[15] and that the 2′-F substituents are responsible. The availability of SFM4-3 should enable further exploration of these and other 2′ modifications and possibly the identification of aptamers with activities and properties tailored for specific applications.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b13132.

Methods and supporting tables and figures (PDF)

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Notes

The authors declare no competing financial interest.

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