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A Potent Inhibitor of Protein Sequestration by Expanded Triplet (CUG) Repeats that Shows Phenotypic Improvements in a *Drosophila* Model of Myotonic Dystrophy

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Abstract

Myotonic dystrophy is the most common form of the adult-onset muscular dystrophy, originating in a CTG repeat expansion in the *DMPK* gene. The expanded CUG transcript sequesters MBNL1 a key regulator of alternative splicing, leading to the misregulation of numerous pre-mRNAs. We report an RNA-targeted agent as a possible lead compound for the treatment of DM1 that reveals both the promise and challenges for this type of small molecule approach. The agent is a potent inhibitor of the MBNL1-rCUG complex with an inhibition constant, K_I of 25 ± 8 nM, and is also relatively non-toxic to HeLa cells, able to dissolve nuclear foci, and correct the IR splicing defect in DM1 model cells. Moreover, treatment with this compound improves two separate disease phenotypes in a DM1 *Drosophila* model, the adult external eye degeneration and larval crawling defect. However, the compound has a relatively low maximum tolerated dose in mice and its cell uptake may be limited, providing insights into directions for future development.

Graphical abstract

A potential RNA-targeted lead therapeutic agent for the treatment of myotonic dystrophy type 1 (DM1) is described. Two bisamidinium ligands are linked using "click" chemistry. The heterodimer is a potent inhibitor of the MBNL1-rCUG complex ($K_I = 25 \pm 8$ nM), relatively non-toxic to HeLa cells, dissolves nuclear foci, fully corrects the IR misregulated alternative splicing in

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DM1 model cells, and shows significant improvement of disease phenotypes in a DM1 *Drosophila* model.

Keywords

RNA recognition; Click chemistry; Myotonic Dystrophy; *Drosophila* model; RNA-protein inhibitor

Introduction

Myotonic dystrophy type 1 (DM1) is an autosomal dominant neuromuscular disorder characterized by a range of symptoms that include muscle weakness (myopathy), difficulty relaxing muscles (myotonia), progressive muscle wasting (atrophy), cataracts, cardiac defects, and insulin dependent diabetes.^[1,2] There is an urgent need to discover lead agents for treating DM1 because it affects about 1 in 8,000 people yet remains incurable with no direct therapeutic options.

DM1 results from a progressive expansion of the trinucleotide CTG repeat in the 3'-untranslated region of the dystrophin myotonia-protein kinase (*DMPK*) gene on chromosome 19q13.3.^[3] The number of CTG repeats is less than 35 in healthy people, and ranges from 50 to thousands in DM1 patients.^[2] The molecular origin of DM1 was previously attributed to three possible mechanisms:^[4] (1) *DMPK* haploinsufficiency, (2) decreased expression of neighboring genes, including *SIX5* and *DMAHP*, and (3) a gain-of-function for the expanded RNA transcript (rCUG^{exp}). Recent studies have argued against the first two hypotheses, leaving the third mechanism as the favored one for therapeutic intervention.^[5-8]

The gain-of-function model involves expanded rCUG repeats forming stable stem-loop structures with U-U mismatches flanked by G-C and C-G base pairs,^[9,10] and sequestering important proteins. Key among these proteins is the muscleblind-like 1 protein (MBNL1), a key alternative splicing regulator.^[11-13] The loss of MBNL1 results in the abnormal alternative splicing of more than 100 pre-mRNAs, including the cardiac troponin T (cTNT), insulin receptor (IR), and chloride channel 1 (ClC-1).^[14] Supporting the toxic RNA model is the finding that overexpression of MBNL1 protein in the skeletal muscle of a DM1 mouse model relieved the myotonia and abnormal RNA splicing.^[15] The MBNL1-rCUG^{exp} complex formation has emerged as a key therapeutic target for DM1 with reports of inhibitors ranging from small molecules^[16] to antisense oligonucleotides.^[17]

We reported the rational design of several acridine-containing ligands that strongly and selectively bind rCUG^{exp} by intercalation.^[16b,18,19] More recently, we discovered **1a**, a second-generation ligand designed with two triaminotriazine recognition units linked to a bisamidinium moiety, a known rCUG groove binder.^[20] Ligand **1a** exhibited excellent water solubility and low cytotoxicity. Importantly, it partially dissolved the MBNL-rCUG^{exp} nuclear foci that formed in DM1 model cells, thereby partially rescuing the mis-splicing of *cTNT* and *IR* pre-mRNA. However, **1a** is only a weak inhibitor of the MBNL1-(CUG)₁₂ complex ($K_I = 8 \pm 2 \mu\text{M}$) so more effective agents were sought.

Our previous work,^[19] along with earlier examples by Disney^[21] and Miller^[16a] demonstrated that dimeric ligands can lead to enhancements in both the binding affinity and selectivity of rCUG-binding units. Herein, we report the synthesis of compounds **1b-f**, alkyne- and azide-containing analogs of **1a**, that might be conveniently elaborated using the click reaction, ultimately allowing for a fragment-based drug discovery strategy. The initial interest in these compounds, and the focus of this article, is on linking two units of **1a** to create a bivalent ligand. Dimer **2a** is indeed shown to be a potent MBNL1-rCUG^{exp} complex inhibitor, with activity in DM1 model cells and in a DM1 *Drosophila* model. However, the increased molecular weight of the dimer (MW = 1166.4 Da) means that its cellular efficacy is not what might be expected for such a strong inhibitor and the compound has a significantly lower MTD in mice than does the monomeric ligand. These important observations point to a need to access the potency of the dimer while retaining some of the favorable attributes of the monomer.

Results and Discussion

Design and synthesis of dimeric ligands 2a-b

The rational design of monomer **1a** was based on a known complex formed between a frame-shifting bisamidinium stimulator ligand and its HIV-1 frameshift site (FS) RNA stem-loop target.^[22] An overlay of the (CUG)₁₂ X-ray and the NMR-determined HIV-1 FS RNA

structures showed striking similarities and the possibility of the bisamidinium unit of **1a** similarly binding the A-form CUG^{exp} RNA major groove.^[20] Thus, the bisamidinium unit of **1a** was designed to cover a central CUG with the two triaminotriazine units recognizing the adjacent CUG sites.

There are a number of considerations in designing a dimeric ligand based on **1a**, but the two most important clearly are synthetic accessibility and the ability to span adjacent binding sites without disrupting the primary interactions between the monomeric ligand and RNA. Although no structural data is available yet regarding the actual complex formed between **1a** and rCUG^{exp}, both considerations suggested that the logical functionalization point is the triaminotriazine amino group of **1a**. Thus, substituted analogs of dicyanobenzene and 1,4-diaminobutane are quite limited in availability and would require long linker groups to span adjacent sites. In contrast, unsymmetrically substituted triaminotriazines are readily accessible synthetically.

The key question was whether *N*-substitution of one or both of the triaminotriazine units of **1a** would affect its ability to bind rCUG^{exp}. In our first generation series of rCUG^{exp}-targeting ligands containing acridine intercalators, methylation of the amino groups in some cases had a profound effect on both affinity and selectivity.^[23] However, we recently discovered that the binding affinity toward (CUG)₁₂ of compound **1b** ($K_D = 7.7 \mu\text{M}$) and **1c** ($K_D = 4.8 \mu\text{M}$) are comparable with that of compound **1a** ($K_D = 8 \mu\text{M}$).^[24] This finding enables the rapid development of a wide range of functional derivatives of ligand **1a** using the [3+2] copper-assisted alkyne-azide cycloaddition of **1b** and **1c**.

Synthesis of dimeric ligands using the alkyne-azide cycloaddition

Compounds **1d-f** bearing one clickable group were prepared as described in Scheme 1. Triaminotriazine **3** reacted with a large quantity of **4**, which was obtained from the Pinner reaction of 1,4-dicyanobenzene with hydrochloric acid in anhydrous ethanol, to give **5** and a small amount of **1a**. The mixture was treated with the appropriate **6** to give compounds **1d-f** in moderate yields. Because hydrochloric acid was used in the purification, each compound **1** was a tetrahydrochloride salt.

Dimeric ligands **2a-b** were prepared from the click reaction of **1d-e** and **1f**. The azide-alkyne cycloaddition of **1d-e** and **1f** was assisted by the *in situ* formation of copper(I) from the reduction of copper(II) sulfate by sodium ascorbate.^[25] The reaction was run in the presence of tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) to protect Cu(I) from oxidizing to Cu(II).^[26] Dimers **2a** and **2b** were purified by Sephadex CM-25 column and isolated as their hydrochloride salts. The desired compounds were judged to be >95% pure by ¹H NMR and analytical HPLC (Figure S3) showed peaks corresponding to 100% and 97% of the total chromatogram signal for **2a** and **2b**, respectively.

Inhibition of the MBNL1-(CUG)₁₆ complex by dimeric ligands

An electrophoretic mobility shift assay (EMSA) was employed to determine the *in vitro* inhibition of MBNL1-rCUG^{exp} complex by monomer **1a** and dimers **2a-b** (Figure 1 and S5). This study used (CUG)₁₆, which can form a hairpin structure containing a maximum of 8 U-

U mismatches. Monomer **1a** was designed to span 3 UU mismatches suggesting that the (CUG)₁₆ target provides at least one binding site for dimers **2a** and **2b** assuming a 1:1 complex is formed.^[20] In this study, MBNL1N, a truncated MBNL1 containing four zinc finger motifs of MBNL1 and a hexahistidine tag (C-terminus) was employed (see Experimental Section for additional details). This MBNL1 construct and the full-length MBNL1 have the similar affinities to rCUG^{exp}.^[27]

From the EMSA binding study, an apparent K_D value of 5.1 ± 1.6 nM was determined for the MBNL1 complex to radiolabeled-(CUG)₁₆ (Figure S4), which is consistent with reported K_D values by EMSA and other techniques.^[18,20] To determine the inhibition constant of potential bivalent ligands **2a** and **2b**, the MBNL1-(CUG)₁₆ mixture, in which ca. 50% of the RNA is free, was incubated with various concentrations of ligand (Figure 1a and S5). The dimer concentrations were not raised to higher than 10 μ M due to the aggregation on the gel. As the concentration of ligand increased, more RNA was observed to be released from the complex with MBNL1. By fitting the plot of bound RNA fractions versus ligand concentrations with an appropriate equation (see SI for more details), the IC₅₀ value was determined (Figure 1b).

Dimer **2a** exhibited an IC₅₀ value of 290 ± 20 nM, and a calculated K_I value of 25 ± 8 nM. Similarly, compound **2b** gave an inhibition constant $K_I = 37 \pm 12$ nM. In comparison, the inhibition of MBNL1-(CUG)₁₆ complex by monomeric compound **1a** was also determined by EMSA under identical conditions, giving an IC₅₀ value of 189 ± 32 μ M, and a K_I value of $16,000 \pm 6,000$ nM, which is almost 1000-fold higher than that for **2a**. The significant improvement in inhibition potency likely results from bivalent binding of (CUG)₁₆ by the dimeric ligands. In fact, the binding of **2a** to r(CUG)₁₆ produced a significantly greater heat than that of **1a** in an isothermal titration calorimetry experiment (Figure S6). The binding isotherm indicates a positively cooperative system, however, additional experiments are needed to fully elucidate the binding mode and determine quantitatively the affinity of the dimeric ligands. We previously showed that the first generation acridine-based ligands and MBNL1 can form a ternary complex with rCUG repeat indicating that inhibition may be noncompetitive.^[28] Whether **2a** and **2b** sterically block MBNL1 binding or operate by a noncompetitive mechanism is not known, but beyond their greater RNA affinity, these ligands cover a greater surface area of the rCUG^{exp} target than does monomer **1a**.

Ligand **2a** was selected for cellular and animal assays for two main reasons. First, it exhibited a slightly lower K_I value than did **2b**, although both were within the experimental error of one another. More importantly, the lower cost and ready availability of propargylamine compared to 1-amino-3-butyne made it the choice for synthesis. In particular, the lower cost made it easier to scale up the synthesis and prepare a sufficient quantity of **2a**.

Study of MBNL1-rCUG^{exp} nuclear foci dispersion in DM1 model cells

To evaluate the ability of dimer **2a** to disrupt the MBNL1-(CUG)₁₆ foci in DM1 model cells, a confocal microscopy experiment was conducted, using immunofluorescence staining and fluorescent *in situ* hybridization (Figure 2a).^[29] Normal and DM1 model cells were

generated by transfecting HeLa cells with, respectively, GFP-DT0 and GFP-DT960 plasmids containing (CTG)₀ and (CTG)₉₆₀, respectively, in exon 15 of a truncated *DMPK* gene. The endogenous MBNL1 was visualized by Alexa Fluor 647 dye-labeled antibody (column 1), while r(CUG)₉₆₀ was detected by a complementary sequence (CAG)₁₀ conjugated with a Cy3 dye and represented by the red color (Column 2). The nucleus was stained by Hoechst 33342 as a blue area (Column 3).

In normal cells, MBNL1 is observed throughout the nucleus, whereas in the DM1 model cells, it is concentrated in bright green spots that colocalized with the (CUG)₉₆₀ as indicated by the red spots (Cy3-(CAG)₁₀). The yellow spots in the merge column of Figure 2a are the nuclear foci. A reduction to approximately 72% of the original number of foci was observed after 48 h with 1 μ M treatment of **2a**. In contrast a similar reduction with **1a** required 100 μ M over the same time (Figure 2a-b). We previously found that 100 μ M **1a** was more effective in foci dispersion, reducing the number of foci to ca. 20% the original number.^[20] That study used different plasmids for transfection and different HeLa cells. Because we have found changes such as these can lead to noticeable changes in compound performance, it is essential to perform head to head comparisons as was done with **1a** and **2a** in this study.

Dimer **2a** did not exhibit a full dose-dependent effect (Figure 2b). Incubation of **2a** at 10 μ M decreased the foci area to ca. 36%, but a similar level was also observed for the 100 μ M treatment. Although, it is encouraging that dimeric ligand **2a** is as active at 1 μ M as monomeric ligand **1a** is at 100 μ M, it is surprising that full foci dispersion is not observed at the highest concentration. This observation and the lack of an obvious dose dependence may indicate a cellular penetration issue for **2a**. These foci dispersion experiments were also carried out using exogenous GFP-MBNL1 with similar results (Figure S7).

Cellular and mouse toxicity of ligand 2a

Although ca. 10% HeLa cell death was observed after the treatment of **2a** at 100 μ M for 96 h, the dimer showed negligible cellular toxicity at lower concentrations in a sulforhodamine B (SRB) colorimetric assay (Figure S8). In contrast, a series of acridine-based dimeric ligands was found to exhibit a comparatively higher level of cytotoxicity.^[19] This finding suggests an advantage of the bisamidinium-based ligands over the acridine-containing ligands. However, the maximum tolerated dose (MTD) of **2a** in mice was a relatively low 3 mg/kg as determined via daily intraperitoneal injections.

Ability of ligand 2a to correct the IR splicing defect

Various studies have demonstrated the abnormal alternative splicing of pre-mRNAs that are directly associated with disease phenotypes in DM1 transgenic mouse models and DM1 patients. For example, mis-splicing of human cardiac troponin T (cTNT),^[30] insulin receptor (IR) in skeletal muscle tissue,^[31] and muscle-specific chloride channel (ClC-1)^[32] are correlated with, respectively, reduced myocardial function/conduction abnormalities, insulin resistance, and myotonia, respectively. In a mouse model, the overexpression of MBNL1 leads to a correction of some of these splicing defects and reverse the disease phenotypes (e.g., myotonia, myopathy).^[33]

Because **2a** is capable of inhibiting MBNL1-rCUG^{exp} both *in vitro* and in DM1 model cells, we evaluated its ability to correct the splicing defects in DM1 model cells using an *IR* minigene. During the alternative splicing of *IR*, two products are generated: isoforms IR-A and IR-B with the exclusion and inclusion of exon 11, respectively (Figure 3a). In normal skeletal muscle, IR-B is the dominant isoform, whereas IR-A is more abundant in DM1 skeletal muscle.^[31] We found that normal HeLa cells and the DM1 model cells contained ca. 49% and 26% isoform B, respectively (Figure 3b). The treatment of **2a** in DM1 model cells showed a dose-dependent reversal of *IR* splicing. Importantly, compound **2a** at 100 μ M fully recovered the *IR* defect. However, the significantly improved K_i did not allow the full recovery at a lower concentration given that a similar effect was observed in the treatment with **1a** at 100 μ M. As with the foci inhibition studies these results point to a limited cellular uptake of the dimer.

***In vivo* testing for phenotypic improvement upon ligand 2a treatment in two DM1 *Drosophila* models**

Determining drug efficacy in a living organism is a critical step in drug development. The *Drosophila* line with tissue-specific expression of 480 interrupted CTG repeats, *i(CTG)₄₈₀*, shows many key characteristics of DM1, such as nuclear accumulation of rCUG^{exp} and MBNL proteins, CUG-induced eye phenotypes, muscle degeneration, and abnormal alternative splicing of muscle genes.^[34] In contrast, the fly line expressing 60 uninterrupted CTG repeats, *(CTG)₆₀*, does not exhibit severe pathologies. Several small molecules were reported to improve the CUG-induced phenotypes in the DM1 *Drosophila* model,^[35,36] including our previous studies of **1a**.^[20] The comparative effectiveness of **1a** and **2a** in suppressing the external eye degeneration phenotype and improving the larva's mobility was examined in the DM1 *Drosophila* model.

Similar to reported *Drosophila* models,^[34] our transgenic fly model with the *gmr-GAL4* driven expression of *i(CTG)₄₈₀* repeats (*gmr-GAL4>UAS-i(CTG)₄₈₀*) showed external eye morphological phenotypes, including disorganized ommatidia and mechanosensory bristles, resulting in a glossy eye (Figure 4a). The microscopic images showed that treatment with 50 μ M of **2a** led to a partial reversal of the degenerative phenotype, whereas the same effect was only achieved when the treatment with monomer **1a** was raised to 400 μ M (Figures 4a, S9, and S10). On the other hand, treatments of *(CTG)₆₀* *Drosophila* flies with either **1a** or **2a** at 400 μ M did not change the fly's eye structure.

Recently, Jagla and coworkers demonstrated the mobility or locomotor defect of DM1 *Drosophila* larvae using a contraction and righting assay.^[37] We found that compounds **1a** and **2a** significantly improved the locomotor ability of *i(CTG)₄₈₀* DM1 *Drosophila* larvae using a larval crawling assay.^[38] In this assay, an individual larva generated from transgenic flies was placed on a Petri dish over graph paper, and the number of grid lines crossed by the larva in 1 min was recorded. It was found that the *(CTG)₆₀* control larvae were able to crawl ca. 13 lines per min, whereas the *i(CTG)₄₈₀* DM1 larvae could cross only 9 lines (Figure 4b), demonstrating their locomotor defect. When the control larvae were raised in the presence of 400 μ M aqueous solution containing fly food of either **1a** or **2a**, the mobility was not affected (Figure 4c), suggesting a low toxicity of the bis-amidinium ligands.

Interestingly, treating the DM1 larvae with **1a** at 100, 200, 400 μ M demonstrated a dose response with ca. 14%, 54%, 84% of the crawling defect rescued, respectively (Figure 4b). Compared with **1a**, dimer **2a** showed a greater efficacy. The crawling movement of the *i(CTG)₄₈₀* DM1 larvae was nearly fully recovered at 100 μ M of **2a**. Even at concentrations of as low as 20 μ M, **2a** induced a 37% improvement in the DM1 larva's mobility. To our knowledge, this is the first example of a small molecule showing a substantial improvement in locomotor behavior or muscle performance defect of the DM1 *Drosophila* larvae.^[29]

Conclusions

In summary, the high potency of the benzamidinium-based dimeric ligand **2a**, prepared by copper-catalyzed click reaction, was demonstrated by *in vitro* inhibition of the MBNL1-rCUG^{exp} interaction, nuclear foci disruption in model DM1 cell culture, correction of IR mis-splicing defect, and a dose-dependent reversal of rCUG-induced phenotypes in a DM1 *Drosophila* model. Although **2a** exhibited a 1000-fold lower K_i for inhibiting MBNL1-rCUG^{exp} complexation and promising effects in the DM1 *Drosophila* model, its inability to both fully disrupt nuclear foci formation and recover the *IR* splicing defect at lower concentration than the monomer suggests potential issues with cell permeability. This outcome is unsurprising given the ligands high molecular weight and the increased number of hydrogen bond donor and acceptor groups.^[39]

The observation of clear dose dependent phenotypic improvement in the *Drosophila* models supports the notion that these compounds may be useful if delivery and toxicity issues can be addressed. Two obvious solutions involve the use of a delivery vehicle or through the use of *in vivo* self-assembly of the clickable monomers. The latter approach is particularly appealing because monomer **1a** is non-toxic to mice at 50-100 mg/kg.^[20] Efforts along these lines will be reported in due course.

Experimental Section

Synthetic methods

All non-aqueous reactions were carried out under a dry N₂ atmosphere with oven-dried (115 °C) glassware. All solvents and reagents were of reagent quality, purchased commercially, and used without further purification unless otherwise indicated. Reactions were monitored by thin layer chromatography using EMD pre-coated silica gel 60 F₂₅₄ plates. Flash chromatography was carried out using Silicycle SiliaFlash P60 (230–400 mesh) silica gel. Unless otherwise noted, ¹H and ¹³C NMR spectra were recorded on a 500 MHz Varian Unity Inova spectrometer. All NMR measurements were carried out in DMSO-*d*₆ at ambient temperature. Chemical shifts are in parts per million (ppm), relative to the residual peaks of DMSO-*d*₆ (¹H: 2.50; ¹³C: 39.52). Coupling constants (*J*) were reported in Hertz. Low resolution electrospray ionization mass spectra (LR-ESI-MS) were obtained by the Mass Spectrometry Laboratory, School of Chemical Sciences, University of Illinois at Urbana-Champaign.

Compound 10

To a white suspension of 5.0 g (17.1 mmol) of **4**^[22] in 30 mL of anhydrous EtOH was added 7.5 mL (53.8 mmol) of anhydrous Et₃N, resulting in a colorless solution. A suspension of 1.1 g (5.7 mmol) of **3**^[16b] in 10 mL of anhydrous EtOH was added slowly to the reaction mixture. The reaction was stirred at room temperature for 24 h. The white solid was filtered, washed extensively with dichloromethane, dried under nitrogen overnight to give 2.1 g (97%) of compound **10** as a white solid. Compound **10** was used for the next step without further purification because the amide ester is water sensitive. LR-ESI-MS (*m/z*) calculated for [M + H]⁺: 372.2; found 372.2.

Compound 1d

A suspension of 4.0 g (10.8 mmol) of **5** and 2.8 g (11.9 mmol) of *N*²-(4-aminobutyl)-*N*⁴-(prop-2-yn-1-yl)-1,3,5-triazine-2,4,6-triamine (**6a**)^[24] in 50 mL of anhydrous EtOH was stirred at room temperature overnight. The solvent was removed using a rotary evaporator. The resulting white solid was purified by Silica gel column chromatography with a gradient mixture of CH₂Cl₂ and MeOH from 9:1 to 1:9 (v/v). After using 1L of DCM/MeOH (1:9, v/v), the eluent was acidified with a gradient of 0.10–0.2 mL of 4 M dioxane solution of HCl (per liter of eluent). The product-containing fractions were combined, filtered, and concentrated using a rotary evaporator to give 5.4 g (71%) of **1d** as a white tetra-HCl salt. ¹H NMR: δ 10.11 (s, *NH*, 2H), 9.73 (s, *NH*, 2H), 9.30 (s, *NH*, 2H), 7.95 (s, *ArH*, 4H), 6.87–5.95 (m, *NH* and *NH*₂, 9H), 3.97 (s, *CCH*₂, 2H), 3.45 (t, *J* = 7.1, *NHCH*₂, 4H), 3.25–3.21 (m, *NHCH*₂, 4H), 3.01 (t, *J* = 2.4, *CCH*, 1H), 1.70–1.53 (m, *CH*₂, 8H). LR-ESI-MS (*m/z*) calculated for [M + H]⁺: 561.3; found 561.4.

Compound 1e

Using the procedure described above, 0.3 g (0.8 mmol) of **10** and 5 mL (1.0 mmol) of 0.2 M ethanolic solution of *N*²-(4-aminobutyl)-*N*⁴-(but-3-yn-1-yl)-1,3,5-triazine-2,4,6-triamine (**6b**)^[24] afforded 0.3 g (52%) of **1e** as a white tetra-HCl salt. LR-ESI-MS (*m/z*) calculated for [M + H]⁺: 575.4; found 575.5.

Compound 1f

Using the procedure described above, 3.5 g (9.4 mmol) of **10** and 2.9 g (10.4 mmol) of *N*²-(4-aminobutyl)-*N*⁴-(3-azidopropyl)-1,3,5-triazine-2,4,6-triamine (**6c**)^[24] afforded 4.3 g (61%) of **1f** as a white tetra-HCl salt. ¹H NMR: δ 10.13 (s, *NH*, 2H), 9.74 (s, *NH*, 2H), 9.32 (s, *NH*, 2H), 7.95 (s, *ArH*, 4H), 6.63–5.95 (m, *NH* and *NH*₂, 9H), 3.45 (t, *J* = 7.2, *NHCH*₂, 4H), 3.38–3.54 (m, *NHCH*₂, 4H), 3.24–3.21 (m, *NHCH*₂ N₃CH₂, 4H), 1.74–1.53 (m, *CH*₂, 10H). LR-ESI-MS (*m/z*) calculated for [M + H]⁺: 606.4; found 606.2.

Compound 2a

To a solution of 34 mg (0.049 mmol) **1d** and 37 mg (0.049 mmol) of **1f** in 11 mL of MeOH was added 148 μL (0.015 mmol) of 0.1 M DMSO solution of TBTA, 246 μL (0.025 mmol) of 0.1 M aqueous solution of copper sulfate. To the resulting solution was added 985 μL (0.099 mmol) of 0.1 M aqueous solution of sodium ascorbate. The mixture was stirred at room temperature for 24 h. Solvents were removed using a rotary evaporator. The resulting

solid was suspended in 10 mL water. The suspension was filtered using a micro-filter to obtain a clear solution. Concentrated aqueous hydrochloric acid solution was added to adjust to pH of the solution to 5–6. The resulting solution was loaded onto the Sephadex CM-25 column, and purified using an aqueous solution of ammonium bicarbonate from 0.1 M to 0.9 M. Fractions containing the product was collected, concentrated using a rotary evaporator at 60 °C. The solid was dissolved in 5 mL of 1 M aqueous solution of hydrochloric acid. The resulting solution was frozen in an dry-ice bath, and dried in a lyophilizer for 2 days, gave 27 mg (38%) of **2a** as a white solid. ¹H NMR: δ 10.30 (s, NH, 4H), 9.83 (s, NH, 4H), 9.50–9.46 (m, NH, 4H), 8.58–7.86 (m, ArH and NH, 26H), 4.57–4.52 (m, CCH₂, 2H), 4.42–4.39 (m, NCH₂, 2H), 3.51–3.47 (m, NHCH₂, 8H), 3.38–3.27 (m, NHCH₂, 10H), 2.08–2.06 (m, CH₂, 2H), 1.72–1.59 (m, CH₂CH₂, 16H). ¹³C NMR: δ 161.61, 132.84, 128.70, 128.68, 47.08, 42.46, 29.52, 25.98, 24.61. LR-ESI-MS (*m/z*) calculated for [M + H]⁺: 1166.7; found 584.0 ([M + 2H]²⁺, 35%), 389.7 ([M + 3H]³⁺, 60%), 292.6 ([M + 4H]⁴⁺, 100%). HPLC (0.1% TFA in H₂O/MeOH (1:1, v/v), flow rate = 3 mL/min): t = 3.9 min (100%).

Compound 2b

Using the general procedure described above, 39 mg (0.054 mmol) of **1e** and 40 mg (0.054 mmol) of **1f** afforded 26 mg (32%) of **2b** as a white solid: ¹H NMR: δ 10.30 (s, NH, 4H), 9.83 (s, NH, 4H), 9.50–9.47 (s, NH, 4H), 8.51–7.87 (m, ArH and NH, 26H), 4.39 (t, *J* = 7.2, NCH₂, 2H), 3.56–3.47 (m, NHCH₂, 10H), 3.36–3.26 (m, NHCH₂, 10H), 2.90–2.86 (m, CH₂, 2H), 2.07–2.04 (m, CH₂, 2H), 1.71–1.59 (m, CH₂CH₂, 16H). ¹³C NMR: δ 161.56, 155.70, 132.79, 128.63, 122.90, 47.01, 42.43, 25.93, 24.56. LR-ESI-MS (*m/z*) calculated for [M + H]⁺: 1180.7; found 591.1 ([M + 2H]²⁺, 15%), 394.4 ([M + 3H]³⁺, 35%), 296.1 ([M + 4H]⁴⁺, 45%), 237.1 ([M + 5H]⁵⁺, 60%), 197.7 ([M + 6H]⁶⁺, 100%). HPLC (0.1% TFA in H₂O/MeOH (1:1, v/v), flow rate = 3 mL/min): t = 3.8 min (97%).

Protein Expression and Purification

An expression vector for MBNL1N was obtained from Maurice S. Swanson (University of Florida, College of Medicine, Gainesville, FL). MBNL1N is comprised of the four zinc finger motifs of MBNL1 and contains a hexahis tag. The protein was expressed and purified as described previously.^[20] The molecular weight of MBNL1N was confirmed by MALDI mass spectrometry and the concentration was determined by an amino acid analysis.

Biological experiments

ITC, EMSA, IR splicing were conducted as described previously.^[20] The foci dispersion experiments using immunofluorescence staining was performed as reported recently.^[29] See Supporting Information for more details. The cytotoxicity of **2a** was assessed using a published protocol.^[40]

Drug treatment in *Drosophila*

Fly lines bearing *UAS-(CTG)60* and *UAS-(CTG)480*^[34] were kind gifts of Prof. Rubén Artero Allepuz (Universitat de València, Estudi General, Spain). The external eye and larval crawling assays were performed as previously described.^[29]

Determination of murine maximum tolerated dose (MTD)

All experimental procedures were reviewed and approved by the University of Illinois Institutional Animal Care and Use Committee (Protocol # 15232). Compound was formulated in sterile water and administered to C57BL/6 female mice (12-14 week old, Charles River) intraperitoneally for 5 consecutive days. All the mice were monitored over the course for signs of toxicity and weight loss.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Determination of inhibition constants, K_I , by EMSA. (a) Representative gels show the release of MBNL1N from the complex with (CUG)₁₆ at different concentrations of **1a** and **2a**. (b) The IC₅₀ value was obtained by plotting the normalized fraction RNA bound versus the ligand concentration in Prism and fitting the curve with the equation: $y = ax^b / (IC_{50}^b + x^b)$, where y is the fraction of RNA bound, x is the concentration of compound, $a = y_{\max} - y_{\min}$, b is a Hill slope. Error bars represent the standard deviation from three independent experiments. The inhibition constant K_I was calculated using the equation: $K_I = IC_{50} \times K_D / [\text{protein}]_{\text{total}}$, where $[\text{protein}]_{\text{total}} = 0.06 \mu\text{M}$.

Figure 2.

Foci disruption in DM1 model cells with ligand **2a**. (a) Representative confocal microscope images showing the MBNL-rCUG^{exp} foci disruption by monomer **1a**, and dimer **2a** in DM1 model cells. (b) Percent of foci area per cell after treatment of ligands for 48 h. The dispersion of endogenous MBNL following treatment of ligand were quantitatively measured. Error bars represent standard errors of mean from at least four independent experiments, two-tailed *t*-test **P*<0.005. Note: This was a head-to-head comparison between **1a** and **2a**.

Figure 3.

a) Schematic representation of IR alternative splicing. b) Correction of IR splicing by **2a** and **1a** determined by conventional RT-PCR. Error bars represent standard error of the mean from at least three independent experiments, two-tailed *t*-test **P* < 0.05, ****P* < 0.001

Figure 4.

Efficacy of **1a** and **2a** in DM1 *Drosophila* fly model. a) Improvement in adult external eye degeneration phenotype. Each experiment was conducted in triplicate. b) Dose-dependent effect of ligands on the DM1 larvae (*24B-GAL4 > UAS-i(CTG)₄₈₀*). (c) Effect of ligands on the control *Drosophila* larvae (*24B-GAL4 > UAS-(CTG)₆₀*). Each experiment was conducted in triplicate, 10 individual larvae for each trial. Error bars represent standard deviation, two tailed *t*-test **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Scheme 1.
Synthesis of ligands **1d-f**.