Targeting the r(CGG) Repeats That Cause FXTAS with Modularly Assembled Small Molecules and Oligonucleotides

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Supporting Information

ABSTRACT: We designed small molecules that bind the structure of the RNA that causes fragile X-associated tremor ataxia syndrome (FXTAS), an incurable neuromuscular disease. FXTAS is caused by an expanded r(CGG) repeat (r(CGG)exp) that inactivates a protein regulator of alternative pre-mRNA splicing. Our designed compounds modulate r(CGG)exp toxicity in cellular models of FXTAS, and pull-down experiments confirm that they bind r(CGG)exp in vivo. Importantly, compound binding does not affect translation of the downstream open reading frame (ORF). We compared molecular recognition properties of our optimal compound to oligonucleotides. Studies show that r(CGG)exp’s self-structure is a significant energetic barrier for oligonucleotide binding. A fully modified 2′-O-Methyl phosphorothioate is incapable of completely reversing an FXTAS-associated splicing defect and inhibits translation of the downstream ORF, which could have deleterious effects. Taken together, these studies suggest that a small molecule that recognizes structure may be more well suited for targeting highly structured RNAs that require strand invasion by a complementary oligonucleotide.

Oligonucleotides are the gold standard for targeting RNA as they have broad utility and can be designed using simple Watson–Crick base pairing rules.1 Oligonucleotides, however, have several suboptimal properties as therapeutic modalities or chemical genetics probes of function, including poor cellular permeability and nonspecific stimulation of the immune system.2–4 As an alternative to oligonucleotide-based therapeutics, we have been investigating RNA-small molecule interactions in an effort to establish design principles analogous to base pairing rules. In particular, we identify and characterize the preferred RNA secondary structural elements (motifs) of various small molecules.5–7 Indeed, we have shown that these interactions can inform drug design.8–11 That is, we compare the motifs within a cellular RNA to our set of RNA motif-small molecule interactions to identify lead small molecules. Lead small molecules can be optimized using various medicinal chemistry approaches such as definition of structure–activity relationships, structure-based design,12–14 or chemical similarity searching.15 Ideal RNA targets have more than one targetable motif, such that a multivalent compound can be designed to increase affinity and selectivity.16–20

Various diseases are caused by RNAs, including microsatellite disorders such as fragile X-associated tremor ataxia syndrome (FXTAS).21 FXTAS is an incurable neurological disorder that is associated with multisystem atrophy, parkinsonism, dysautonomia, neuropathy, and dementia. The disease is caused via a gain-of-function by expanded r(CGG) repeats (r(CGG)exp)22 located in the 5′ untranslated region (UTR) of the fragile X mental retardation 1 (FMR1) mRNA (encodes fragile X mental retardation protein; FMRP).23–25 The repeat folds into a hairpin with an array of 1×1 nucleotide GG internal loops (Figure 1A).9,22,26 The loops bind and sequester proteins involved in RNA biogenesis including DiGeorge syndrome critical region gene 8 protein (DGCR8), Src-associated in mitosis, 68 kDa protein (Sam68), and heterogeneous nuclear ribonucleoprotein (hnRNP).22,26 Protein sequestration causes dysregulation of alternative pre-mRNA splicing in FXTAS model cellular systems and patient-derived tissue.26 Although oligonucleotides have been employed successfully to improve defects associated with other microsatellite disorders,27,28 they may not be ideal for r(CGG)exp because it is highly structured and because depletion or inactivation of the FMR1 mRNA via antisense or RNAi pathways could exacerbate disease; fragile X syndrome is caused by loss of FMRP.29 Herein, we describe our studies to investigate the therapeutic potential of modularly assembled compounds and oligonucleotides that are complementary to r(CGG)exp (Figure 1B). It is likely that certain types of RNAs are more suitable for small molecules while others are more suitable for oligonucleotides.
RESULTS AND DISCUSSION

Design of Small Molecules That Bind r(CGG)exp. In order to identify lead small molecules that bind r(CGG)exp, the causative agent of FXTAS,\textsuperscript{23–25} we compared its secondary structure to a set of RNA motif–small molecule interactions that have been identified and characterized by our laboratory.\textsuperscript{5–7} In particular, we were interested in compounds that (i) bind to the repeating motifs in r(CGG)exp (1×1 nucleotide GG internal loops; Figure 1) and (ii) can be modularly assembled to afford multivalent compounds that recognize the repeating structure of r(CGG)exp, rather than a singular motif. Six small molecules including five aminoglycosides and the bis-benzimidazole Ht-N3, which are well-known nucleic acid binders,\textsuperscript{30,31} were identified as lead RNA-binding modules.\textsuperscript{20}

We chose Ht-N3 to pursue for further investigation because of its drug-like properties, cellular permeability in mammalian cells, and high affinity binding to 5′CGG/3′GQC (K_d = 375 ± 26 nM).\textsuperscript{9} Ht-N3 also contains an orthogonal azide moiety that can be used for polyvalent display on a polymeric backbone (Figure 2B). A peptoid backbone was chosen as the modular display scaffold because (i) valency and the distance between RNA-binding modules can be easily controlled, (ii) their synthesis is facile and various RNA-binding modules can be easily incorporated,\textsuperscript{32,33} and (iii) they are cell-permeable.\textsuperscript{34}

In order to determine the distance between RNA-binding modules that most closely mimics the distance between 5′CGG/3′GQC motifs that periodically repeat in r(CGG)exp, we screened a library of H-dimers\textsuperscript{20} for disrupting a r(CGG)12–protein complex \textit{in vitro} (where H indicates the conjugated form Ht-N3; Figure 2B). The following nomenclature is used for modularly assembled compounds: 2H-n where 2H indicates two H RNA-binding modules and n indicates the number of propylamine spacers (or distance) that separate Hs (Figure 2B). The \textit{in vitro} potencies of Ht-N3 and the library of dimers were measured using a previously reported time-resolved fluorescence resonance energy transfer (TR-FRET) assay (Table 1).\textsuperscript{9} It has been previously shown that DGCR8 binds
Table 1. IC₅₀’s of Modularly Assembled Small Molecules for Inhibition of r(CGG)₁₂-DGCR8Δ Complexes in Vitro and in Vivo

| compound | IC₅₀ (μM) | IC₅₀ (μM) | % improvement of SMN2 splicing |
|----------|----------|----------|-------------------------------|
| Ht-N₁    | 33 ± 1   | 130 ± 4  | 2 ± 3                         |
| 2H-1     | 18 ± 0.2 | 31 ± 2   | 4 ± 12                        |
| 2H-2     | 17 ± 0.7 | 40 ± 4   | 11 ± 10                       |
| 2H-3     | 18 ± 0.7 | 30 ± 0.7 | 30 ± 2                        |
| 2H-4     | 20 ± 0.2 | 28 ± 1   | 25 ± 10                       |
| 2H-5     | 18 ± 0.8 | 23 ± 3   | 34 ± 8                        |
| 2H-6     | 23 ± 1   | 24 ± 2   | 27 ± 7                        |
| 2H-7     | 35 ± 0.2 | 37 ± 1   | 33 ± 3                        |

*IC₅₀’s were measured for disruption of a preformed RNA–protein complex. Studies were completed in the presence of 65-fold excess tRNA over r(CGG)₁₂. Compounds were tested at 20 μM concentration.

r(CGG)₆⁰ and forms a scaffold for the binding of other proteins such as Sam68 and hnRNP. Therefore, the TR-FRET assay measures the amount of r(CGG)₁₂-DGCR8Δ complex present.

Potencies and Affinities of Small Molecules. We completed IC₅₀ measurements in the presence and absence of competitor tRNA (65-fold excess) (Table 1 and Supplementary Figure S-1). In the absence of competitor tRNA, 2H-1–2H-6 have similar potencies with IC₅₀’s ranging from 17 to 23 μM, an ~1.6-fold increase in potency over monomer Ht-N₁ (Table 1). In contrast, there is a wider range of IC₅₀’s in the presence of competitor tRNA, from 23 to 130 μM (Table 1). The most potent compounds are 2H-5 and 2H-6 with IC₅₀’s of 23 ± 1 and 24 ± 2 μM, respectively, which vary little from their IC₅₀’s in the absence of competitor and indicate that they are selective for r(CGG)₁₂. In contrast, the IC₅₀ for Ht-N₁ increases by ~4-fold in the presence of competitor tRNA (33 ± 1 μM vs 130 ± 4 μM). Taken together, 2H-5 and 2H-6 are more selective than Ht-N₁ (as evidenced by no change in their IC₅₀’s in the presence and absence of tRNA) and are ~6-fold more potent than Ht-N₁ in the presence of competitor (Table 1). The enhancement in potency is somewhat less than expected and may be due to a lack of preorganization of the modular assembly scaffold, which can be further optimized.

The affinity of 2H-5 for various RNAs was determined by using a fluorescence-based assay. 2H-5 forms a 1:1 complex (1.1 ± 0.2:1) with an RNA with one copy of the 5’CGG/3’CGG motif that periodically repeats in r(CGG)₆⁰ (Supplementary Figure S-2) and binds with an affinity of 16 ± 5 nM. The affinity of the compound to an RNA with 12 copies of the 5’CGG/3’CGG motif (Supplementary Figure S-2) is enhanced by ~3-fold (55 ± 10 nM). Moreover, 2H-5 occupies each 5’CGG/3’CGG binding site when statistical effects are taken into account as the stoichiometry is 4.6 ± 1.1 (2H-5-RNA). The enhancement in affinity is not as large as expected, likely because the peptoid backbone has a great deal of conformational flexibility and thus is not preorganized for binding. Such effects have been previously observed.

We studied the selectivity of 2H-5 in vitro by investigating its binding to (CUG) repeats and bulk tRNA. Importantly, 2H-5 binds ~5-fold more tightly to an RNA with 12 copies of a 5’CGG/3’CGG motif than to an RNA with 12 copies of a 5’CUG/3’CUG motif (K₅ = 280 ± 76 nM; Supplementary Figure S-2). Moreover, 2H-5 binds weakly to competitor tRNA as no binding saturation was observed when up to 2 μM tRNA (K₅ ≫ 2 μM) was added (4-fold excess over 2H-5), in agreement with IC₅₀ values in the presence and absence of competitor tRNA, which do not change (Table 1). We previously reported that a similar compound, 2H-4, is optimal for targeting 5’CUG/3’CUG repeats. 2H-4 binds an RNA with 12 copies of a 5’CGG/3’CGG motif 4-fold more weakly than 2H-5 with a K₅ of 212 ± 35 nM (Supplementary Figure S-2). Thus, the distance between RNA-binding modules contributes to the specificity of the small molecule.

In a previous report, we identified another small molecule, 9-hydroxy-5,11-dimethyl-2-(2-(piperidin-1-yl)ethyl)-6H-pyrindo-[4,3-b]-carbazol-2-ium (1a) by screening small molecule libraries for compounds similar to Ht-N₁. 1a binds r(CGG) repeats with high affinity (K₅ = 76 ± 4 nM) and disrupts the binding of DGCR8Δ in vitro with an IC₅₀ of 13 ± 0.4 μM.

Potencies and Affinities of Oligonucleotides. Previous structural studies of r(CGG)₆⁰ model systems have shown that the repeat forms a stable structure in which the 1x1 nucleotide GG internal loops adopt a syn-anti conformation with three hydrogen bonds. Because of the stability of the loops and because r(CGG) repeats fold into an intramolecular hairpin (Supplementary Figure S-3), we hypothesized that the self-structure of the repeats poses a significant barrier for duplex formation with a complementary oligonucleotide. The significance of this barrier was probed using gel mobility shift assays in which r(CGG)₁₂ and a complementary oligonucleotide were folded either separately or together (Supplementary Figure S-4). These studies showed that the IC₅₀ is 7-fold lower when oligonucleotides are folded with r(CGG)₁₂ than when they are folded separately (Supplementary Figure S-4). This large difference cannot be traced to oligonucleotide self-

Table 2. Oligonucleotide IC₅₀’s for Inhibition of a r(CGG)₁₂-DGCR8Δ Complex

| oligonucleotide | IC₅₀ (μM) | IC₅₀ (μM) | IC₅₀ (μM) |
|----------------|----------|----------|----------|
| d(CCG)₉        | >100⁰    | 50 ± 17  | 5 ± 1    |
| d(CCG)₁₂       | 65 ± 6°   | 37 ± 2   | 1.6 ± 0.6 |
| 2‘OMe-PS-(CCG)₁₂ | 0.32 ± 0.04° | 0.39 ± 0.02 | 0.16 ± 0.02 |
| 0.57 ± 0.05 (5 min incubation) | 0.35 ± 0.04 (30 min incubation) | 0.32 ± 0.01 (45 min incubation) |

*Experiments were completed by disrupting a preformed r(CGG)₁₂-DGCR8Δ complex using the same conditions to collect the data in Table 1 including the presence of 65-fold excess of bulk tRNA. Samples were incubated for 60 min. Oligonucleotides and r(CGG)₁₂ folded separately and then allowed to equilibrate prior to addition of DGCR8Δ. Oligonucleotides and r(CGG)₁₂ were mixed together and folded by heating at 95 °C and slowly cooling to RT. DGCR8Δ was then added.
structure as both d(CCG)_{8} and d(CCG)_{12} form weak hairpin structures (as determined by optical melting experiments; \(\Delta G^\circ \approx -1.5\) kcal/mol), but rather to the inherent stability of r(CGG)_{12} (\(\Delta G^\circ = -5.6\) kcal/mol) (Supplementary Figure S-3 and Supplementary Tables S1−S3). We predicted the contributions of the RNA’s and oligonucleotide’s self-structures to duplex formation using the OligoWalk program,^{38} which confirmed that the self-structure of the RNA is a large energetic barrier (Supplementary Table S-4). (Please note that Oligo-Walk predicts duplex stability based on experimental measurements completed in 1 M Na+, which is much different than conditions used herein (193 mM Na+).)

The two DNA oligonucleotides were also studied for inhibition and disruption of the r(CGG)_{12}-DGCR8Δ complex (Table 2 and Supplementary Figure S-5). In the first set of experiments, the oligonucleotide and r(CGG)_{12} were folded separately, mixed together, and incubated for 15 min prior to addition of DGCR8Δ. Under these conditions, d(CCG)_{12} is only slightly more potent than d(CCG)_{8}, with IC_{50}’s of 37 and 50 \(\mu\)M, respectively. In the second set of experiments, the oligonucleotide and r(CGG)_{12} were folded together and incubated for 15 min prior to addition of DGCR8Δ. Not unexpectedly, the IC_{50}’s of both compounds improve at least 10-fold, indicating that the self-structure of r(CGG)_{12} significantly decreases oligonucleotide potency (Table 2).

Lastly, the d(CCG)_{n} oligonucleotides were studied for disrupting a preformed r(CGG)_{12}-DGCR8Δ complex in the presence of competitor tRNA, the same conditions under which the IC_{50}’s for 2H-n compounds were measured (Table 2). Not unexpectedly, d(CCG)_{12} and d(CCG)_{8} are poor inhibitors of the preformed complex, with IC_{50}’s of 65 and >100 \(\mu\)M, respectively (Table 2). (It should be noted that d(CCG)_{12} and d(CCG)_{8} do not bind DGCR8Δ as determined by gel mobility shift assays.)

Figure 3. 2H-5 and 2′-OMe-PS-(CCG)_{12} improve FXTAS-associated defects in alternative pre-mRNA splicing in a cell culture model. (A, top) Alternative splicing of SMN2 pre-mRNA is regulated by Sam68.19 When Sam68 is sequestered and inactivated by r(CGG)\^{exp}, SMN2 splicing is dysregulated. (A, bottom) When an FXTAS cell culture model is treated with 2H-5, SMN2 splicing patterns are restored as determined by RT-PCR. (B, top) Alternative splicing of Bcl-x pre-mRNA is also regulated by Sam68 and hence dysregulated in FXTAS.19 (B, bottom) When a FXTAS cell culture model is treated with 2H-5, normal Bcl-x splicing patterns are restored as determined by RT-PCR. (C) 2′OMe-PS-(CCG)_{12} improves the SMN2 pre-mRNA splicing defect when transfected into an FXTAS model system. Complete reversal of the splicing defect is not observed, however, even when cells are treated with 500 nM oligonucleotide. * denotes \(p < 0.05\) as determined by a two-tailed Student’s \(t\) test (\(n \geq 2\)). Error bars indicate standard deviation.

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Various studies have shown that the thermodynamic stability and other properties of complementary oligonucleotides can be improved by base and sugar modifications, which could provide more potent modalities. For example, RNA-RNA duplexes are more thermodynamically stable than DNA-RNA duplexes.\textsuperscript{40,41} 2′-OMe modification increases the thermodynamic stability of the resulting duplex by \lowercase{o}.01 kcal/mol per substitution, and locked nucleic acids (LNAs) provide even greater enhancements. In our case, we used a 2′-OMe oligonucleotide modified with a phosphorothioate (PS) backbone, or 2′-OMe-PS-(CCG)\textsubscript{12}. Phosphorothioates have been well studied.\textsuperscript{44} Although they decrease duplex stability and selectivity in some cases,\textsuperscript{44} they are resistant to nuclease cleavage, thereby increasing metabolic stability, and are generally nontoxic to animals.\textsuperscript{37} Indeed, 2′-OMe-PS-(CCG)\textsubscript{12} is significantly more potent \textit{in vitro} than d(CCG)\textsubscript{12} with an IC\textsubscript{50} of \~350 nM (Table 2).

Taken together, these studies establish that modularly assembled small molecules that target r(CGG)\textsuperscript{exp} structure are more potent inhibitors of the r(CGG)\textsubscript{12}-DGCR8\textsubscript{Δ} complex than DNA oligonucleotides that recognize sequence but less potent than an oligonucleotide with 2′-OMe modifications. Moreover, self-structure of the repeat is an impediment to complex formation with oligonucleotides.

**Improvement of FXTAS-Associated Splicing Defects.** Next, Ht-N\textsubscript{4} and the 2H-\textit{n} compounds (20 μM) were screened for improving FXTAS-associated defects in a cell culture model (Table 1). The sequestration of Sam68 by r(CGG)\textsuperscript{exp} (via DGCR8) causes dysregulation of alternative pre-mRNA splicing regulated by Sam68.\textsuperscript{26} COS7 cells were co-transfected with a plasmid that encodes r(CGG)\textsubscript{88} in the 5′ UTR of GFP (in-frame). As shown in Figure 4A, treatment with up to 20 μM 2H-5 does not affect the amount of GFP produced nor does it change the amount of transcript as determined by qRT-PCR. In contrast, 2′-OMe-PS-(CCG)\textsubscript{12} severely decreases translation of GFP (Figure 4A) and slightly increases the amount of (CCG)\textsubscript{386}-GFP transcript (Figure 4B). These data suggest that 2′-OMe-PS-(CCG)\textsubscript{12} acts as a road block, impeding the translational machinery. Collectively, our \textit{in vivo} studies suggest that oligonucleotides may not be ideal therapeutic modalities for FXTAS. 2′-OMe-PS-(CCG)\textsubscript{12} is incapable of restoring splicing patterns to wild type (Figure 3C), and even low nanomolar concentrations that have no effect on splicing outcomes significantly inhibit translation of the downstream ORF (50% inhibition at 4 nM; 90% inhibition at 100 nM) (Figure 4A). Since many oligonucleotide modifications are available,\textsuperscript{39} it is possible that an oligonucleotide could be developed that improves splicing defects but does not inhibit translation of the downstream ORF. For example, peptide nucleic acids (PNAs) have been shown to possess enhanced strand invasion properties,\textsuperscript{53} which will be particularly important for highly structured targets.

**Oligonucleotides That Target Other Repeating RNAs.** Oligonucleotides have been studied for reducing toxicity of other repeating RNAs, in particular r(CUG)\textsuperscript{exp} (causative agent of myotonic dystrophy type 1; DM1) and r(CAG)\textsuperscript{exp} (associated with Huntington’s disease (HD) and spinocerebellar ataxia (SCA)). In general, they are more potent than oligonucleotides that target r(CGG)\textsuperscript{exp} despite the fact that the oligonucleotides studied herein are much longer (36 nucleotides compared to 14–21 nucleotides). For example, (CAG)\textsubscript{7}, a fully phosphorothioate modified 2′-OMe oligonucleotide, silences transcripts containing r(CUG)\textsuperscript{exp} with an IC\textsubscript{50} of \~0.4 nM.\textsuperscript{27} Interestingly, a phosphorothioate DNA complementary to r(CUG)\textsuperscript{exp} has an IC\textsubscript{50} in cells of \~300 nM (as measured by a 50% reduction in r(CUG)\textsuperscript{exp} levels),\textsuperscript{54} 1000-fold less potent than a phosphorothioate DNA-2′-OMe gapmer.\textsuperscript{54} Other gapmers that are complementary to r(CUG)\textsuperscript{exp} have similar potencies. A LNA-2′-O-methoxy ethyl (2′-MOE) gapmer significantly knocks down expression of r(CUG)\textsubscript{360} when cells are transfected with 0.3 nM oligonucleotide.\textsuperscript{55} The gapmer also

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is the compounds can be easily modified to display a chemical handle for affinity-based purification. To identify the RNA targets of 2H-5, we synthesized a 2H-5-biotin derivative by coupling biotin carboxylate to the imino terminus of 2H-5 (Figure 5A, Supplementary Figures S-8 and S-9, and Supplementary Methods). 2H-5-Biotin was then added to streptavidin resin to afford an affinity matrix. Likewise, we added 5′-biotin-d(CCG)12 to streptavidin resin to identify the cellular RNA targets of the oligonucleotide.

Total RNA from a FXTAS model cellular system was applied to the affinity matrix displaying 2H-5 or the oligonucleotide. The resin was washed to remove unbound biomolecules, and the cellular targets were harvested and separated by gel electrophoresis. Although both 2H-5 and d(CCG)12 bind r(CGG)exp (confirmed by Northern blot; Supplementary Figure S-10), it appears that 2H-5 pulls down more of the target and that r(CGG)exp comprises a larger percentage of all cellular targets as compared to d(CCG)12 (Figure S5B and C). In order to confirm that these observations are indeed the case, we completed qRT-PCR of the pull-down with primers specific for r(CGG)exp or 18S rRNA. There is 64-fold more r(CGG)exp present in the RNAs pulled down by 2H-5 than by d(CCG)12. Moreover, 2H-5 pulls down 16-fold more r(CGG)exp than 18S rRNA, a 1.6-fold increase compared to the oligonucleotide. Taken together, this pull-down approach could be a general method that provides insight into the targets and off-targets of small molecules.

**Summary and Conclusions.** In summary, it is likely that different types of RNAs are more amenable to targeting by small molecules while other types are more amenable to targeting by oligonucleotides. Both classes of compounds show promise as therapeutic modalities that modulate the function of disease-associated RNAs. In the case of highly structured r(CGG)exp residing in a 5′ UTR, we have shown that small molecule modalities may be better as they do not inhibit translation of the downstream ORF while also improving alternative pre-mRNA splicing defects.

## METHODS

**Synthesis of 2H-5-Biotin.** Please see the Supporting Information for synthetic methods and compound characterization (Supplementary Figures S-8 and S-9).

**Oligonucleotide Preparation and Purification.** The RNA used in the protein displacement assay (5′-biotin-r(CGG))12 was purchased from Dharmacon and deprotected per the manufacturer’s recommended protocol. The RNA was desalted using a PD-10 gel filtration column (GE Healthcare). Concentration was determined by absorbance at 90 °C using a Beckman Coulter DU800 UV–vis spectrophotometer equipped with a Peltier temperature controller unit. The extinction coefficient (at 260 nm) was calculated using the HyTHER server, which uses nearest neighbors parameters. DNA oligonucleotides were purchased from Integrated DNA Technologies (IDT) and used without further purification.

**DGCR8Δ Expression and Purification.** His-tagged DGCR8Δ was expressed and purified as previously described.

**Protein Displacement Assays.** The protein displacement assay used to measure the IC50 of 2H-n compounds and oligonucleotides inhibitors of the (r(CGG))12-DGCR8Δ complex was previously described (n = 2 for 2H-n compounds; n = 3 for oligonucleotides). The IC50s were determined by plotting percent inhibition of the r(CGG)12-DGCR8Δ complex as a function of compound or oligonucleotide concentration. The resulting curves were fit to Sigma Plot’s four parameter logistic curve fit. Representative IC50 curves are provided in Supplementary Figures S-1 and S-5.
Figure 5. Identifying the cellular RNA targets of 2H-5-biotin and S′-Biotin-d(CCG)12 via a pull-down assay. 2H-5-Biotin and S′-Biotin-d(CCG)12 were complexed with streptavidin-functionalized resin to afford an affinity matrix. (A) Structure of 2H-5-biotin. (B) Representative gel image of the nucleic acids pull-downed by 2H-5-biotin and S′-Biotin-d(CCG)12. Lanes: 1, DNA ladder; 2, total RNA isolated from an FXTAS cellular model system; 3, final wash of S′-biotin-d(CCG)12-functionalized resin; 4, elution of RNA bound to S′-biotin-d(CCG)12; 5, total RNA isolated from an FXTAS cellular model system; 6, final wash of 2H-5-biotin-functionalized resin; 7, elution of RNAs bound to 2H-5-biotin-functionalized resin; and 8, DNA ladder. (C) Quantitation of the gel image shown in B. Both 2H-5-biotin and S′-biotin-d(CCG)12 recognize r(CGG)35. However, 2H-5-biotin pulls down a larger amount of the target, which is also a larger percentage of the bound RNAs as confirmed by qRT-PCR.

Optical Melting Experiments. Optical melting experiments of S′-biotin-r(CGG)12, d(CCG)12, and d(CCG)35 were completed in 1X Melting Buffer (8 mM NaH2PO4 pH 7.0, 185 mM NaCl, and 1 mM EDTA) using a Beckman Coulter DU800 UV–vis spectrometer with an attached peltier heater. Melting curves of absorbance versus temperature were acquired at 260 nm with heating rate of 1 °C/min from 35 to 92 °C for r(CGG)12 and 15 to 80 °C for d(CCG)35 and d(CCG)12. Melting curves were fit to a two-state model using the MeltWin program (http://www.meltwin.com) as previously described. The results of optical melting experiments including normalized melting curves are provided in Supplementary Tables S-1–S-3 and Figure S-3.

Affinity Measurements. The affinities of 2H-5 and 2H-4 for various RNAs were determined as previously described (Supplementary Figure S-2).20

Treatment of a FXTAS Cell Culture Model with Small Molecules. In order to determine if Ht-N1 and 2H-n compounds improve FXTAS-associated splicing defects in vivo, a cell culture model system was used as previously described. Briefly, COS7 cells were maintained in growth medium (1X DMEM, 10% (v/v) FBS, and 1X GlutaMax (Invitrogen)). Cells were transfected using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s standard protocol using equal amounts of a plasmid expressing 60 CGG repeats ((CGG)60X)35 and a mini-gene of interest. Approximately 5 h post-transfection, the transfection cocktail was removed and replaced with growth medium containing 20 μM Ht-N1 or 2H-n compound.

Treatment of an FXTAS Cell Culture Model with Oligonucleotides. COS7 cells were seeded in a 96-well plate and transfected with (CGG)60X and SMN2 mini-genes as described above. Approximately 5 h post-transfection, the transfection cocktail was removed and replaced with growth medium containing 20 μM Ht-N1 or 2H-n compound.

Quantification of Alternative pre-mRNA Splicing by RT-PCR. RT-PCR amplification of alternative pre-mRNA splicing isoforms derived from mini-genes was completed as previously described.9 Supplementary Table S-5 lists the RT-PCR primers used for each mini-gene construct. Splicing isoforms were separated using a 2–3% (w/v) agarose gel or a denaturing 5% (w/v) polyacrylamide gel stained with SYBR gold and quantified using QuantityOne software (BioRad). Statistical significance was determined by comparing splicing patterns in treated cells to untreated cells with a two-tailed Student’s t-test (n = 2).

Western Blotting. After treatment, cells were lysed in the plate using 50 μL/well of M-Per Mammalian Protein Extraction Reagent containing 0.5 μL of Halt Protease Inhibitor cocktail (Thermo Scientific). A 5-μL aliquot of the cell lysate was boiled in 1X Laemmlı Buffer. Cellular proteins were separated by SDS-PAGE and then transferred to PVDF membrane using a Trans-Blot Turbo Transfer System (Bio-Rad). Western blotting was completed using anti-GFP (Roche) or anti-β-actin (Sigma Aldrich) as primary antibodies and anti-IgG-horseradish peroxidase conjugate as the secondary antibody. Chemiluminescent signal was generated by adding 10 mL of SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific), and the blot was imaged using Molecular Imager Gel Doc XR+ System (Bio-Rad). Statistical significance was determined by comparing the amount of GFP in treated cells to the amount in untreated cells using a two-tailed Student’s t-test (n = 2).

qRT-PCR. Real time quantitative RT-PCR was completed to quantify the relative expression levels of 18S, β-actin, (CGG)60X or (CGG)35-GFP as previously described.91 Briefly, 400 ng of total RNA was reverse transcribed (25 μL total volume) to generate cDNA as previously described.9 qPCR was completed in 10 μL using Power SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer’s protocol. PCR reactions contained 5 μL of 2X Power SYBR Green PCR Master Mix, 2 μL of the RT reaction, and 2 μM concentration of each primer. qPCR was performed on an ABI 7900 HT Real-Time PCR System (Applied Biosystems). The sequences of RT-PCR primers are provided in Supplementary Table S-6. Statistical significance was determined by comparing the amount of RNA of interest in treated cells to the amount of RNA of interest in untreated cells using a two-tailed Student’s t-test (n = 2 biological replicates; n = 3 technical replicates).
Synthetic methods, characterization of 2H-5-biotin, methods for gel shift assays and Northern blotting, results of optical melting experiments, representative IC_{50} plots, representative gel images of gel mobility shift assays and RT-PCR analysis of alternative pre-mRNA splicing, and plots of controls for alternative splicing. This material is available free of charge via the Internet at http://pubs.acs.org.

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