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Design of a “Mini” Nucleic Acid Probe for Cooperative Binding of an RNA-Repeated Transcript Associated with Myotonic Dystrophy Type 1

Wei-Che Hsieh^{†,‡,§}, Raman Bahal^{†,‡}, Shivaji A. Thadke^{†,‡,§}, Kirti Bhatt^{||}, Krzysztof Sobczak^{||,§}, Charles Thornton^{||}, and Danith H. Ly^{†,‡,§,*}

[†]Department of Chemistry, Carnegie Mellon University, 4400 Fifth Avenue, Pittsburgh, Pennsylvania 15213, United States

[‡]Institute for Biomolecular Design and Discovery (IBD), Carnegie Mellon University, 4400 Fifth Avenue, Pittsburgh, Pennsylvania 15213, United States

[§]CNAST, Carnegie Mellon University, 4400 Fifth Avenue, Pittsburgh, Pennsylvania 15213, United States

^{||}Department of Neurology, Box 645, University of Rochester Medical Center, 601 Elmwood Avenue, Rochester, New York 14642, United States

Abstract

Toxic RNAs containing expanded trinucleotide repeats are the cause of many neuromuscular disorders, one being myotonic dystrophy type 1 (DM1). DM1 is triggered by CTG-repeat expansion in the 3'-untranslated region of the *DMPK* gene, resulting in a toxic gain of RNA function through sequestration of MBNL1 protein, among others. Herein, we report the development of a relatively short miniPEG- γ peptide nucleic acid probe, two triplet repeats in length, containing terminal pyrene moieties, that is capable of binding rCUG repeats in a sequence-specific and selective manner. The newly designed probe can discriminate the pathogenic rCUG^{exp} from the wild-type transcript and disrupt the rCUG^{exp}-MBNL1 complex. The work provides a proof of concept for the development of relatively short nucleic acid probes for targeting RNA-repeat expansions associated with DM1 and other related neuromuscular disorders.

*Corresponding Author: dly@andrew.cmu.edu. Telephone: (412) 268-4010. Fax: (412) 268-5579.

Present Addresses

R.B.: School of Pharmacy, University of Connecticut, 69 N. Eagleville Rd., Storrs, CT 06269.

#K.S.: Department of Gene Expression, Institute of Molecular Biology and Biotechnology, Adam Mickiewicz University, Umultowska 89, 61-614 Poznan, Poland.

ORCID

Danith H. Ly: 0000-0002-8760-0070

Author Contributions

W.-C.H. and R.B. contributed equally to this work.

Notes

The authors declare no competing financial interest.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.7b01239. UV-melting, MALDI-TOF, and HPLC data; fluorescent image of P4-RNA samples; and experimental procedures (PDF)

Genetic disorders generally occur as a result of an aberrant protein function due to mutation in the DNA coding sequence or dysregulation at the transcriptional or translational level, resulting in the loss or gain of protein function. However, over the past three decades, a preponderance of evidence has emerged showing that a large number of neuromuscular disorders, more than 20 and counting,¹ including myotonic dystrophy type 1 (DM1) and type 2 (DM2), occur as the result of an unstable repeat expansion. An expansion in the coding region of a gene can lead to an altered protein function, whereas that occurring in the noncoding region can cause a disease without interfering with a protein sequence through a toxic gain of RNA function and, in certain cases, inadvertent production of deleterious polypeptides through repeat-associated non-ATG (RAN) translation.²

A prototype of the latter class of genetic disorders is DM1, a debilitating muscular atrophy that affects one in every 8000 adults worldwide for which there is no effective treatment. DM1 is caused by a CTG-repeat expansion in the 3'-untranslated region (3'-UTR) of the dystrophin myotonia protein kinase (*DMPK*) gene, from a normal range of 5–35 repeats to a pathogenic range of 80 to >2500.³ The etiology of DM1 is largely attributed to RNA toxicity.⁴ Upon transcription, the expanded rCUG repeats (rCUG^{exp}) adopt an imperfect hairpin structure that sequesters muscleblind-like protein 1 (MBNL1), a key RNA splicing regulator. Their association results in an rCUG^{exp}–MBNL1 complex that is trapped in the nucleus, precluding its export to the cytoplasm for the production of DMPK protein, as well as preventing MBNL1 from performing its normal physiological function. Accumulated evidence has suggested that therapeutic intervention could be developed for DM1, and possibly for other related neuromuscular conditions as well, by targeting the mutant transcript.⁵ The challenge, however, is in how to design molecules that would target the expanded transcripts without interfering with the wild type (wt) and would be able to displace the noncognate proteins such as MBNL1 from rCUG^{exp}.

The pursuit of this goal has led to the development of several classes of molecules for targeting rCUG^{exp}, including pentamidines,⁶ triaminotriazines,⁷ and peptidomimetics.⁸ Recently, Disney and co-workers reported the development of modular peptoids,^{9,10} as well as the identification of several small molecules with high affinity and potency.^{11,12} The antisense approach, utilizing morpholino¹³ and 2'-*O*-methoxyethyl gapmer,¹⁴ has also been explored and has been shown to be effective in disrupting the rCUG^{exp}–MBNL1 complex and in degrading the toxic RNA, and in reversing the DM1 phenotypes in an animal model. More recently, an antigene strategy directed at modification of the affected alleles, employing TALEN¹⁵ and CRISPR/Cas9,¹⁶ has been investigated as a possible remedy for DM1 and related medical conditions. Despite the promising outlook, a considerable challenge associated with recognition specificity and/or selectivity, and cellular delivery, to a certain extent, still remains for many of these classes of designer molecules, in particular antisense agents. The low to moderate affinity, along with the lack of substantial binding cooperativity, of most synthetic oligonucleotide molecules developed to date has prevented the application of shorter probes for greater ease of cellular delivery and for better discrimination of the expanded (diseased) RNA-repeated transcripts from the wt.

To date, we have shown that peptide nucleic acid (PNA), a randomly folded nucleic acid mimic comprising a pseudopeptide backbone originally developed by Nielsen and Buchardt,

¹⁷ can be preorganized into a right-handed helical motif,¹⁸ and that its water solubility and biocompatibility can be improved by installing an (*R*)-diethylene glycol (miniPEG, or MP) unit at the γ -backbone (Chart 1A, I).¹⁹ Such a molecular foldamer exhibits ultra-high affinity and sequence specificity for DNA or RNA. The unveiling of these thermodynamic properties has prompted the suggestion that it might be feasible to develop relatively short miniPEG- γ peptide nucleic acid (MP γ PNA) probes for targeting rCUG^{exp}. We envisioned that, in addition to terminal base stacking, binding cooperativity could be augmented through intermolecular π - π interaction by covalent attachment of terminal aromatic pendant groups. An improvement in binding cooperativity should enable the development of shorter probes with enhanced recognition specificity and selectivity and provide greater synthetic flexibility for further lead optimization in drug development.

To test this hypothesis, we synthesized a series of MP γ PNA probes, six nucleotides in length, containing terminal pyrenes [P2–P6 (Chart 1B)], along with the P1 control, and characterized their binding properties. P2–P4 comprised different linker lengths connecting pyrene to the probes' backbone (Chart 1A, II). P5 and P6 contained the corresponding single and double base mismatches, designed to test recognition specificity. We selected a tandem triplet repeat because a prior study showed that MP γ PNA of a similar length was able to transiently interact with the RNA target at a physiological temperature.²⁰ Pyrene was adopted as a model compound for promoting binding cooperativity because of its expanded aromatic surface and large bathochromic shift in the emission upon dimerization,²¹ and the fact that it has been successfully demonstrated in the cooperative binding of polyamides to DNA by Sugiyama and co-workers.²² The latter photochemical property provides a convenient means for monitoring probe hybridization and pyrene–pyrene interaction. The monomers were prepared according to the published protocols.¹⁹ Probes were synthesized on HMBA-resin, purified by reverse phase high-performance liquid chromatography, and verified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (Figures S1–S6). A series of model RNA targets containing different numbers of hexameric r(CUGCUG) repeats were chosen for the binding study (Chart 1C).

All the experiments were conducted at a physiologically relevant ionic strength (10 mM NaP_i, 150 mM KCl, and 2 mM MgCl₂ at pH 7.4).²³ RNA concentrations were prepared such that the number of r(CUGCUG) binding sites in each sample was the same. A preliminary study revealed that among the three linker lengths (Chart 1A, II, P2–P4), lysine yielded the highest degree of binding cooperativity (Figure S7). The diaminopropionic (Dap) and ornithine (Orn) linkers of probes P2 and P3, respectively, might be too short to enable effective intermolecular pyrene–pyrene interaction, which is evident in the smaller enhancement in the T_m of the probe–RNA complex and the absence of an inverse ultraviolet (UV) absorption profile as a function of temperature in comparison to that of P4. On the basis of this finding, we selected P4 and performed an UV melting study with the various RNA targets. Our result showed that the melting transitions (T_m s) of the P4–RNA series monotonically increased with the number of binding sites in the targets (Figure S8). Pyrene–pyrene interaction is evident from the inverse absorption profiles of P4–T6 and P4–T8 in the 40–70 °C temperature regime. When samples are heated, the solvophobic effect becomes more pronounced because of the inverse intensity distribution of the vibronic transition of the pyrene excimer relative to that of the monomer ($A_e^{0\rightarrow0}/A_m^{0\rightarrow0} \sim 0.6$),²⁴ prior to their

dissociation upon further heating. This phenomenon has been well-documented with perylene and other thermophilic foldamers.²⁵ Comparing the T_m s of the three series, P4–RNA, P1–RNA, and RNA, revealed a distinct pattern. The T_m s of the P4–RNA series follow a positive linear correlation, $Y = 66 + 2X$, with X being the number of binding sites in the targets (Figure 1). However, for the latter two series, the T_m s plateaued at $X \sim 3$, indicating that increasing the number of binding sites in RNA beyond three does not necessarily make the corresponding hairpin structures more thermodynamically stable.²⁶ In contrast to the perfectly matched sequence, no discernible differences in the T_m s of RNAs were observed with the mismatched P5 and P6 probes (Figure 1, inset). Together, these results show that P4 binds cooperatively and sequence-specifically to RNA-repeated targets. This phenomenon has also been observed with other PNA–ligand conjugates.²⁷

To further corroborate these findings, we performed fluorescent measurements under identical conditions. The samples were excited at 340 nm, and the fluorescent signals were recorded from 345 to 650 nm. Characteristic of the pyrene–pyrene excimer formation is the emission at ~ 480 nm (Figure 2). Consistent with the UV melting data, the degree of P4 binding cooperativity increases with the number of binding sites in RNAs, as observed in the gradual increase in the fluorescence intensities at 480 nm. The samples were markedly different under UV illumination, such that they could be distinguished with the naked eye (Figure S9). Kinetic measurements further revealed that the hybridization of P4 to T8 was nearly complete within 10 min (Figure 2, inset). Addition of a competing T1 strand at an equimolar ratio of binding sites resulted in a hybridization lag time of ~ 2 min, after which complete fluorescence recovery and binding of P4 to T8 were observed. This result shows that the interaction of P4 with single-binding site RNA is weak and transient under a physiologically simulated condition and that a complete recovery and binding of the probe to RNA repeats is achieved within a similar time frame.

To assess the recognition selectivity of the P4 probe, we performed a competitive binding assay. Equimolar binding sites of the normal-length T6 and the pathogenic T48 [r(CUG)₉₆, prepared according to our published protocol¹³] were incubated with different concentrations of P4 at 37 °C for 16 h. The resulting mixtures were analyzed on an agarose gel and stained with SYBR-Gold for visualization. Inspection of Figure 3 reveals that P4 was able to discriminate the pathogenic T48 from wt T12 (compare lane 6 to lane 3). No evidence of binding was observed with the single-base mismatched P5 probe (compare lane 7 to lane 3). These results indicate that P4 can discriminate the expanded T48 transcript from wt T6 and that probe binding occurs in a sequence-specific manner.

Next, we determined whether P4 can disrupt the rCUG^{exp}–MBNL1 complex by performing a gel-shift assay. The RNA–protein complex was prepared by incubating 5′-³²P-labeled T48 with MBNL1 under a physiologically relevant condition. Upon confirmation of their binding, P4 was added and the resulting mixtures were incubated at 37 °C for 4 h prior to their analysis by nondenaturing polyacrylamide gel electrophoresis and autoradiography. Formation of the T48–MBNL1 complexes is evident in the smeared patterns observed in lanes 2–4 of Figure 4.¹³ Addition of P4 resulted in the formation of a shifted band, which became more pronounced with increasing probe concentrations (lanes 5–7). We take this result as evidence of P4 being able to disrupt the rCUG^{exp}–MBNL1 complex, resulting in

the formation of the T48–P4 heteroduplex and in the displacement of all MBNL1 proteins from the RNA transcript. Such a capability is critical to the interference of the DM1 disease pathway.

Compared to the conventional antisense agents, which are typically in the length range of 15–30 nucleotides, or to a shorter version comprising all-locked nucleic acid (LNA),²⁸ MP γ PNA is synthetically more flexible. Its structure and chemical functionality can be easily modified to meet the application requirements at hand. The smaller probe size offers several distinct benefits for biological and biomedical applications, including greater ease of chemical synthesis and scale-up and improvements in recognition specificity and selectivity (and possibly pharmacokinetic properties).²⁹ Pyrene was chosen as a model compound to induce intermolecular π – π interaction because of its appealing chemical and photophysical properties. However, in the actual biological and biomedical applications, such an aromatic pendant group can be readily replaced with a more biologically benign, or health-benefit natural products, such as riboflavin (vitamin B₂),³⁰ mangostin,³¹ and mangiferin,³² all of which can promote π – π interaction (Chart 2). These are natural antioxidants, present in fruits and vegetables, commonly used as dietary supplements to combat oxidative stress, inflammation, cancer, aging, and other ailments.

In summary, we have shown that a relatively short nucleic acid probe, two triplet repeats in length, containing terminal aromatic moieties can discriminate the pathogenic rCUG^{exp} from the short CUG-repeat-containing transcript and can disrupt the rCUG^{exp}–MBNL1 complex. In addition to the inherent benefits of being small, the modular design and high recognition specificity and selectivity of the MP γ PNA probe provide a general strategy for targeting RNA-repeat expansions that can be applied not only to rCUG^{exp} but also to a broad range of other repeated sequences, as a possible means for treating DM1 as well as a number of other related neuromuscular and neurodegenerative disorders.¹ We will perform more rigorous, quantitative characterizations of the binding property, cellular uptake, and biological activity of probes with biologically relevant pendant groups and report the results in due course.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Ultraviolet melting transitions of RNAs and the corresponding probe–RNA heteroduplexes containing the perfectly matched and mismatched sequences as a function of the number of r(CUGCUG)_n binding sites in the targets. The inset shows ultraviolet melting profiles of RNA with P5 and P6 containing single and double base mismatches, respectively.

Figure 2.

Fluorescence spectra of P4–RNA duplexes at equimolar concentrations ($1\ \mu\text{M}$ P4, $1\ \mu\text{M}$ T1, $\frac{1}{2}\ \mu\text{M}$ T2, $\frac{1}{4}\ \mu\text{M}$ T4, $\frac{1}{6}\ \mu\text{M}$ T6, and $\frac{1}{8}\ \mu\text{M}$ T8) following incubation at $37\ ^\circ\text{C}$ for 1 h and excitation at 345 nm. The inset shows fluorescent signals at 480 nm as a function of time following the addition of P4 ($1\ \mu\text{M}$) to T8 ($\frac{1}{8}\ \mu\text{M}$), and P4 ($1\ \mu\text{M}$) to T1 ($1\ \mu\text{M}$) and T8 ($\frac{1}{8}\ \mu\text{M}$) at $37\ ^\circ\text{C}$.

Figure 3.

Selective binding of $r(\text{CUGCUG})_n$ -RNA transcripts by P4. The samples were prepared by mixing preannealed RNAs with probes at 37 °C for 4 h. The ratios of P4 to the total RNA binding sites were 0 (lane 3), 1/4 (lane 4), 1/2 (lane 5), and 1/1 (lane 6) and for the mismatched P5 1/1 (lane 7).

Figure 4.

Displacement of MBNL1 from T48 by P4. [32 P]T48 was allowed to form complexes with GST-MBNL1-F1 prior to the addition of P4. The samples were prepared in a physiologically relevant buffer at final T48 and GST-MBNL1-F1 concentrations of 25 and 400 nM, respectively.

(A) Chemical building blocks **(B) $\text{MP}_{\gamma}\text{PNA}$ oligomers**

- P1: $\text{H}-^{\text{L}}\text{Arg}-\text{CAGCAG}-^{\text{L}}\text{Arg}-\text{NH}_2$
 P2: $\text{H}-^{\text{L}}\text{Arg}-^{\text{L}}\text{Dap}(\text{Pyr})-\text{CAGCAG}-^{\text{L}}\text{Dap}(\text{Pyr})-^{\text{L}}\text{Arg}-\text{NH}_2$
 P3: $\text{H}-^{\text{L}}\text{Arg}-^{\text{L}}\text{Orn}(\text{Pyr})-\text{CAGCAG}-^{\text{L}}\text{Orn}(\text{Pyr})-^{\text{L}}\text{Arg}-\text{NH}_2$
 P4: $\text{H}-^{\text{L}}\text{Arg}-^{\text{L}}\text{Lys}(\text{Pyr})-\text{CAGCAG}-^{\text{L}}\text{Lys}(\text{Pyr})-^{\text{L}}\text{Arg}-\text{NH}_2$
 P5: $\text{H}-^{\text{L}}\text{Arg}-^{\text{L}}\text{Lys}(\text{Pyr})-\text{CATCAG}-^{\text{L}}\text{Lys}(\text{Pyr})-^{\text{L}}\text{Arg}-\text{NH}_2$
 P6: $\text{H}-^{\text{L}}\text{Arg}-^{\text{L}}\text{Lys}(\text{Pyr})-\text{CTGCTG}-^{\text{L}}\text{Lys}(\text{Pyr})-^{\text{L}}\text{Arg}-\text{NH}_2$

(C) RNA targets

Primary sequence	Predicted secondary structure
5'-G(CUGCUG) _n C-3'	5'-G[CUG]CUG
T1, n = 1	3'-C[GUC]GUC T1, m = 0
T2, n = 2	T2, m = 1
T4, n = 4	T4, m = 3
T6, n = 6	T6, m = 5
T8, n = 8	T8, m = 7

Chart 1.

Chart 2.