Published in final edited form as:

ChemMedChem. 2014 November ; 9(11): 2455–2462. doi:10.1002/cmdc.201402095.

Small Molecules that Target the Toxic RNA in Myotonic Dystrophy Type 2

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Abstract

Myotonic dystrophy type 2 is caused by an expansion of CCTG repeats in the zinc-finger protein gene (ZNF9). Transcribed CCUG repeats sequester MBNL1, an important alternative splicing regulator, preventing its normal function, leading to the disease phenotype. We describe a series of ligands that disrupt the MBNL1-r(CCUG)_n interaction as potential lead agents for developing DM2 therapeutics. A previously reported triaminopyrimidine-acridine conjugate was a moderate inhibitor in vitro, however it proved to be poorly water-soluble and not cell-permeable. To improve its therapeutic potential, the new set of ligands maintained the key triaminopyrimidine recognition unit but replaced the acridine intercalator with a bisamidinium groove binder. The optimized ligands exhibit low micromolar inhibition potency to MBNL1-r(CCUG)₈. Importantly, the ligands are the first to show the ability to disrupt the MBNL1-r(CCUG)_n foci in DM2 model cell culture and exhibit low cellular cytotoxicity.

Keywords

Myotonic dystrophy; triaminopyrimidine; bisamidinium; DM2

Introduction

Myotonic dystrophy type 1 and type 2 (DM1 and DM2) are the most common forms of muscular dystrophy, both estimated to affect 1 in 8000.^[1] DM1 is caused by an expansion of CTG repeats (CTG^{exp}) in the 3'-untranslated region of the *DMPK* gene on chromosome 19, whereas DM2 is associated with an expansion of CCTG repeats (CCTG^{exp}) in the intron 1 of the *ZNF9* gene on chromosome 3.^[1] The minimum lengths of the repeats indicating the presence of DM1 and DM2 diseases are 50 CTG and 75 CCTG repeats, respectively. At this length the sequences become unstable, progressively expanding and reaching thousands of CTG or CCTG repeats in the most severe cases of the disease.

Transcribed CUG and CCUG repeats (abbreviated rCUG^{exp} and rCCUG^{exp}, respectively) sequester muscleblind-like proteins including an important alternative splicing regulator

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MBNL1. A decrease in cellular MBNL1 levels results in splicing defects of >100 pre-mRNAs, leading to the disease symptoms, which include myotonia, muscle weakness, and cataracts.^[2] In addition, the rCCUG transcript appears to interact with other proteins that are involved in translation.^[2–4] Thus, it was reported that the CUG binding protein (CELF1) and a translation initiation factor (eIF2) form a complex and both are sequestered by rCCUG^{exp} as are the proteasome, both events leading to a reduction in the global rate of protein synthesis.^[3–5] The rCCUG^{exp} transcript may effect the expression levels of the ZNF9 protein, and this may contribute to the DM2 phenotype, however the reports on this point are somewhat contradictory.^[4–8]

An important potential approach to treating DM2 involves molecular intervention, the discovery and development of either oligonucleotides or small molecules that selectively disrupt the interaction of rCCUG^{exp} with MBNL1 and other proteins. This same general approach has been applied to DM1 and is considerably more advanced.^[9–19] To our knowledge there are only two reports of small molecules that selectively target the toxic rCCUG^{exp} transcript causing DM2.^[20,21] In 2009, Disney and coworkers described a series of multivalent ligands displaying kanamycin A units with varying separations (see **1**, $n = 1–3$, $m = 0–19$), the best was the trimer which inhibited the r(CCUG)₄₇-MBNL1 interaction with nanomolar IC₅₀ values in vitro.^[20]

Recently, we described ligand **2**, a rationally designed “stacked-intercalator” featuring a triaminopyrimidine recognition unit linked to an acridine intercalator and showed that this simple ligand bound rCCUG repeat sequences with high selectivity and micromolar K_D values.^[21] Although it inhibited formation of the MBNL1-r(CCUG)₆ complex with a moderate inhibition potency (IC₅₀ = 52 μ M) we found it to be toxic in cellular assays. Herein we report a new series of triaminopyrimidine-bisamidinium conjugates as potential DM2 ligands that maintain the low micromolar inhibition potency for MBNL1-rCCUG^{exp} complexes but also have improved water solubility, cell-uptake, and cytotoxicity. Importantly, the optimized DM2 ligands are the first documented to dissolve the foci of MBNL1 and rCCUG^{exp} in DM2 model cell culture.

Results and Discussion

Design of Bisamidinium based DM2 Ligands

The current design combines knowledge gained in several recent efforts at targeting both DM1 and DM2. As illustrated in Figure 1, rCCUG^{exp} targeting ligand **2**, was based on ligand **3**, a triaminotriazine-acridine conjugate, designed to bind UU and TT mismatches selectively by formation of a Janus-wedge, base triplet.^[16] Thus, the higher pK_a of the pyrimidine unit was proposed to result in its protonation leading to a Janus-wedge moiety that is complementary to the CU mismatch.^[21] Support for this idea comes from the finding that despite the structural similarities of the triaminotriazine and triaminopyrimidine moieties, ligands **2** and **3** hold their selectivity toward rCUG^{exp} and rCCUG^{exp}, respectively. The selective targeting of rCCUG^{exp} by the triaminopyrimidine recognition unit is key to the current DM2 ligand design.

Both ligands **2** and **3** contain an acridine intercalator, which was expected to stack on the Janus wedge unit and provide a hydrophobic driving force for binding, while reducing the nonspecific intercalation. This “stacked-intercalator” approach was successful in so far as the off-target binding of duplex DNA was significantly suppressed. However, ligand **3** exhibited unacceptable toxicity and similar studies with **2** in cell culture experiments revealed poor water-solubility, low cell-permeability, and high toxicity (see SI).

Concern about the acridine unit led to its replacement using ligand **4**, a groove binding bisamidinium ion. The selection of **4** was based on a number of reports showing bisamidinium compounds to possess excellent water-solubility, high cellular up-take,^[22] and low cytotoxicity.^[23] Indeed, with the appropriate substitution pattern the compounds targeted the nucleus.^[22] Equally importantly, Butcher and co-workers reported the NMR structure of bisamidinium ligand **4** (DB213, R = NMe₂, Figure 1) bound within the groove of the HIV-1 frame shift inducing RNA stem-loop.^[24] The groove binding site adopts a remarkably similar A-form conformation to that in r(CUG)₁₂ and the distance between the ammonium ions in bound **4** (DB213, R = NMe₂) is the same as between every third UU mismatch in r(CUG)₁₂.

Ligand **5** containing the bisamidinium groove binder **4** and two UU recognition units was found to exhibit low toxicity in cellular assays, be well tolerated by mice, bind rCUG^{exp}, dissolve nuclear MBNL1-rCUG^{exp} foci in vitro, and show phenotypic improvements in a DM1 *Drosophila* model.^[19] In considering whether a similar strategy might be successful for DM2 (e.g., Figure 1, ligand **6**), it was important to know the rCCUG^{exp} structure, and particularly whether it similarly adopts an A-form. It is predicted that rCCUG^{exp} might adopt two possible structures: one with two consecutive CU mismatches and another with alternating CC and UU mismatches, the latter RNA referred to as the slipped form.^[21,25] The former is predicted to be more stable and Disney and coworkers recently reported the first high resolution x-ray analysis of an r(CCUG)₃ duplex within a larger RNA sequence. The rCCUG sequence indeed adopts an A-form structure with two CU mismatches separated by two GC base pairs.^[26]

Synthesis of Bisamidinium based DM2 Ligands

Following the prior success^[19] with ligand **5** and based on the other considerations above, several analogous bisamidinium ligands for DM2 were synthesized with triaminopyrimidine, Janus wedge units for targeting the CU mismatches in rCCUG^{exp} (Figure 1).^[21] The substitution pattern of the bisamidinium motif may affect both its cellular penetration and localization^[22] and its specific fit to rCCUG^{exp}. Therefore, ligands with both para- and meta-substituted bisamidinium moieties were prepared, with alkyl linkers varying from two to four methylene groups to determine the optimum length.

Ligands **6–11** were prepared from the appropriate meta- or para-bisimidate and the corresponding aminoalkylpyrimidine (Scheme 1, see SI for details). The aminoalkylpyrimidines were prepared using a modification of the preparation reported by Mascari and coworkers.^[27] Briefly, the corresponding dicyanoalkyl phthalimide reacted with guanidine carbonate, followed by the deprotection using hydrazine (see Scheme S1 and SI).

Inhibition of MBNL1-(CCUG)₈ Complex by DM2-Targeting Small Molecules In Vitro

An electrophoretic mobility shift assay (EMSA) was used to determine the inhibition potencies of the ligands toward MBNL1-r(CCUG)₈. To avoid the slipped rCCUG repeat from forming and restrict the r(CCUG)₈ structure to that with two consecutive CU mismatches, an RNA oligomer was used with complementary GC rich regions both at the 3'- and 5'-ends (Figure 2A). The binding affinity of MBNL1 to r(CCUG)₈ was measured by EMSA. Thus, ³²P-labeled r(CCUG)₈ was incubated with MBNL1, serially diluted, and fraction of RNA that is free or complexed measured following their electrophoretic separation. The percent RNA bound by MBNL1 was plotted against the MBNL1 concentration, curve fitting providing K_D = 3.9 ± 0.8 nM (Figure 2B and C).

The inhibitory potency of the ligands was screened qualitatively by incubating each at 100 μM with MBNL1-r(CCUG)₈. Free and protein-complexed r(CCUG)₈ were separated on a gel and their relative ratios were measured. Incubation of MBNL1-r(CCUG)₈ with compounds **6**, **8**, **10**, and **11** that each contain linkers with three or four methylene groups lead to full disruption of the MBNL1-r(CCUG)₈ complex (Figure 3A). In contrast, a significant amount of complex remained with the two methylene linker-containing ligands **7** and **9**, as well as for negative control **4** (DB213, R = NMe₂) lacking the triaminopyrimidine rings. Likewise, the DM1 targeting ligand **5**, its meta-substituted analog and analogs containing longer or shorter linkers failed to inhibit the MBNL1-r(CCUG)₈ complex effectively (Figure S2).

Given these qualitative results, IC₅₀ values were measured for ligands **6**, **8**, **10**, and **11** using quantitative EMSA with varying ligand concentration (Figure 3B and C). The corresponding inhibition constants K_i were in the low micromolar range (Table 1). Interestingly, there is no significant difference in inhibition potency between meta- and para-bisamidinium compounds (**8** vs. **10**, **6** vs. **11**). This result was unexpected because the substitution pattern changes both the distance the recognition units can span as well as the fit of the bisamidinium unit within the RNA groove. In the absence of additional structural data it is difficult to explain the insensitivity to the benzamidinium substitution pattern.

Lead DM2 Compounds Show Low Cytotoxicity Toward HeLa Cells

As noted above, development of the triaminopyrimidine-acridine conjugate, ligand **2**, was limited by its high cytotoxicity. In particular, it was found that treatment of model DM2 cells (see below) with **2** at 50 μ M led to >90% cell death and a change in cell morphology with a presence of disease foci after 48 h (Figure S6). In contrast, a low cytotoxicity was observed when the bisamidinium-based ligands **6**, **8**, **10**, and **11** at 100 μ M were incubated with the DM2 model cell culture for 48 h.

To determine more quantitatively if these ligands exhibited an improved cytotoxicity profile in comparison with ligand **2**, a Sulforhodamine B (SRB) colorimetric assay^[28] with HeLa cells was performed. The percentage of cell death was plotted as a function of ligand concentration (Figure 4 and S5). The para-bisamidinium conjugates **6** and **8** were observed to have low cytotoxicity with between 15–30% of cell death observed. Interestingly, the meta-bisamidinium ligands, **10** and **11**, exhibited essentially no cytotoxicity at concentrations as high as 100 μ M.

Small Molecule Foci Disruption in DM2 Model Cell Culture

The hallmark of DM2 disease is the sequestration of MBNL by rCCUG^{exp}. Immunofluorescence experiments showed a co-localization of MBNL1 protein with rCCUG^{exp} in DM2 patient cells in clearly observable punctate foci.^[29] With a similar pathogenesis to DM1, it is believed that the DM2 phenotype might be reversed by ligands able to disrupt the interaction of MBNL1-rCCUG^{exp} in cells, thereby liberating MBNL1 to function normally. Although a report of downstream splicing rescue is consistent with foci disruption, there are no small molecules directly observed to disrupt foci in DM2 cell culture. It is known in DM1 that the induced overexpression of MBNL1 prevented the muscle degeneration in a DM1 fly model, and reversed the spliceopathy and myotonia in a DM1 mouse model.^[29–31] Importantly, the ligands targeting toxic CUG repeats and disrupting the MBNL-CUG^{exp} interaction in the nucleus reverse DM1 phenotypes.^[9–19]

To examine if the in vitro activity of lead ligands **6**, **8**, **10**, and **11** translates into cellular activity, the ability to dissolve DM2 foci in model cell culture was measured by fluorescence *in situ* hybridization (FISH) experiments. Thus, HeLa cells were co-transfected with GFP-MBNL1 and (CCTG)₁₂₀₀ plasmids to generate a DM2 cell model.^[3,4,26] The location of MBNL1 and the r(CCUG)₁₂₀₀ transcripts were readily tracked by confocal microscopy, monitoring the fluorescent signal of GFP and a Cy3-(CAGG)₈ FISH probe, respectively (Figure 5A). The rCCUG repeats were found to localize in both nuclei and cytoplasm, which is consistent with a report by Timchenko and coworkers.^[4] There was a clear co-localization of GFP-MBNL1 and r(CCUG)₁₂₀₀ within the disease foci, providing evidence of MBNL1 sequestration by rCCUG repeats (Figure 5A, row 2). Additionally, there were some r(CCUG)₁₂₀₀ foci not co-localized with GFP-MBNL1, which may suggest a limited co-transfection of the protein or possibly the r(CCUG)₁₂₀₀ transcript bound by other protein complexes, including the 20S catalytic core and the CELF1-eIF2 complex.^[3,4]

Whereas **4** (DB213, R = NMe₂) showed almost no foci dispersion with the co-localization of Cy3 and GFP signals (Figure 5A, row 3), all of the bisamidinium ligands that inhibited the

MBNL1-r(CCUG)₈ complex in vitro (i.e., **6**, **11**) dissolved the disease foci (Figure 5A, rows 4–7, see SI for more images with a larger field) with a significant reduction of the number and size of the foci. In particular, treatment of DM2 model cells with **6**, **8**, **10**, and **11** at 100 μ M resulted in 50–70% foci disruption (Figure 5B). There was no correlation between the K_i value and the extent of foci disruption, which is understandable given that many other factors govern the cellular activity.

Conclusion

A documented approach for the treatment of myotonic dystrophy is through small molecules that target CUG or CCUG repeats and disrupt their interaction with MBNL1 and other proteins. Herein, we reported a new set of ligands that inhibit the MBNL1-rCCUG^{exp} interaction. The ligands are low molecular weight (all MW < 520) and contain two important units: a triaminopyrimidine moiety for targeting the CU mismatch and a bisamidinium moiety. The bisamidinium unit minimally provides a general A-form RNA targeting ability and confers excellent water-solubility and cellular uptake. The optimized ligands show promising bioactivities: inhibiting MBNL1-r(CCUG)₈ with low micromolar K_i values, very low toxicity in HeLa cells, and the ability to dissolve the disease foci in DM2 model cell culture. Indeed, the low cytotoxicity of ligands **6**, **8**, **10**, **11** in the cells and the binding selectivity of ligand **2** toward a sequence containing CU mismatches^[21] suggest a specificity of bisamidinium ligands towards rCCUG^{exp}. Our current efforts are focused on improving these already promising properties with an eye toward testing a second-generation series in *Drosophila* and mouse models.

Experimental Section

Materials

r(CCUG)₈ (5'-GCCCUGCCUGCCUGCCUGCCUGCCUGCCUGCCUGGC-3') was purchased from Dharmaco RNAi Technologies, Inc. with 2'-deprotection, desalting, and HPLC purification. The r(CCUG)₈ was dissolved in Ambion RNA storage solution (pH 6.4) to give 1 mM stock solution.

Protein expression and purification

An expression vector for a truncated MBNL1 contains amino acid 1–272. This MBNL is comprised of the four zinc-finger motifs of MBNL1 and a His₆, which has a similar affinity as the full-length MBNL1. The protein was expressed and purified following the procedure described previously.^[32]

RNA radioactive labeling

r(CCUG)₈ was labeled with [γ -³²P]-ATP using T4 polynucleotide kinase (New England Biolabs) and purified by phenol extraction and ethanol precipitation. Labeled RNA was heated at 95 °C for 5 min, placed on ice for 10 min, and diluted in a binding buffer (175 mM NaCl, 5 mM MgCl₂, 20 mM Tris (pH 7.5), 1.25 mM 2-mercaptoethanol, 12.5% glycerol (v/v), 2 mg/mL bovine serum albumin (BSA), 0.1 mg/mL heparin, 0.05% Triton-X (v/v)).

Binding experiments

r(CCUG)₈ at a final concentration of 0.22 nM was incubated with MBNL1 at serially diluted concentrations (3.3 μM to 2.1 pM). The reaction mixture was incubated at room temperature for 15 min, and loaded on 6% polyacrylamide gel (80 : 1) at 4 °C. The gels were run for 2 h at 200 V in 0.5 M Tris-borate buffer (pH 8.2). Gels were visualized on a Molecular Dynamics Storm PhosphorImager. The apparent K_D was obtained by fitting fraction RNA bound versus protein concentration using the equation: Fraction RNA bound = $1/(1+K_D/[MBNL1])$. The standard deviation was obtained from three independent experiments.

Screening EMSA experiments

Each ligand at 100 μM was incubated with pre-incubated MBNL1-r(CCUG)₈ complexes at room temperature for 15 min. Final concentrations of MBNL1 and (CCUG)₈ were 33 nM and 0.22 nM, respectively. The reaction mixture was loaded on the gel as described above. Fraction RNA bound was compared to the control samples (water or DMSO) to determine the hits under the testing conditions.

Inhibition EMSA experiments

Inhibition experiments were done as described above except MBNL1-r(CCUG)₈ complexes were incubated with ligands to give a serially diluted concentrations (from 200 μM to 50 nM). The IC_{50} values were obtained by fitting fraction RNA bound versus the log function of ligand concentrations using the equation: $Y = B + (A-B)/(1+10^{(X-\log(IC_{50}))})$, in which Y = fraction RNA bound, B = minimum fraction RNA bound, A = maximum fraction RNA bound, X = log [ligand]. The inhibition constants (K_i) were obtained using the equation: $K_i = IC_{50} \times K_D(rCCUG_8-MBNL1)/[MBNL1]$. Standard deviations were obtained from three independent experiments.

Synthesis of DM2 ligands

Triaminopyrimidine-bisamidinium conjugates were synthesized following Scheme S1. ¹H NMR spectra were recorded on a Varian Unity Inova 500 spectrometer. All NMR measurements were carried out in DMSO-*d*₆ at room temperature unless otherwise stated. Chemical shifts were reported as parts per million (ppm). Mass spectra were obtained in the Mass Spectrometry Laboratory, School of Chemical Sciences, University of Illinois. The analytical HPLC traces was obtained using a C18 column with the eluent of 0.1% TFA (v/v) in 1:1 MeOH-water as eluent.

All reactions were carried out under a nitrogen atmosphere. All solvents and reagents were purchased commercially with a reagent quality and used without further purification. The progress of reaction was monitored by thin layer chromatography (TLC) using Merck pre-coated silica gel 60F₂₅₄.

Ligand 2—Ligand 2 was prepared using the procedure reported by Wong and coworkers, the characterization data was identical to that reported.^[21]

Ligand 6—To 150 mg diethyl terephthalimidate dihydrochloride (0.5 mmol) in 10 mL EtOH cooled in an ice bath, was added 0.14 mL Et₃N (1.0 mmol). A solution of 215 mg 5-

(4-aminobutyl)pyrimidine-2,4,6-triamine (1.1 mmol) in 3 mL EtOH and 3 mL ethylene glycol was added dropwise. The mixture was warmed to room temperature and stirred for 24 h. The reaction was monitored by TLC using 7:3 H₂O-HOAc as eluent. The solvent was removed in vacuo. The residue was dissolved in methanol and loaded onto a silica gel flash column and purified by chromatography (MeOH-CH₂Cl₂ gradient from 1:4 to 4:1). Starting material and a side product were eluted and the desired product was eluted with 4:1 MeOH-CH₂Cl₂ containing 0.04–0.08 mL of a 4M aqueous HCl solution in 1,4-dioxane per liter of solvent. The desired product fractions were combined and the solvent was removed in vacuo to afford 160 mg (48%) of **6** as a light yellow solid, a hydrochloride salt, m.p. > 230 °C (decomp.). ¹H NMR: δ 10.6 (br s, NH₂, 2H), 9.8 (br s, NH₂, 2H), 9.5 (br s, NH₂, 2H), 8.0 (s, ArH, 4H), 7.2 (br s, NH₂, 12H), 3.5 (br, CH₂, 4H), 2.4 (br, CH₂, 4H), 1.8 (br, CH₂, 4H), 1.4 (br, CH₂, 4H). ESI-MS (m/z): 521.1 ([M + H]⁺), 100%.

Ligand 7—The desired compound was prepared using a procedure analogous to that described for **6** using 217 mg diethyl terephthalimide dihydrochloride (0.7 mmol) in 5 mL ethanol, 0.2 mL Et₃N (1.4 mmol), and 309 mg 5-(2-aminoethyl)pyrimidine-2,4,6-triamine (1.8 mmol) to afford 240 mg (52%) of **7**. ¹H NMR: δ 10.37 (br s, NH₂, 2H), 9.69 (br, NH₂, 2H), 9.5 (br s, NH₂, 2H), 8.0 (s, ArH, 4H), 7.2 (br s, NH₂, 12H), 3.5 (br t, CH₂, 4H), 2.4 (br t, CH₂, 4H). ESI-MS (m/z): 465.3 ([M + H]⁺), 100%.

Ligand 8—The desired compound was prepared using a procedure analogous to that described for **6** using 150 mg diethyl terephthalimide dihydrochloride (0.5 mmol) in 10 mL ethanol, 0.14 mL Et₃N (1.0 mmol), and 205 mg 5-(3-aminopropyl)pyrimidine-2,4,6-triamine (1.1 mmol) to afford 150 mg (47%) of **8**. ¹H NMR: δ 10.6 (br s, NH₂, 2H), 10.4 (br s, NH₂, 2H), 9.8 (br s, NH₂, 2H), 8.0 (s, ArH, 4H), 7.40 (br s, NH₂, 8H), 7.2 (br s, NH₂, 4H), 3.5 (br, CH₂, 4H), 1.8 (br, CH₂, 4H), 1.2 (q, CH₂, 4H). ESI-MS (m/z): 493.6 ([M + H]⁺), 100%.

Ligand 9—To 255 mg diethyl isophthalimide dihydrochloride (0.87 mmol) in 10 mL EtOH cooled in iced bath, was added 0.25 mL Et₃N (1.8 mmol). A solution of, 309 mg 5-(2-aminoethyl)pyrimidine-2,4,6-triamine (2.4 mmol) in 2 mL EtOH and 2 mL ethylene glycol was added dropwise. The reaction mixture was warmed up to room temperature and stirred for 24 h. The reaction was monitored by TLC using 7:3 H₂O-HOAc as eluent. The solvent was removed in vacuo. The residue was dissolved in methanol and loaded onto a silica gel flask column and purified by chromatography (MeOH-CH₂Cl₂ gradient from 1:4 to 4:1). Starting material and a side product were eluted and the designed product was eluted with 4:1 MeOH-CH₂Cl₂ containing 0.04–0.08 mL of a 4M aqueous HCl solution in 1,4-dioxane per liter of solvent. The desired product fractions were combined and the solvent was removed in vacuo to afford 265 mg (50%) pale yellow solid of **9** as a HCl salt, m. p. > 230 °C (decomp.). ¹H NMR: δ 10.36 (br s, ArCNH, 2H), 9.82 (br s, ArC=N⁺H₂, 2H), 9.63 (br s, ArC=N⁺H₂, 2H), 8.47 (s, ArH, 1H), 8.13 (dd, *J* = 7.8, 1.8 Hz, ArH, 2H), 7.81 (t, *J* = 7.9 Hz, ArH, 1H), 7.01 (br s, CNH₂C, 8H), 6.77 (br s, NCNH₂C, 4H), 3.48 (br t, *J* = 7.4 Hz, NH₂CH₂, 4H), 2.74 (br t, *J* = 7.6 Hz, CCH₂CH₂, 4H). ESI-MS (m/z): 465.3 ([M + H]⁺, 100%), 233.1 ([M + H]²⁺, 20%).

Ligand 10—The desired compound was synthesized following a procedure analogous to that described for **9** using 150 mg diethyl isophthalimidate dihydrochloride (0.5 mmol) in 10 mL ethanol, 0.14 mL Et₃N (1.0 mmol) and 200 mg 5-(3-aminopropyl)pyrimidine-2,4,6-triamine (1.1 mmol) to afford 120 mg (39%) of **10**. ¹H NMR: δ 10.4 (br s, NH₂, 2H), 9.8 (br s, NH₂, 2H), 9.6 (br s, NH₂, 2H), 8.4 (s, ArH, 1H), 8.1 (dd, ArH, 2H), 7.8 (dt, ArH, 1H), 6.40 (br s, NH₂, 8H), 6 (br s, NH₂, 4H), 3.5 (br t, CH₂, 4H), 2.4 (br t, CH₂, 4H), 1.8 (br q, CH₂, 4H). ESI-MS (m/z): 493.6 ([M + H]⁺), 100%

Ligand 11—The desired compound was synthesized following a procedure analogous that described for **9** using 150 mg diethyl isophthalimidate dihydrochloride (0.5 mmol) in 10 mL ethanol, 0.14 mL Et₃N (1.0 mmol) and 220 mg 5-(4-aminobutyl)pyrimidine-2,4,6-triamine (1.1 mmol) to obtain 130 mg (39%) of **11**. ¹H NMR: δ 10.4 (br s, NH₂, 2H), 9.8 (br s, NH₂, 2H), 9.6 (br s, NH₂, 2H), 8.4 (s, ArH, 1H), 8.1 (dd, ArH, 2H), 7.8 (td, ArH, 1H), 7.2 (br s, NH₂, 12H), 3.5 (br, CH₂, 4H), 2.4 (br, CH₂, 4H), 1.8 (br, CH₂, 4H), 1.4 (br, CH₂, 4H). ESI-MS (m/z): 521.1 ([M + H]⁺), 100%

The synthesis of intermediates can be found in SI. HPLC traces of triaminopyrimidine-bisamidine conjugates show the purity 95% (see SI)

Cytotoxicity study

Toxicity of ligands in HeLa cells was studied followed the protocol reported by Vichai and Kirtikara.^[28] Briefly, In the 96-well plate, the HeLa cells were incubated at 37 °C for 72 hours with the treatment of the ligand. The ligand was treated to each well to have serially diluted concentrations from 100 μM to 5.1 nM. The cells were fixed after 3 d, and stained with Sulforhodamine B (SRB). Bound SRB was dissolved by Tris base solution (pH 10.5) and the OD was measure at 510 nm. IC₅₀ values of toxicity were obtained by plotting the percentage of cell death against concentrations of ligands.

Fluorescence *in situ* hybridization (FISH)

FISH experiments were performed following the protocol described previously.^[12] Briefly, HeLa cells at 80% confluence were co-transfected with (CCTG)₁₂₀₀ and GFP-MBNL1 plasmids. Transfected HeLa cells were treated with ligands **4**, **6**, **8**, **10**, **11** at 100 μM, 48 h CCUG repeats were probed with Cy3-(CAGG)₈, and the nuclei were stained with To-Pro 3. The cells were looked at under confocal microscope LSM 700 (Zeiss). GFP, Cy3 and To-Pro 3 were excited at 488, 564, and 639 nm, respectively. Four random areas were selected to determine the average foci area per cell, using Teton software. The average foci area per cell of untreated cells was set to 100%. Each experiment was repeated at least three times independently.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Partha S. Sarkar for the (CCTG)₁₂₀₀ plasmids. This work was supported by the National Institutes of Health (RO1 AR058361).

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Figure 1.

Center: The designed Janus-wedge recognition of UU mismatch by triaminotriazine (DM1) and CU mismatch by triaminopyrimidine (DM2). Left: Acridine motif and acridine based DM1 and DM2 ligands. Right: Bisamidinium motif and bisamidinium based DM1 and DM2 ligands (**5** and **6**, respectively)

Figure 2.

A) Structure of r(CCUG)₈. B) MBNL1-r(CCUG)₈ binding gel. C) Plotting RNA fraction bound against log[MBNL1]. The error bars represent the standard deviation of three independent experiments

Figure 3.

A) Screening gel of bisamidinium based DM1 and DM2 ligands. In this gel, $[r(CCUG)_8] = 0.22$ nM, $[MBNL1] = 33$ nM, $[ligand] = 100 \mu$ M; B) Inhibition gel of ligand **10**, MBNL1- $r(CCUG)_8$ complex was incubated with **10** at different concentrations (from 200 μ M to 50 nM, 2-fold dilution); C) The inhibition curve of ligand **10**, the RNA fraction bound was normalized using the control. The error bars represent the standard deviation of at least three independent experiments

Figure 4.

Example of toxicity curves for ligands **2** and **10**. See also Fig. S5. The IC_{50} values for the toxicity, the concentration of ligand causing 50% cell death, was estimated from the plots. The error bars represent standard deviations from three independent experiments

Figure 5.

A) Confocal microscopy images of foci dispersion. DM2 model cells were treated with compounds **7–10** at 100 μ M, 48 h. r(CCUG)_n was probed by Cy3-(CAGG)₈, the nucleus was stained by To-Pro 3. B) Plotting percent normalized foci area per against different treatment conditions. The error bar represents standard error of mean of at least three independent experiments. (*) shows a statistically significant difference in comparison to DM2 model cell sample, P<0.001

Scheme 1.
Synthesis of ligands **6–11**.

Table 1

IC₅₀ and inhibition constants of ligands **6**, **8**, **10** and **11** to MBNL1-r(CCUG)₈

Compound	K _D (MBNL1-r(CCUG) ₈) nM	IC ₅₀ (μM)	K _i (μM)
6	3.9 ± 0.8	73.4 ± 6.0	8.7 ± 1.9
8		12.9 ± 1.0	1.5 ± 0.3
10		14.7 ± 1.2	1.7 ± 0.4
11		24.9 ± 2.0	2.9 ± 0.6

The data were obtained from three independent experiments