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Developing Bivalent Ligands to Target CUG Triplet Repeats, the Causative Agent of Myotonic Dystrophy Type 1

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Abstract

An expanded CUG repeat transcript (CUG^{exp}) is the causative agent of myotonic dystrophy type 1 (DM1) by sequestering muscleblind-like 1 protein (MBNL1), a regulator of alternative splicing. Based on a ligand (**1**) that was previously reported to be active in an *in vitro* assay, we present the synthesis of a small library containing ten dimeric ligands (**4-13**) that differ in length, composition and attachment point of the linking chain. The oligoamino linkers gave a greater gain in affinity for CUG RNA and were more effective when compared to oligoether linkers. The most potent *in vitro* ligand (**9**) was shown to be aqueous-soluble and both cell- and nucleus-permeable displaying almost complete dispersion of MBNL1 ribonuclear foci in a DM1 cell model. Direct evidence for the bioactivity of **9** was observed in its ability to disperse ribonuclear foci in individual live DM1 model cells using time-lapse confocal fluorescence microscopy.

Introduction

Myotonic dystrophy type 1 (DM1) is the most common form of adult muscular dystrophy and is a disease for which there is currently no treatment, only palliative therapy.¹ DM1 is a trinucleotide expansion disease (TRED) that is caused by an aberrant expansion of a CTG repeat sequence in the 3'-untranslated region of the dystrophin myotonia protein kinase (DMPK) gene.² The CTG^{exp} leads to a CUG^{exp} transcript that has a unique secondary structure, consisting of repetitive UU mismatches and CG base pairs.^{3,4} This toxic CUG^{exp} transcript sequesters all three paralogs of human MBNL proteins including MBNL1, a key regulator of the alternative splicing process.⁵⁻⁷ Thus, although DM1 has a complex pathogenesis, it is known to be an RNA-gain-of-function disease with the high affinity MBNL1-CUG^{exp} interaction playing a major role.⁸⁻¹⁰ Another proposed mechanism involves the increased steady-state level of CUG-binding protein 1 (CUG-BP1) as a result of the presence of CUG^{exp}.¹¹⁻¹⁴ The CUG^{exp} RNA is a validated drug target,¹⁵⁻¹⁷ having been successfully targeted by several oligonucleotides including synthetic short interfering RNAs,¹⁸ a morpholino antisense oligonucleotide (ASO),¹⁹ 2'-O-(2-methoxyethyl) ASO,¹⁵ a D-amino acid hexapeptide,²⁰ and several small molecules including pentamidine,²¹

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Supporting Information

Experimental procedures, full characterization data for the compounds, supplementary confocal microscopy images and movies. This material is available free of charge via the Internet at <http://pubs.acs.org>.

benzo[g]quinolone-based heterocycles,²² a Hoechst derivative (H1),²³ a modularly assembled Hoechst 33258,^{24,25} and a triaminotriazine-acridine conjugate, reported by our laboratory (ligand **1**).²⁶

Ligand **1** was reported as a highly selective, albeit moderate (CUG)₁₂-MBNL1 inhibitor (IC₅₀ = 46 μ M) in an *in vitro* assay.²⁶ The hydrogen bonding recognition unit, the triaminotriazine ring was found to be essential for recognition and inhibition of the (CUG)₁₂-MBNL1 interaction because acridine derivatives lacking this unit did not exhibit inhibition potency in an *in vitro* assay.²⁶ However, ligand **1** had two shortcomings: modest inhibition potency and poor cell permeability. Recently a conjugate of ligand **1** containing a oligoamine-derivative side chain was found to be cell-permeable and bioactive.²⁷

Considering the repeating nature of CUG^{exp}, one logical approach to increasing the affinity of **1** for CUG^{exp} is through the generation of multivalent ligands.^{28,29} The multivalent effect has proven useful to increase the binding affinity and selectivity of other ligands toward a wide variety of multivalent targets including CUG^{exp}.³⁰⁻³⁸ The increase in affinity of multivalent ligands arises from the thermodynamic advantage inherent in a cooperative binding system.^{29,39} Upon binding of the first module, the overall entropy of the ligand-CUG^{exp} complex is significantly lowered by having the second binding module localized in the vicinity of its binding site.²⁸ However, in nearly all cases examined, the dimeric binding constant rarely approaches the very high level expected based on $\Delta G_{\text{dimer}} \gg 2\Delta G_{\text{monomer}}$ because of entropic and enthalpic costs involved in bivalent binding.²⁸ In particular, conformational rigidity can cause spatial mismatch and diminish the binding enthalpy of the second module, whereas conformational flexibility raises the entropic cost for the binding of the second module. Thus, it is essential to have the right linker to maximize the multivalent effect as both rigidity and flexibility can potentially diminish this effect.

Another advantage of multivalent ligands is their potential to become cell-permeable, by taking advantage of the polarity of appropriate linkers. However, development of bioactive multivalent ligands have some obstacles such as their large size and molecular weight reducing their “drugability.”³⁸ Indeed, a tetrameric and pentameric Hoechst 33528 ligand were developed successfully as highly effective inhibitors of the MBNL1-CUG^{exp} interaction *in vitro* but both were found to be insoluble and cell-impermeable.²⁵ The use of dimeric ligands is an attractive pathway that is becoming more prevalent in drug discovery efforts⁴⁰⁻⁴⁵ and has the added advantage of more moderate molecular weights. This approach seemed particularly well suited to ligand **1** (vide infra). To accomplish this goal with limited structural knowledge of the ligand-binding mode,^{46,47} a small library of dimeric ligands was created with a range of chains linking two units analogous to **1**.

Results and Discussion

Rational Design and Synthesis of Dimeric Ligands

The design of a dimeric CUG^{exp}-binding ligand requires the following: (1) a monomeric ligand with at least a modest affinity and selectivity for CUG^{exp}, (2) an appropriate handle for the attachment of the linker to the monomeric ligand, that would not interfere with the ligand-CUG^{exp} interaction, and (3) an appropriate linker to connect the two monomers so that each module can bind to CUG^{exp}. From the previous studies in our lab, it was determined that **1** is a highly selective and modest monomeric inhibitor for (CUG)₁₂-MBNL1 (IC₅₀ = 46 μ M) with over 50 fold selectivity for CUG repeat relative to a random duplex.²⁶ Ligand **1** was designed to serve as a “stacked intercalator” with the acridine unit inserting between the GC base pair and the U-triaminotriazine-U base triplet (i.e., the acridine stacked on the triaminotriazine).⁴⁷

The covalent attachment of **1** to a linker required an appropriately positioned, reactive functional group. Possible sites for this group on **1** were the acridine ring, the triaminotriazine recognition unit, or the linking chain between these two components. Modification of the recognition unit was considered less desirable because of the potential to block key hydrogen bonding functionality involved in the UU mismatch recognition. However, various bis-acridine intercalators spanning two or more base pairs were synthesized and studied previously.⁴⁸⁻⁵² The most straightforward synthesis involved an acridine ring containing a carboxylic acid group that could be amidated with a linker diamine. Placing the carboxylic acid group in either the 1- or 3-position of the acridine ring is challenging because the synthetic route proceeds through an inseparable mixture of both derivatives.⁵³

The remaining two positions of the acridine ring, i.e., the 2- and 4-positions, were considered more tractable from a synthetic standpoint. Importantly, it was found that the chloro- and methoxy-groups in **1** could be replaced with a 2- or 4-carboxamido group (see **2** and **3** in Figure 1, respectively) without reducing its affinity for CUG repeats or its inhibition of the MBNL1-CUG complex. Although modeling was not carried out, it appeared that dimeric ligands interconnected at the 2- and 4-positions would have the linker and triaminotriazine recognition units located on the same or the opposite sides of the acridine unit, thereby likely requiring a non-threading or threading⁵⁴ mechanism of binding, respectively. This simple analysis suggested that these two isomeric dimers would significantly increase the diversity of our library, especially when considering the ultimate RNA-ligand complex.

Linker units with terminal amino groups were selected as they would form a stable amide bond with the carboxylic acid handle on the acridine ring.^{55,56} Without a firm structural data for the binding mode, the linker chain should be flexible enough to allow bivalent binding. As noted above, interconnection can result in an “all or nothing” effect,⁵⁷ whereby an appropriately designed rigid linker puts the second binding module at the UU mismatch site but an inappropriate linker greatly diminishes the binding of one or possibly even both modules. The polarity of the linker is another important consideration as aqueous solubility issues may arise from use of a polymethylene chain. Because the original monomeric ligand is minimally aqueous-soluble, both polyamide and alkyl chains were ruled out as potential linkers. Thus, all of the diamine linkers used in this study contained either oligoether or oligoamino groups. Oligoethers were either oligoethylene and oligopropylene glycols which are attractive moieties for drug delivery, because of their flexibility and polarity.⁵⁸ Oligoamino groups introduce positive charge to the dimeric ligand, therefore increasing its aqueous-solubility and affinity to the RNA polyphosphate backbone.⁵⁹ Moreover, cells have a polyamine transporting system (PTS) that potentially makes such conjugates cell-permeable.⁶⁰

The heteroatom-rich linkers used here possess various lengths from 10 to 21 atoms and were designed to span at least two central GC base-pairs according to the nearest-neighbor exclusion principle,⁶¹ thereby allowing the two triaminotriazine recognition units to bind minimally to consecutive UU mismatches. The actual linkers used were either commercially available or synthesized as shown in Schemes 3-8 in the Supporting Information. The two resulting series of dimeric ligands that were synthesized from these linkers and evaluated are shown in Figure 2.

Optical Melting Studies

It has been suggested that MBNL1 displays a preference for single stranded (ss) CUG^{exp} and thus destabilizes the double stranded (ds) CUG^{exp} stem loop upon binding.^{62,63} If correct, this model suggests that ligands capable of stabilizing the ds form of CUG^{exp} may

prove to be the most effective inhibitors of the MBNL1-CUG^{exp} interaction. The increase in (CUG)₁₂ melting temperature upon ligand binding correlates both with (CUG)₁₂ stem loop stability and ligand binding strength. Therefore, we studied the effect of ligands on the T_m of (CUG)₁₂, the thermal denaturation study of (CUG)₁₂ being carried out in the presence of one equivalent of select ligands. Monophasic melting curves were obtained in each case with ΔT_m values shown in Table 1. Ligands **4**, **5** and **6** were not fully soluble in the buffer used and a ΔT_m could not be obtained. For the monomeric ligand **2**, the ΔT_m was 1.8 °C. For the oligoether-linker dimeric ligands, **7** and **8**, ΔT_m value was 2.7 and 1.8 °C, respectively. The values of the monomeric and oligoether-linker dimeric ligands are similar, suggesting that **7** and **8** are binding to (CUG)₁₂ only with one of their binding modules. This can be explained by the possible coil-like conformation of oligoethylene glycol linkers, preventing the desired distance between binding modules.⁶⁴ Oligopropylene glycol linker in **6** was designed to avoid this coil-like conformation, however it was aqueous-insoluble.

The dimeric ligands **9**, **10** and **11** containing oligoamino linkers had much higher ΔT_m values of 9.3, 9.6, and 8.4 °C, respectively. This finding suggests that **9**, **10**, and **11**, unlike **7** and **8**, have a more optimized linker to bind to (CUG)₁₂ and are better able to stabilize the ds form of (CUG)₁₂ than the corresponding monomer. These results suggest that the composition of the linker is more important than the number of atoms it contains. For example, **7** contains the same number of atoms in its linker as does **11**, yet the latter gives a >3-fold higher ΔT_m value. Overall, the thermal denaturation data suggest that ligands **9**, **10** and **11** have the greatest potential given their higher ΔT_m values, but more quantitative binding data was sought to corroborate these preliminary results. Ligands **2** and **3** (data not shown) showed similar binding affinity and inhibition potency so **2**, was used as the representative monomeric ligand throughout this study.

Steady State Fluorescence Studies

To measure the binding affinity of the ligands to CUG^{exp}, a steady state fluorescence titration method using 5'-TAMRA-(CUG)₆-3' was utilized.⁴⁶ It is possible that binding of a ligand to a UU mismatch, close to 5'-TAMRA, could change the structure from ss to ds, and thus lead to quenching of the 5'-TAMRA by the 3'-G through photoinduced electron transfer.^{65,66} Ligands were titrated into TAMRA-(CUG)₆ solution and the TAMRA fluorescence intensity was observed to gradually decrease with increasing ligand concentration as a result of fluorophore quenching by the bound ligand. A plot of normalized fluorescence intensity versus concentration of each ligand yielded a binding isotherm and K_D value (Figure 3) that showed a trend paralleling that of the ΔT_m study.

Two of the oligoether-linker dimeric ligands, **7** and **12**, gave a K_D value close to the monomeric ligand **2**. On the other hand, dimeric ligands, **11**, **10** and **9**, showed a 14, 17 and 206- fold decrease in K_D compared to **2**, respectively. Although **9** and **10** have the same number of atoms in the linker group, the latter has an additional amino group, yet it is ligand **9** that is a 10-fold tighter binder. The origin of this difference is difficult to identify with certainty without knowing the exact protonation state and preferred conformation of the linking chain. There may also be a role for a specific placement of the amino groups in the linker of **9** for a favorable electrostatic interaction with the polyphosphate backbone of (CUG)₆.⁵⁹

Inhibition of MBNL1-CUG Interaction Using a SPR-Based Biosensor

We recently described a simple SPR-based method capable of directly measuring MBNL1 complexation of (CUG)₁₂ in real time under equilibrium conditions and in a label-free format. Further, we were able to use this technique to quantify the inhibition potency of selected ligands.²⁷ To rule out non-specific inhibition due to aggregation and non-selective

RNA binding, the assays were performed in the presence of both Tween-20 and an excess of competitor tRNA.⁶⁷ As described previously, biotinylated (CUG)₁₂ was immobilized on a streptavidin coated biosensor chip and incubated with different concentrations of each ligand to reach a steady state response unit (RU) over 150 s. The response to the binding of each ligand was found to be negligible in comparison to MBNL1 binding, so the effect of the ligand could be ignored. The method involved successive injections of a 0.65 μ M solution of MBNL1 and enough ligand to keep its concentration identical to that of pre-incubation with the varying responses correlating with ligand concentration. Because the SPR signal directly reflects the binding of MBNL1 to the biotinylated (CUG)₁₂, the differences in the response curves are a result of inhibition by the ligand. The maximum RU at 150 s was recorded for each ligand concentration and converted to the % (CUG)₁₂ bound by MBNL1, with all values normalized to that measured in the absence of each ligand. Fitting the data points in the plot of % (CUG)₁₂ bound by MBNL1 versus increasing concentrations of each ligand (Figure 4a) gave an apparent IC₅₀ value for each ligand (Figure 4b).

Normalized IC₅₀ values can be calculated by dividing the actual IC₅₀ value by the number of binding modules in the ligand. The ratio between normalized IC₅₀ values of the representative monomeric ligand, **2**, and the dimeric ligand is called bivalent effect. The IC₅₀ values (Figure 4b) show that more than the linker length, its composition is critical for an optimal bivalent effect. The oligoether-linker dimeric ligands, **13**, **8**, **12** and **7**, show a small bivalent effect of 3, 4, 5 and 8, respectively, whereas oligoamino-linker dimeric ligands, **9**, **10**, **11**, show a much bigger bivalent effect of 133, 24 and 29, respectively.

Do the increases in affinity for dimeric ligands **9**, **10**, **11** originate in bivalent binding of the acridine-triaminotriazine units? Caution must be exercised in interpreting simple increases in K_D or improvements in IC₅₀ values, because the linker chain may also contribute directly to binding and inhibition. Indeed, the recently reported monomeric ligand **33** containing an analogous oligoamine side chain to that in **9** gave $\Delta T_m = 2.5$ °C and IC₅₀ = 15 ± 2 μ M using the same RNA substrate and analytical methods.²⁷ In comparison, the monomeric ligand **2** studied here gave $\Delta T_m = 1.8 \pm 0.6$ °C, K_D = 66 ± 1 μ M and IC₅₀ = 293 ± 19 μ M. These ΔT_m values are nearly the same but the improvement in the IC₅₀ value suggests that the oligoamine does provide additional binding and inhibitory strength. Perhaps the best evidence ligand **9** is a bivalent binder is the increased melting temperature it affords ($\Delta T_m = 9.3 \pm 0.6$ °C) which is significantly above that for **33** ($\Delta T_m = 2.5$ °C). The significant melting temperature increase for **9** relative to **2** and **33** is attributed in large part to the intercalation of the second acridine unit consistent with a bivalent interaction.

These *in vitro* experiments demonstrate that **9** with $\Delta T_m = 9.3 \pm 0.6$ °C, K_D = 0.32 ± 0.02 μ M and IC₅₀ = 1.1 ± 0.1 μ M, is the optimal dimeric ligand. Thus, **9** binds to (CUG)₁₂ with a high affinity, stabilizing the stem loop structure and inhibiting the (CUG)₁₂-MBNL1 interaction selectively. The evidence collected is consistent with the improved binding affinity and inhibition potency of **9** resulting from a bivalent binding mode with a positive contribution from oligoamine linker. All of the *in vitro* experiments were performed in 1X PBS buffer, which was chosen because it most closely mimics physiological conditions.

Bioactivity of **9** in a DM1 Cell Model

Following the successful *in vitro* studies described above, the optimized dimeric ligand **9** was examined in a cell-based assay. Dimeric ligands **5** and **11** and the corresponding monomeric ligand **2** served as controls. Spermine was studied as a negative control because its structure is analogous to the linker in **9**. The cytotoxicity of **2** and **9** in HeLa cells and examined and both showed less than 20% cytotoxicity at concentrations up to 75 μ M by a sulforhodamine B assay in 24 h.⁶⁸ The cellular and nuclear penetration of ligands could be

monitored by the inherent fluorescence of the acridine unit. It was found that **5** and **9** are cell-permeable and nucleus-permeable whereas **2** and **11** are not (Figure 5, column 2).

It is a distinguishing characteristic of DM1 cells that MBNL1 aggregates with CUG^{exp} in nuclear foci.⁶⁹ To visualize the effect of these ligands on DM1 ribonuclear foci, confocal microscopy was applied to a DM1 cell model, HeLa cells that are transfected with a plasmid containing a truncated DMPK mini-gene with CTG₉₆₀.⁷⁰ As a negative control cell, HeLa cells were transfected with a truncated DMPK-CTG₀ (i.e., no CTG repeat) containing plasmid.⁷⁰ To label (CUG)₉₆₀ foci, Cy3-(CAG)₁₀ was used as a fluorescence *in situ* hybridization (FISH) probe. TO-PRO-3 was used to stain the nucleus. This particular dye was chosen because it does not interfere with the fluorescence of the acridine ring of the ligands and or that from Cy3 used in the Cy3-(CAG)₁₀ FISH probe.

DM1 model cells were treated with three different concentrations (20, 35 and 50 μ M) of ligands **2**, **5**, **11** and **9** for 36 h, followed by a fluorescence *in situ* hybridization (FISH) study with the Cy3-(CAG)₁₀ probe that labels CUG₉₆₀ nuclear foci. Over a hundred cells were counted and classified as having foci or no foci for each. Representative images for each sample are shown in Figures 1-14 in the Supporting Information. The fraction of cells with (CUG)₉₆₀ foci was measured at various concentrations of each of ligand (Figure 6). Ligands **2**, **5**, **11** and spermine showed no significant foci dispersion in any concentration (p-value > 0.05). However for **9**, partial foci dispersion was observed at 20 μ M and 35 μ M (p-value < 0.009) and full foci dispersion was observed at 50 μ M concentration.

MBNL1 Foci Dispersion in Live DM1 Cell model by **9**

Although the reduction in the foci-containing cells upon treatment with **9** was statistically significant, this approach provides only indirect evidence of the foci dispersion. With fixed-cell microscopy the dispersion of ribonuclear foci upon addition of **9** is not directly observed over time in the same cell. Thus, the effect of **9** in a live DM1 cell model was investigated using time-lapse confocal microscopy analogous to the method described recently.²⁷

The DM1 cell model consisted of HeLa cells transfected with the same plasmid used above with a truncated DMPK-CTG₉₆₀ minigene and also a plasmid containing a GFP-MBNL1 minigene, to track the nuclear foci by monitoring the GFP tag on MBNL1.⁷⁰ To monitor ligand uptake and MBNL1 foci dispersion in real time, the model DM1 cells were incubated with **9** at 50 μ M and individual live cells were examined by confocal microscopy over time. The observation made at t = 0 was just prior to addition of **9** and MBNL1 nuclear foci were clearly present (Figure 7a, t = 0). It was necessary to use a Petri dish with an imprinted 500 μ m grid to obtain the absolute position of each individual cell. This way each cell could be relocated following the incubation interval using differential interference contrast (DIC) microscopy. DIC images of **9**-treated and untreated DM1 model cells corresponding to cells in Figures 7a and 7b, used to relocate each individual cell, are shown in Figures 15 and 16 in the Supporting Information.

Over time, ligand **9** was observed to penetrate the cellular and nuclear membrane and the intense foci were fully dispersed with the green fluorescence of MBNL1 spread throughout the nucleus (Figure 7a, t = 3, 6, and 10 h). To validate this observation, a Z-stacked image of 1 μ m-separated slices from the whole **9**-treated DM1 model cell was obtained. This confirmed that upon treatment with **9** at 50 μ M, almost all the foci are dispersed over the entire nucleus at t = 10 h (Supplementary Movie 1 and Supplementary Figure 17). As a negative control, to rule out spontaneous MBNL1 foci dispersion over time, untreated DM1 model cells were studied in the same way. The presence of MBNL1 foci at all time points confirmed the stability of the foci (Figure 7b). A Z-stacked image of 1 μ m-separated slices from the whole untreated DM1 model confirmed the presence of multiple MBNL1 foci at

various Z planes of the cell (Supplementary Movie 2 and Supplementary Figure 18). It is noteworthy that the increase in cell size and number of MBNL1 foci is caused by natural growth of live cells and continuous expression of GFP-MBNL1 in real time. These results provide direct evidence of the ability of **9** to penetrate the cellular and nuclear membrane and disperse almost all of the MBNL1·CUG^{exp} aggregates over a 10 h period.

Conclusion

We report here an approach to targeting CUG^{exp} using a dimeric ligand based on previously reported *in vitro* active ligand **1**.²⁶ Limited by a firm structural knowledge on the ligand·CUG^{exp} complex,^{46,47} we designed and synthesized a small library of dimeric ligands to bind to neighboring CUG sites. This library of 10 dimeric ligands varied in composition, length and attachment point of linker. The *in vitro* activity of ligands was studied by three methods: (1) optical melting to measure the stabilization level of ds (CUG)₁₂, (2) steady state fluorescence to measure the binding affinity to (CUG)₆ and (3) SPR to measure inhibition of the (CUG)₁₂·MBNL1 complex.

The bivalent effect was found to depend more on the composition of the linker than its length. The dimeric ligands containing oligoamines as linkers were the most potent and they exhibited excellent water solubility. Among the four dimeric ligands with oligoamine linkers, **9** was the most potent ligand in *in vitro* assays. In comparison to monomeric ligand **2** it had a 206-fold greater affinity to (CUG)₆ and 266-fold greater inhibition potency in inhibiting formation of the (CUG)₁₂·MBNL1 complex. Comparison with previously reported monomeric ligand **33**²⁷ supports the formation of a bivalent complex with a portion of the gain in affinity and inhibition potency originating in the polyamine-derivative linker of **9**. In DM1 cell model, **9** was bioactive as partial foci dispersion was observed at 20 and 35 μ M (p-value < 0.009) and full foci dispersion was observed at 50 μ M concentration. To obtain direct evidence of its bioactivity, ligand **9** was examined by time-lapse confocal fluorescence microscopy and gradual MBNL1 foci dispersion was observed in individual live DM1 model cells at 50 μ M over a 10 h period. To validate that foci are dispersed over the entire nucleus, a Z-stacked image containing 1 μ m-separated slices from the whole DM1 model cell was obtained. These positive results suggest that dimeric ligand **9** is a good candidate for further development. Indeed, the next step will be to measure downstream effects of **9** on splicing as well as its toxicity in animals, positive results in these two areas pointing toward future studies in animal models of DM1. These efforts are underway and will be reported in due course.

Experimental Section

General Synthetic Procedure for Compounds 18-27

A round-bottom flask, equipped with a stir bar, was charged with **14** or **15** (1 equivalent) and freshly distilled thionyl chloride (16 equiv.). Synthetic schemes for Compounds **14** and **15** are shown in Scheme 2 of the Supporting Information. A catalytic amount of DMF was added and the mixture heated gently under reflux at 70 °C, stirring until homogeneous and then for 2 h. The excess thionyl chloride was distilled off and the last traces were removed azeotropically via co-evaporation with DCM (3 $\times$ 50 mL). The residue was left under vacuum (minimally) for 1 h to afford the crude intermediate as a yellow powder. The crude intermediate was dissolved in anhydrous DCM. Anhydrous triethylamine was added to the solution until the pH was 11 and it was cooled to 0 °C. The corresponding diamine (0.45 equiv.), compounds **34-43** shown in Supporting Information, or methylamine (1.1 equiv) was added and the solution was stirred at 0 °C for 2 h and slowly warmed to room temperature overnight. The solvent was removed by rotary evaporation and the crude mixture was purified via flash chromatography (SiO₂; CH₂Cl₂:MeOH, generally from 98:2

to 95:5) to yield **16-27** as a yellow solid. The yield of each compound is shown in the Supporting Information.

General Synthetic Procedure for Compounds **4**, **6-8**, **12**, **13**, **29-32**

A round-bottom flask, equipped with a stir bar, was charged with one of compounds **18-27** (0.5 equivalent) or **16-17** (1 equiv.) and **28** (1.1 equivalent). Synthetic scheme for compounds **28** is shown in Scheme 1 of Supporting Information. DIPEA (1.1 equivalent) and anhydrous DMF (25 mL) were added. The solution was heated at 80 °C for 6 h. The solvent was removed by rotary evaporation and the product purified via flash chromatography (basic alumina; DCM:methanol:NH₄OH, generally from 95:4.9:0.1 to 90:9.5:0.5) to yield the corresponding compound, **2-4**, **6-8**, **12-13**, or **29-32** as a yellow solid. The yield of each compound is shown in the Supporting Information.

General Synthetic Procedure for Compounds **5**, **9-11**

A round-bottom flask, equipped with a stir bar, was charged with one of compounds **29-32** (1 equivalent). Anhydrous TFA (30 mL) and anhydrous DCM (70 mL) were added and stirred at room temperature for 6 h. The solvents were removed to yield compounds **5**, **9-11** as a yellow solid in quantitative yield.

Compound Purification and Characterization

Following chromatographic purification, compounds were observed as single spots on thin layer chromatography plates. The key monomeric and dimeric ligands were estimated to be 95% based on the ¹H NMR spectra that showed single homogeneous compounds as judged by the single set of peaks. This estimate was confirmed quantitatively for control ligand **2** and dimeric ligands **5-7**, **9**, and **11** using HPLC (LC-MS) with traces shown in the Supporting Information. HPLC showed ligand **9**, which was studied in cell culture, to be ca. 99% pure and its elemental analysis (CHNF) showed it to be ca. a hexa-ammonium ion trifluoroacetate salt. See Supporting Information for additional details.

Bioassays, Methods, and Origin of Biological Materials

The MBNL1N protein expression and purification procedures and origin of CTG₀ and CTG₉₆₀ plasmids and RNAs were described previously.²⁷ The optical melting experiments, surface plasmon resonance (SPR) analysis, and steady state fluorescence-based binding assays followed directly from the previously reported.²⁷

FISH (Fluorescence *In Situ* Hybridization)

A total of ca. 120,000 HeLa cells were seeded in each well of a 6-well plate on coverslips. After a day, the cells were transfected with 500 ng DMPK-CTG₀ or DMPK-CTG₉₆₀ plasmid, as described previously.²⁷ Ligands were added to wells at different concentrations (20, 35 and 50 μM). After 36 h, cells were fixed and processed and slides were imaged and analyzed, as described previously. The following table indicates the excitation filters used in these experiments.

Fluorophore	Component	Excitation wavelength (nm)
Acridine	Ligands	405
Cy3	CUG ₉₆₀	555
TO-PRO-3	Nucleus	639

The p-values were calculated using two-tailed Student t test by Microsoft® Excel 2010.

Live Cell Imaging

Cells were prepared as described previously.²⁷ Ligand **9** was added to give a final concentration of 50 μ M. Live-cell, time-lapse images were taken before addition of **9** as well as at 3, 6 and 10 h time points, as described previously.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS USED

ASO	antisense oligonucleotide
CUG-BP1	CUG-binding protein 1; myotonic dystrophy type 1
DM1	dystrophia myotonica protein kinase
ds	double stranded
DMPK	dystrophia myotonica protein kinase
FISH	fluorescence <i>in situ</i> hybridization
MBNL1	muscleblind-like 1 protein
PTS	polyamine transporting system
RU	response unit
ss	single stranded
SPR	surface plasmon resonance
TAMRA	carboxytetramethylrhodamine
T_m	melting temperature
TRED	trinucleotide expansion disease

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Figure 1.
Library of synthesized monomeric (1-3) and dimeric (4-13) ligands.

^aReagents and conditions: (a) SOCl₂, DMF (Cat.), 70 °C, 2 h; (b) DCM, NEt₃, 0 °C to RT, 15 h; 60-78%; (c) DMF, DIPEA, 80 °C, 6 h; 55-75%; (d) CF₃COOH, DCM, RT, 6 h, 100%.

Figure 2.
General synthetic scheme for dimeric ligands

Figure 3.

a) Fluorescence titrations of TAMRA-(CUG)₆ with ligands. Comparison of normalized fluorescence intensity change of TAMRA-(CUG)₆ in the presence of increasing concentrations of ligands. TAMRA was excited at 560 nm and its emission was recorded at 590 nm. Error bars represent mean $\pm$ s.d. of three independent experiments. b) K_D was derived by fitting the Fluorescence intensities at different concentrations of ligands into the

following equation:
$$F = \frac{F_{max} - F_0}{1 + \left(\frac{K_D}{[L]}\right)^n} + F_0$$
 where [L] is the concentration of each ligand and F_{max} and F_0 are the maximum and minimum of normalized fluorescence intensities, respectively.

Figure 4.

a) Fitting the response unit data in the plot of % (CUG)₁₂ bound by MBNL1 versus increasing concentrations of each ligand to a dose-response curve by SPR assay. b) IC₅₀ values and bivalent effects were derived. Error bars represent mean ± s.d. of three independent experiments.

Figure 5.

Confocal fluorescent images show CUG^{exp} foci in DM1 model cells are present in rows 2-6, no ligand treatment, treated with spermine (negative control), **2**, **9** and **5**, respectively. CUG^{exp} foci are not present in negative control cells, row 1, as well as row 7 where DM1 cell model is treated with **9**, for 36 h. Corresponding fluorophores in columns 1-3 are TO-PRO-3, Acridine ring and Cy3-(CAG)₁₀ FISH probe, respectively.

Figure 6.

Plot of CUG^{exp} foci-containing cell fraction at various concentrations of ligands. It is noteworthy that data for ligand **5** (50 μM) was not measurable due to solubility issues. These data are gathered from counting more than one hundred cells. The cells were not counted in a blinded manner, but loss of foci was very apparent. Cells with a single foci were not counted as containing nuclear foci. The error bars represent mean $\pm$ standard error of at least three independent experiments. The symbol indicates a p-value < 0.009 , otherwise giving a p-value > 0.05 .

Figure 7.

Live cell microscopy demonstrates a direct evidence for MBNL1 foci dispersion with **9**. a) Live DM1 cell model are treated with **9** (50 μM) at t = 0, immediately after the first image is taken. Fluorescence of **9**, confirms its penetration to the nucleus. MBNL1 nuclear foci are gradually dispersing over time in two cells. b) Two live cell show stability of foci in DM1 cell model, in the absence of **9**, over the period of 10 h. Each box shows 120 μM X 120 μM.

Table 1 ΔT_m value of (CUG)₁₂ upon adding ligand in a 1:1 ratio.^a

Ligand	Solution	$\Delta T_m(^{\circ}\text{C})$
2	5% DMSO	1.8 ± 0.6^1
7	5% DMSO	2.7 ± 0.4
4	N.S.	_____
12	5% DMSO	N.D.
13	5% DMSO	N.D.
5	N.S.	_____
8	5% DMSO	1.8 ± 0.2
6	N.S.	_____
9	Aqueous	9.3 ± 0.6
10	Aqueous	9.6 ± 1.3
11	Aqueous	8.4 ± 2.1

N.S. – not sufficiently soluble.

^aEach value is mean $\pm$ s.d. of three independent experiments.