Supporting Information

Targeting Toxic RNAs that Cause Myotonic Dystrophy Type 1 (DM1) with a Bisamidinium Inhibitor

Chun-Ho Wong,[†] Lien Nguyen,[†] Jessie Peh,[†] Long M. Luu,[†] Jeannette S. Sanchez,[†] Stacie L. Richardson,[†] Tiziano Tuccinardi,[||] Ho Tsoi,[‡] Wood Yee Chan,[§] H. Y. Edwin Chan,[‡] Anne M. Baranger,[†,1] Paul J. Hergenrother*,[†,2] and Steven C. Zimmerman*,[†,2]

[†]Department of Chemistry, University of Illinois at Urbana-Champaign, 600 South Mathews Avenue, Urbana, Illinois 61801, United States

[||]Dipartimento di Scienze Farmaceutiche, Università di Pisa, via Bonanno 6, 56126 Pisa, Italy

[‡]Laboratory of Drosophila Research and School of Life Sciences, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong SAR

[§]School of Biomedical Sciences, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong SAR

[1]Current address: Department of Chemistry, 213 Lewis Hall, University of California, Berkeley, CA 94720

[2]To whom correspondence may be addressed. E-mail: hergenro@illinois.edu or sczimmer@illinois.edu

Materials

Anhydrous solvents and starting materials were purchased by the Aldrich Company and used without further purification unless otherwise specified.

Instrumentation

High and low resolution resolution mass spectra were obtained by the Mass Spectrometry Laboratory, School of Chemical Science, University of Illinois. Mass spectra were obtained by field desorption (FD) on a Waters 70-VSE-A and by ESI on a Waters Micromass Q-Tof. Elemental analyses were performed at the University of Illinois, School of Chemical Sciences.

High performance liquid chromatography (HPLC) was performed by a Dynamax SD-200 system with a UV detector set at 260 nm using an Alltech Denali C-18 column (250×10 mm) with a dual solvent system of 0.1% formic acid/H_2O (Solvent A) and 0.1% formic acid/MeCN (Solvent B). Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Unity 500 or Varian INOVA 500NB spectrometer at 21±3 °C unless otherwise mentioned. Chemical shifts (δ) are reported in parts per million (ppm). Coupling constants ($\mathcal{J}$) were reported in Hertz. 1H NMR chemical shifts were referenced to the residual solvent peak at 7.26 ppm in chloroform-*d* ($CDCl_3$) and at 2.50 ppm in DMSO-d_6. ^{13}C NMR chemical shifts were referenced to the center solvent peak at 77.16 ppm for $CDCl_3$ and at 40.45 ppm for DMSO-d_6. Analytical thin-layer chromatography (TLC) was performed on 0.2 mm silica 60 coated on aluminum plates with F254 indicator. Flash column chromatography was performed on 40–63 μm silica gel (SiO_2). Solvent mixtures used for chromatography are reported as percent volume (%) or as volume ratio (v/v).

Synthesis

The syntheses of the bisamidine ligands (**2**–**4**) are based on a modified literature procedure as shown in Scheme 1 (Fig. S1a) (1). Syntheses of compound **6**–**8** were previously reported (2). Compound **9** was synthesized according to a literature procedure (1). The purity of all ligands was estimated to be >95% by ^1H NMR, in which the purity of ligand **3** was >99% by HPLC (Fig. S1b); ^1H NMR of ligand **3** (Fig. S1c).

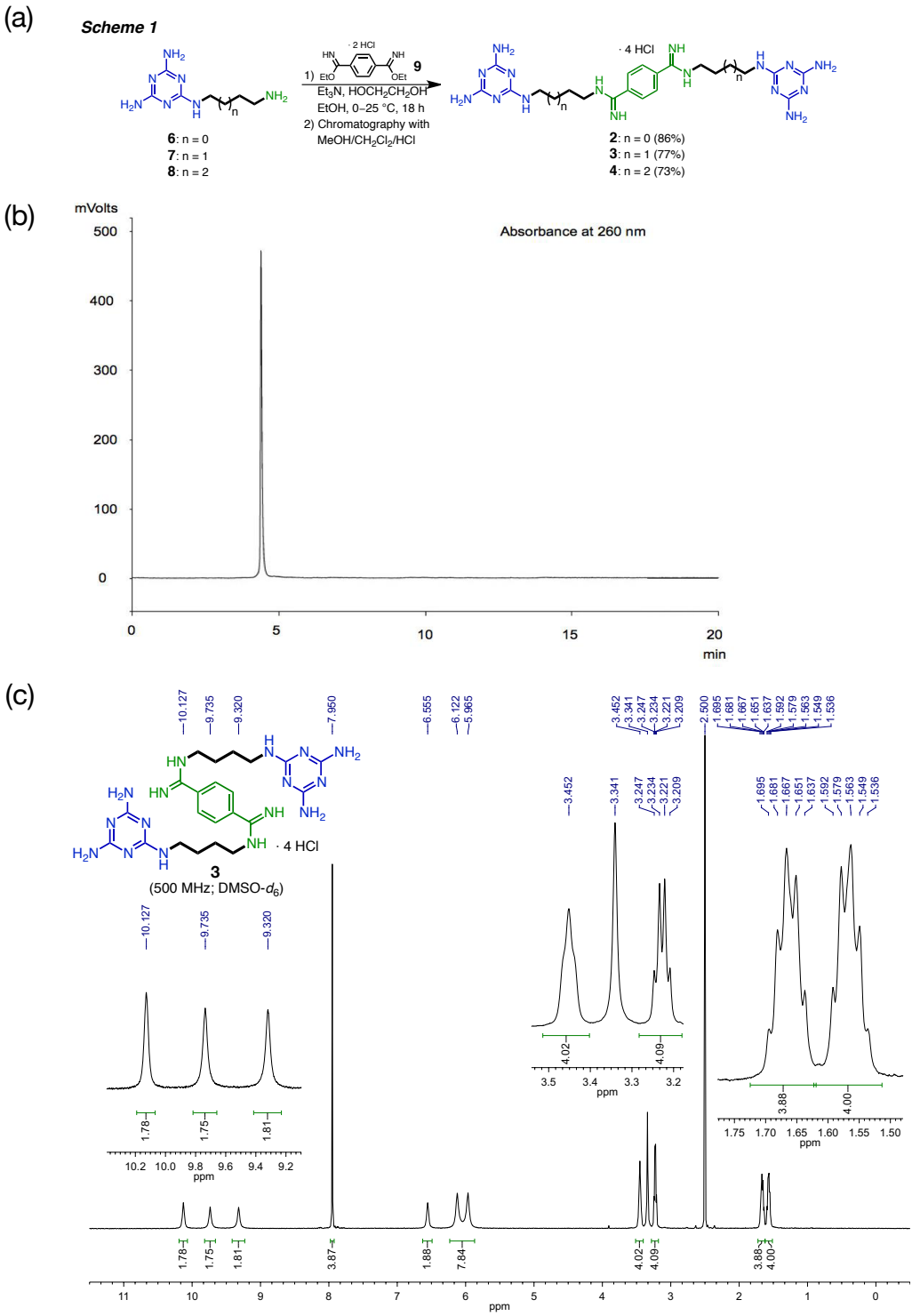


Figure S1. Synthesis and characterization of ligands **2**–**4**. (a) Synthesis of ligands **2**–**4**. (b) HPLC Analysis of ligand **3**. The sample was analyzed with the Dynamax® Ranin system equipped with a binary pump and an absorbance detector set at 260 nm. The LC separation was performed on a Grace Vydac C18 monomeric 120A column (250×10 mm) with 50% mobile phase A (0.1% TFA in water) and 50% mobile phase B (0.1% TFA in methanol) for 20 min. The flow rate was 3 mL/min. The relative peak area in the UV chromatogram was used to determine the purity of the compounds. The purity of **3** was ≥99%. (c) ^{1}H NMR of ligand **3**.

Ligand 2

A 25-mL, two-necked, round-bottomed flask equipped with a magnetic stir bar containing a white suspension of 0.124 g (0.423 mmol) of compound **9** in 10 mL of anhydrous EtOH is cooled in an ice-water bath under a nitrogen atmosphere. To the suspension was added 0.30 mL (1.722 mmol) of DIPEA to produce a colorless clear solution. A solution of 0.175 g (0.953 mmol) of compound **6** in 5 mL of ethanol was added dropwise using a pipette. The reaction mixture was allowed to warm to 25 °C slowly and stirred for 18 h. The reaction was monitor by TLC (*n*-BuOH:AcOH:H_2O = 3:1:4; R_f = 0.15). The white suspension was filtered and washed with EtOH (20 mL). The white solid was dissolved in 15 mL of 1:9 (v/v) H_2O/MeOH mixture and chromatographed on short silica gel (~200 mL) column starting with CH_2Cl_2:*n*-BuOH:MeOH mixture (gradient from 1:5:4 to 1:3:6). Once the impurities were removed, the eluent was acidified with a gradient of 0.10–0.15 mL of 4 M HCl in dioxane (per liter of eluent) to afford the desired product. The product-containing fractions were combined, filtered, and concentrated *in vacuo* to afford 0.180 g (86%) of product as a white tetra·HCl salt. R_f(AcOH:H_2O:MeOH = 3:6:1) = 0.25. ^{1}H NMR (DMSO-d_6): 10.20 (s, 2 H, NH), 9.76 (s, 2 H, NH), 9.36 (s, 2 H, NH), 7.99 (s, 4 H, ArH), 7.34 (br s, 2 H, Het-NH), 6.77 (br s, 8 H, Het-NH), 3.49 (br s, 4 H, N=CNHCH_2), 3.32 (overlap with H_2O, 4 H, Het-NHCH_2), 1.89 (q, 4 H, $\mathcal{J}$ = 10 Hz, CH_2). ESI-MS $[M+H]^+$: 10% $[M+2H]^{2+}$: 100%.

Ligand 4

A 25-mL, two-necked, round-bottomed flask equipped with a magnetic stir bar containing a white suspension of 0.435 g (1.48 mmol) of compound **9** in 10 mL of anhydrous EtOH is cooled in an ice-water bath under a nitrogen atmosphere. To the suspension was added 0.60 mL (3.44 mmol) of DIPEA to produce a colorless clear solution. A solution of 0.658 g (3.11 mmol) of compound **8** in 10 mL of ethanol was added dropwise using a pipette. The reaction mixture was allowed to warm to 25 °C slowly and stirred for 18 h. The reaction was monitor by TLC (*n*-BuOH:AcOH:H_2O = 3:1:4; R_f = 0.1). The white suspension was filtered and washed with EtOH (20 mL). The white solid was dissolved in 15 mL of 1:9 (v/v) H_2O/MeOH mixture and chromatographed on short silica gel (~200 mL) column starting with CH_2Cl_2:*n*-BuOH:MeOH mixture (gradient from 1:5:4 to 1:3:6). Once the impurities were removed, the eluent was acidified with a gradient of 0.10–0.15 mL of 4 M HCl in dioxane (per liter of eluent) to afford the desired

product. The product-containing fractions were combined, filtered, and concentrated *in vacuo* to afford 0.751 g (73%) of product as a white tetra·HCl salt (m.p. > 260 °C (decomp.)). R_f(AcOH:H_2O:MeOH = 3:6:1) = 0.35. ^{1}H NMR (DMSO-d_6): 10.20 (s, 1 H, NH), 9.74 (s, 1 H, NH), 9.36 (s, 1 H, NH), 8.21 (br s, 2 H, Het-NH), 7.97 (s, 4 H, ArH), 7.89 (s, 8 H, Het-NH), 3.45 (q, 4 H, $\mathcal{J}$ = 5 Hz, N=CNHCH_2), 3.29 (q, 4 H, $\mathcal{J}$ = 10 Hz, Het-NHCH_2), 1.68 (q, 4 H, $\mathcal{J}$ = 10 Hz, CH_2), 1.54 (q, 4 H, $\mathcal{J}$ = 10 Hz, CH_2), 1.40 (br s, 4 H, CH_2). HR-ESI-MS for $C_{24}H_{39}N_{16}$: 551.3544 found: 5551.3549. ESI-MS $[M+H]^+$: 30% $[M+2H]^{2+}$: 100%.

Molecular Dynamics (MD) Simulations

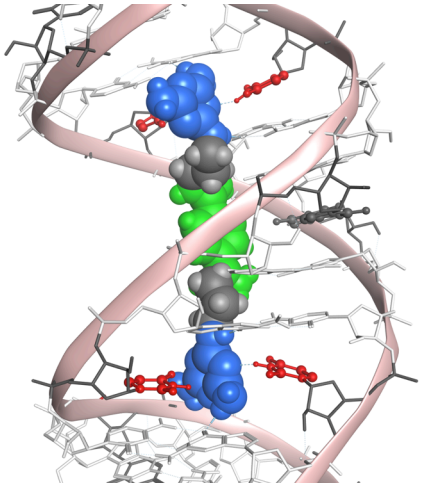


Figure S2. Snapshot of energy minimized structure (Amber) of ligand **2** with r(CUG)$_6$. U-U pairs (in red) interact with the triaminotriazine moieties (in blue). Non-triaminotriazine interacting U-U pairs are in gray.

The structure of r(CUG)$_{15}$ repeat was developed starting from the r(CUG)$_6$ crystal structure published in 2009 (PDB code: 3GM7) (3). Ligands were manually docked into the major of the RNA structure. All graphical manipulations were carried out using Maestro 9.0 (4). All simulations were performed using AMBER, version 10 (5). MD simulations were carried out using the parm99 force field at 300 K. The complex was placed in a rectangular parallelepiped water box. An explicit solvent model for water, TIP3P, was used, and the complex was solvated with a 15 Å water cap. Sodium ions were added as counterions to neutralize the system. Prior to MD simulations, two steps of minimization were carried out. In the first stage, we kept the complex fixed with a position restraint of 500 kcal/(mol·Å^2) and we solely minimized the positions of the water molecules. In the second stage, we minimized the entire system through 5000 steps of steepest descent followed by conjugate gradient (CG) until a convergence of 0.05 kcal/(mol·Å^2) was attained. Particle mesh Ewald (PME) electrostatics and periodic boundary conditions were used in the simulation (6). The MD trajectory was run using the minimized structure as the starting conformation. The time step of the simulations was 2.0 fs with a cutoff of 10 Å for the nonbonded interaction, and SHAKE was employed to keep all bonds involving hydrogen atoms rigid. Constant-volume periodic boundary MD was carried out for 500 ps, during which the temperature was raised from 0 to 300 K. Then 9.5 ns of constant pressure periodic boundary MD was carried out at 300 K using the Langevin thermostat to maintain constant the temperature of our system. In the first 1.7 ns, all the P atoms of the nucleic acid were blocked with a harmonic force constant, which decreased during these 1.7 ns from 10 to 1 kcal/(mol·Å^2), while in the last 7.3 ns, no constraints were applied. General AMBER force field (GAFF) parameters were assigned to the ligand, while partial charges were calculated using the AM1-BCC method as implemented in the

Antechamber suite of AMBER 10. The final structure of the complexes was obtained as the average of the last 7.3 ns of MD minimized by the CG method until a convergence of 0.05 kcal/(mol·Å^2). The average structures were obtained using the ptraj program implemented in AMBER 10.

Preparation of RNA for Isothermal Titration Calorimetry (ITC)

RNA oligonucleotides were purchased from Dharmacon RNAi Technologies, Inc. with 2'-deprotection, desalting, and HPLC purification. The RNA oligonucleotides were dissolved in Ambion RNA Storage Solution (pH 6.4) to give 1–2 mM stock solutions. DNA sequences were dissolved in TE buffer (pH 7.6) to give 1–2 mM stock solutions. The oligonucleotide concentration was determined by its absorbance at λ_{max} = 260 nm at 25 °C on a Shimadzu UV-2501PC spectrophotometer. The concentration of each single-stranded sequence was calculated using Beer's law with extinction coefficient (ε_{260}) provided by the supplier. RNA and DNA duplexes were freshly prepared by mixing the required volumes of the corresponding ssRNA and ssDNA, respectively. The solution was annealed by heating in a water bath at 95 °C for 2 min and slow cooling to 25 °C. Final duplex solutions were prepared by adding MOPS buffer (100 mM; pH 7.0), NaCl solution (5.0 M) and diluting to the required concentrations by adding AccuGENE Molecular Biology Grade Water to give 20 mM MOPS and 300 mM NaCl solutions.

MBNL1 Expression and Purification

An expression vector for a truncated MBNL1 comprised of amino acids 1–272 was obtained from Maurice S. Swanson (University of Florida, College of Medicine, Gainesville, FL). This MBNL1 construct is comprised of the four zinc finger motifs of MBNL1 and a hexahistidine tag (C-terminus) and binds RNA with similar affinity as the full length MBNL1. The protein was expressed and purified as described previously (7). The molecular weight was confirmed by MALDI-TOF mass spectrometry, the concentration was determined by Bradford protein assay (Bio-Rad), and the purity determined by silver-stained SDS-PAGE.

Equilibrium Binding Assays

r(CUG)$_{12}$ RNA was labeled with [γ-^{32}P]-ATP using T4 poly-nucleotide kinase (New England Biolabs) and labeled RNA was purified by phenol extraction and ethanol precipitation. Labeled RNA and unlabeled RNA was heated at 95 °C for 5 min, then placed on ice for 10 min and diluted to protein binding buffer (175 mM NaCl, 5 mM $MgCl_2$, 20 mM Tris-HCl (pH 8), 1.25 mM 2-mercapto-ethanol (BME), 12.5% glycerol, 2mg/mL bovine serum albumin (BSA), 0.1 mg/mL heparin, 0.05% or 0.1% Triton X). For MBNL1-r(CUG)$_{12}$ binding assay, MBNL1 was serially diluted in protein binding buffer and labeled and unlabeled RNAs were added to final concentration of 0.22 nM (unlabeled RNA:labeled RNA = 10:1). The reaction mixture was incubated at room temperature for 20 min and loaded onto a 6% polyacrylamide gel (80:1) at 4 °C. The gel was run for 1.5 h at 150 V in Tris-borate buffer (pH 8). Gels were dried, developed overnight in phosphor cassette and visualized on a Molecular Dynamics Storm PhosphorImager. The K_d (1:1 stoichiometry assumption) was obtained fitting fraction RNA bound versus protein concentration (Prism) using the equation: Fraction RNA bound = $B_{max} \times [MBNL1]_{total}^h / (K_d^h + [MBNL1]_{total}^h)$, where B_{max} is maximum fraction RNA bound, h is Hill slope. Protein concentration in reaction mixture was larger 10-fold excess over RNA concentration. The average K_d and standard deviation were obtained from three independent experiments.

Inhibition Assays

Ligands **3–5** were dissolved in the protein binding buffer with 10% DMSO (v/v) at the highest concentration (3.4 mM) and the resulting stocks were serially diluted. Pentamidine was dissolved in water. *Para*- and *meta*-H1 were dissolved in DMSO. The inhibition assay was performed with the same procedure as in K_d of MBNL1-r(CUG)$_{12}$ binding assay except adding of small molecule to MBNL1-RNA complex. A mixture of RNAs (unlabeled and labeled RNAs with the ratio of 10) and MBNL1 was incubated at room temperature. After 15 min of incubation, the MBNL1-RNA complex solution was added to ligand solution to give final RNA and MBNL1 concentrations of 0.22 nM and 0.1 μM, respectively. The mixture was incubated 15 min and loaded onto a 6% polyacrylamide gel (80:1) at 4 °C. The gel was run for 1.5 h at 150 V in Tris-borate buffer (pH 8). Gels were dried, developed overnight in phosphor cassette and visualized on a Molecular Dynamics Storm PhosphorImager. The fraction RNA bound versus log[compound] was fit (Prism) using the equation: $y = ax^b/(IC_{50}^b + x^b)$ where y =bound RNA fraction, x = ligand concentration, a

= $y_{max} - y_{min}$, b = hill slope. The average IC_{50} and standard deviation were obtained from three independent experiments.

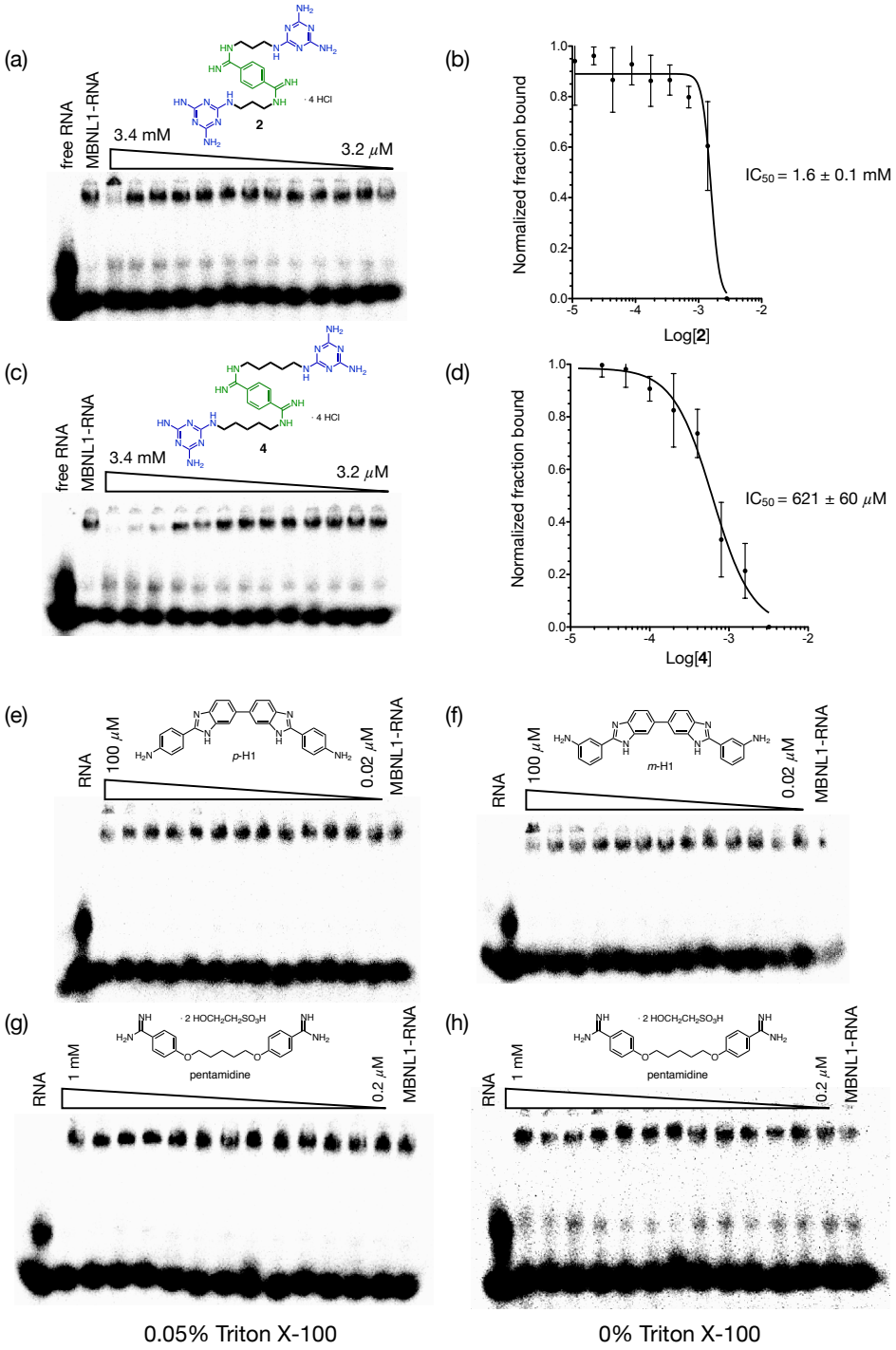
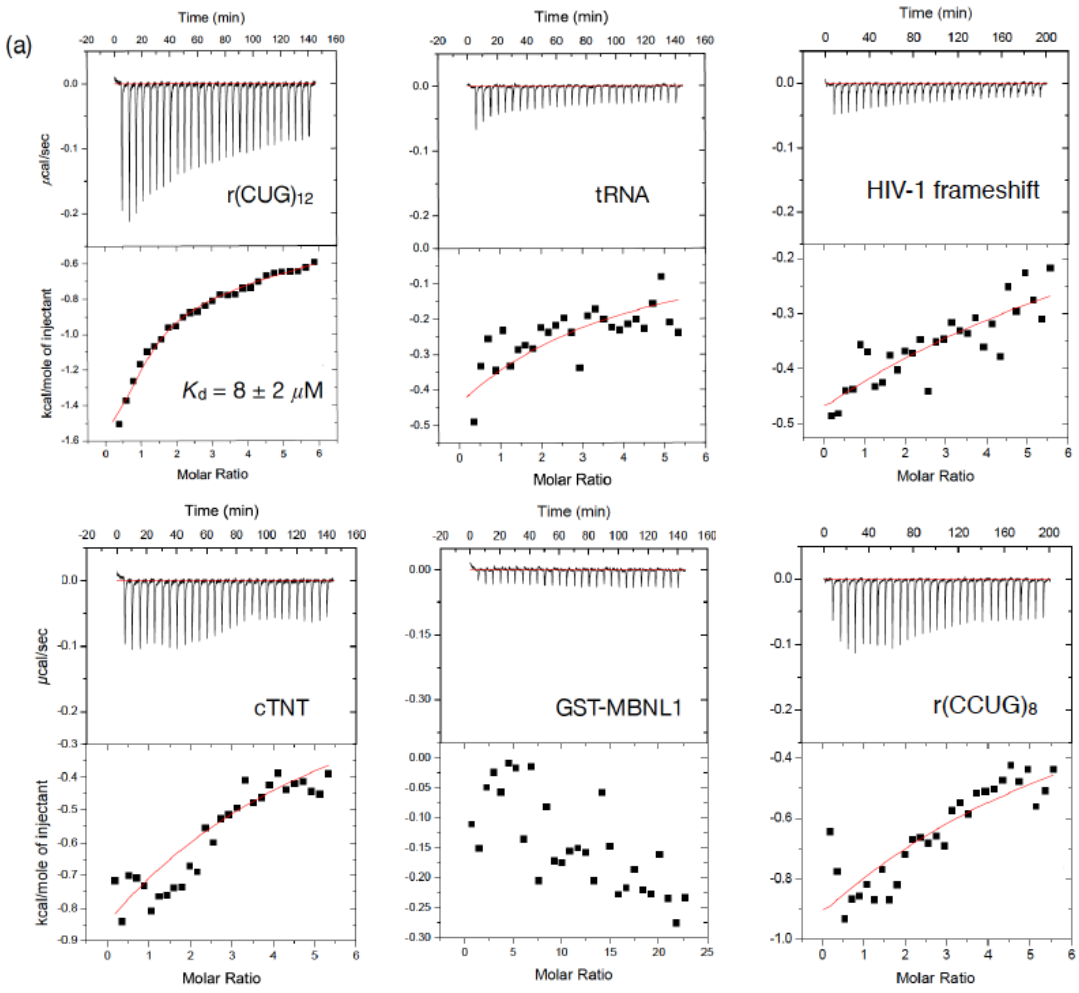


Figure S3. EMSA data for (a) ligand **2** with r(CUG)$_{12}$ RNA and (b) the corresponding IC_{50} curve. EMSA data for (c) ligand **4** with r(CUG)$_{12}$ RNA and (d) the corresponding IC_{50} curve. First lane: RNA only; second lane: MBNL1-RNA complex with 10% DMSO. Error bars indicate SEM of at least three independent measurements. EMSA data of (e) *para*-H1; (f) *meta*-H1 with r(CUG)$_{12}$ RNA; pentamidine with r(CUG)$_{12}$ RNA (g) in the presence of 0.05% Triton X-100 and (h) without Triton X-100. First lane: RNA only; last lane: MBNL1-RNA complex with 10% DMSO. EMSA data

of First lane: RNA only; last lane: MBNL1-RNA complex. Conditions: [MBNL1] = 0.1 μM; [r(CUG)$_{12}$] = 0.22 nM; [Tris·HCl] = 20 mM; pH = 8.

ITC

ITC measurements were performed at 25 °C on a MicroCal VP-ITC calorimeter. A typical experiment consisted of titrating 10 μL of a ligand solution (500 μM) from a 250 μL syringe (stirred at 300 rpm) into a sample cell containing 1.42 mL of a RNA, DNA or GST-MBNL1 solution (10–20 μM) with a total of 30 injections (1 μL for the first injection and 10 μL for the remaining injections). The initial delay prior to the first injection was 60 s. The duration of each injection was 24 s and the delay between injections was 400 s. All experiments were performed in triplicate. Data analysis was carried out with Origin 5.0 software (MicroCal). Binding parameters, such as the dissociation constant (K_d), enthalpy change (ΔH), and entropy change (ΔS), were determined by fitting the experimental binding isotherms with appropriate models. The ligand stock solutions were 50 mM in water. The buffer solutions for ITC experiments contained 20 mM MOPS (pH 7.0) and 300 mM NaCl.



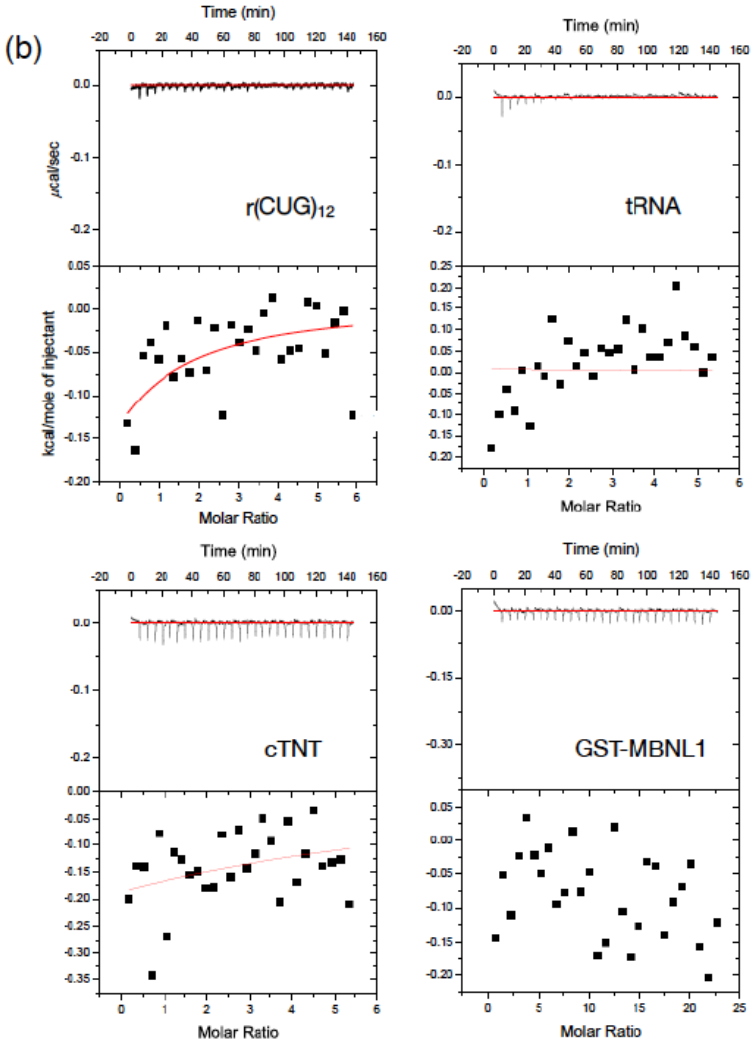


Figure S4. ITC binding studies of (a) ligand **3** with r(CUG)$_{12}$, yeast tRNA, cTNT, GST-MBNL1, HIV-1 frameshift site RNA, and r(CCUG)$_8$; (b) ITC binding studies of ligand **5** (DB213) with r(CUG)$_{12}$, yeast tRNA, cTNT, and GST-MBNL1. When insufficient heat was generated for accurate K_d measurement, an upper limit of the K_d is assigned (>200 μM). Concentration of r(CUG)$_{12}$, tRNA, cTNT RNA and GST-MBNL1 = 10 μM; Concentration of r(CCUG)$_8$, and HIV-1 frameshift RNA = 20 μM; [NaCl] = 300 mM; [MOPS] = 20 mM; pH = 7.

Materials

Wild type cTNT, DT960 and DT0 minigenes were obtained from the lab of Thomas Cooper (Baylor College of Medicine). The insulin receptor (IR) minigene was obtained from the lab of Nicholas Webster (University of California, San Diego).

Confocal Microscopy

Approximate 120,000 HeLa cells were plated in each well of a 6-well plate onto coverslips maintained in complete growth media [DMEM (4.5g/L glucose) supplemented with L-glutamine and 10% FBS (Gemini)]. Cells were transfected at 70–80% confluence with 1 μg of DT960 plasmid with Lipofectamine 2000 (Invitrogen, Life Technologies) according to the manufacturer's protocol. After 4 h, the transfection media was removed and the compound was added to the complete growth media if being assayed. For time course experiments, cells were treated with 100 μM **3** or **5** for 24 h, 48 h, or 72 h. For dose dependence experiments, cells were treated with **3** at concentrations of 5, 10, 20, 35, 50, 75 and 100 μM, for 48 h.

Cells were fixed for 10 min at room temperature with 4% PFA and washed 5 times for 10 min in 1X PBS at room temperature. Cells were stored at 4 °C if not probed immediately. For the fluorescence in situ hybridization (FISH) procedure, cells were permeabilized with 0.5% triton X-100 in 1X PBS at room temperature for 5 min. Cells were then prewashed with 30% formamide, 2X SSC for 10 min at room temperature. Cells were then probed for 2 h at 37 °C, with 1 ng/μL of Cy3-CAG_{10} probe (Integrated DNA Technologies) in 30% formamide (Fisher Scientific), 2X SSC, 20 μg/mL BSA, 66 μg/mL yeast tRNA (Sigma-Aldrich). Cells were then washed for 30 min in 30% formamide, 2X SSC at 37 °C, and then with 1X SSC for 30 min at room temperature.

For immunofluorescent (IF) staining, cells were washed twice in 1X PBS, 10 min at room temperature , and then probed overnight at 4 °C with anti-MBNL1 antibody (1:5000 dilution, clone 3A4 antibody, Millipore) in 1X PBS. Cells were washed 2 times for 10 min at room temperature with 1X PBS. Next, cells were incubated with goat anti-mouse AlexaFluor 488 antibody (1:500 dilution, Invitrogen) for 2 h at room temperature. Cells were then washed 2 times for 10 min at room temperature with 1X PBS and then treated with 10 μg/mL Hoechst 33342 (Sigma-Aldrich) for 5 min. Finally, cells were washed with 1X PBS for 5 min and mounted onto glass slides with fluorescent mounting medium (Dako) before sealing with nail polish. Cells were imaged on Zeiss confocal laser scanning microscope (LSM) 700 at 20× or 40× magnification.

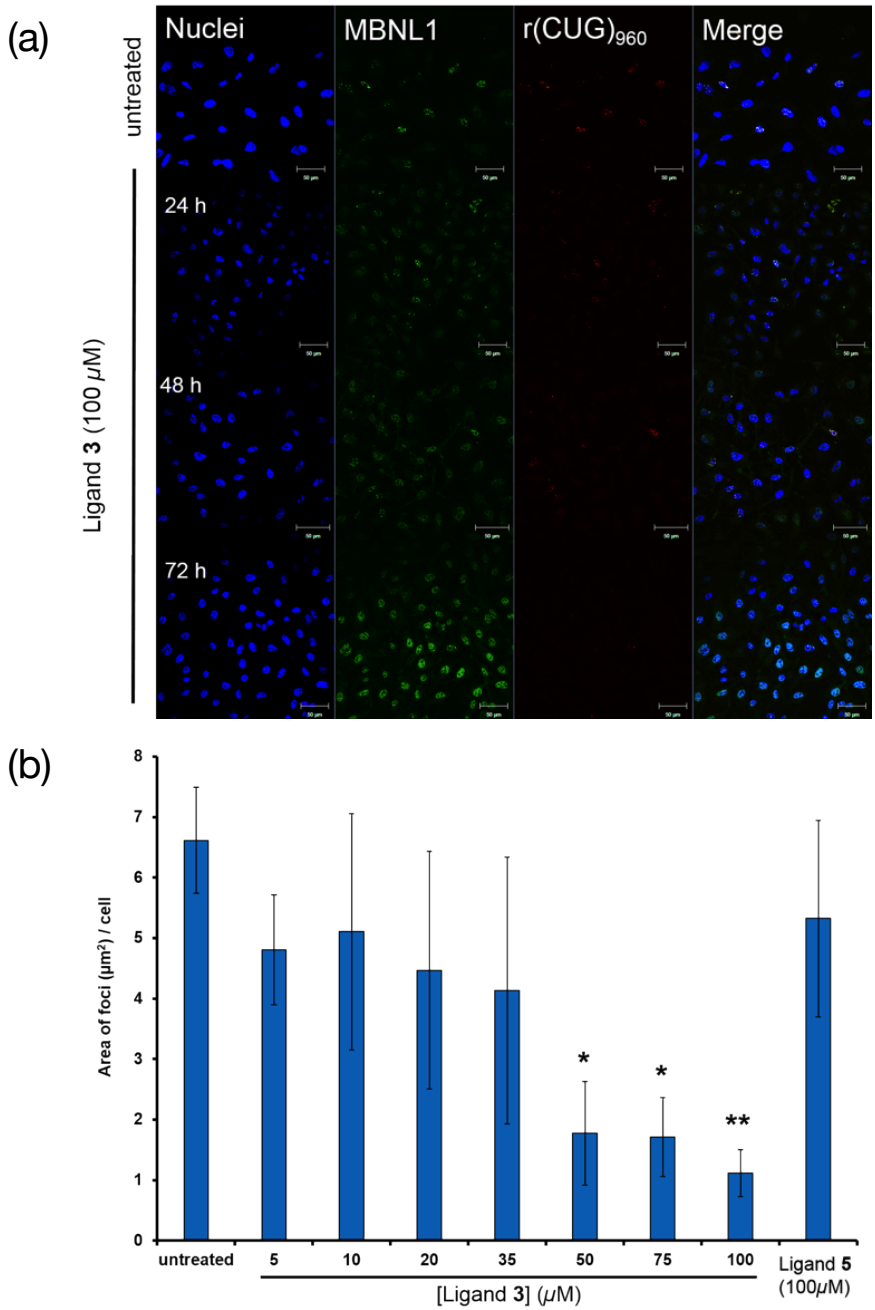


Figure S5. Effect of ligand 3 in dissolving MBNL1-r(CUG)^{exp} foci in the DM1 cell culture model. (a) Wide field confocal images of DT960-transfected HeLa cells treated with ligand **3** in a time dependent manner. Images acquired at 20× magnification. MBNL1 was visualized using mouse anti-MBNL1 and goat anti-mouse AlaxaFluor488 antibodies; r(CUG)$_{960}$ was imaged using FISH with 1 ng/μL Cy3-(CAG)$_{10}$. Nuclei were stained with 10 μg/mL Hoechst 33342. Scale bar = 50 μm. (b) Quantification of ribonuclear foci area in DT960-transfected HeLa cells treated with varying concentrations of ligand **3** and 100 μM of ligand **5** for 48 h. Values are expressed as a ratio of ribonuclear foci area (μm^2) to the total number of cells analysed in three independent experiments. Error bars are standard error of mean. (* $p < 0.05$; ** $p < 0.01$)

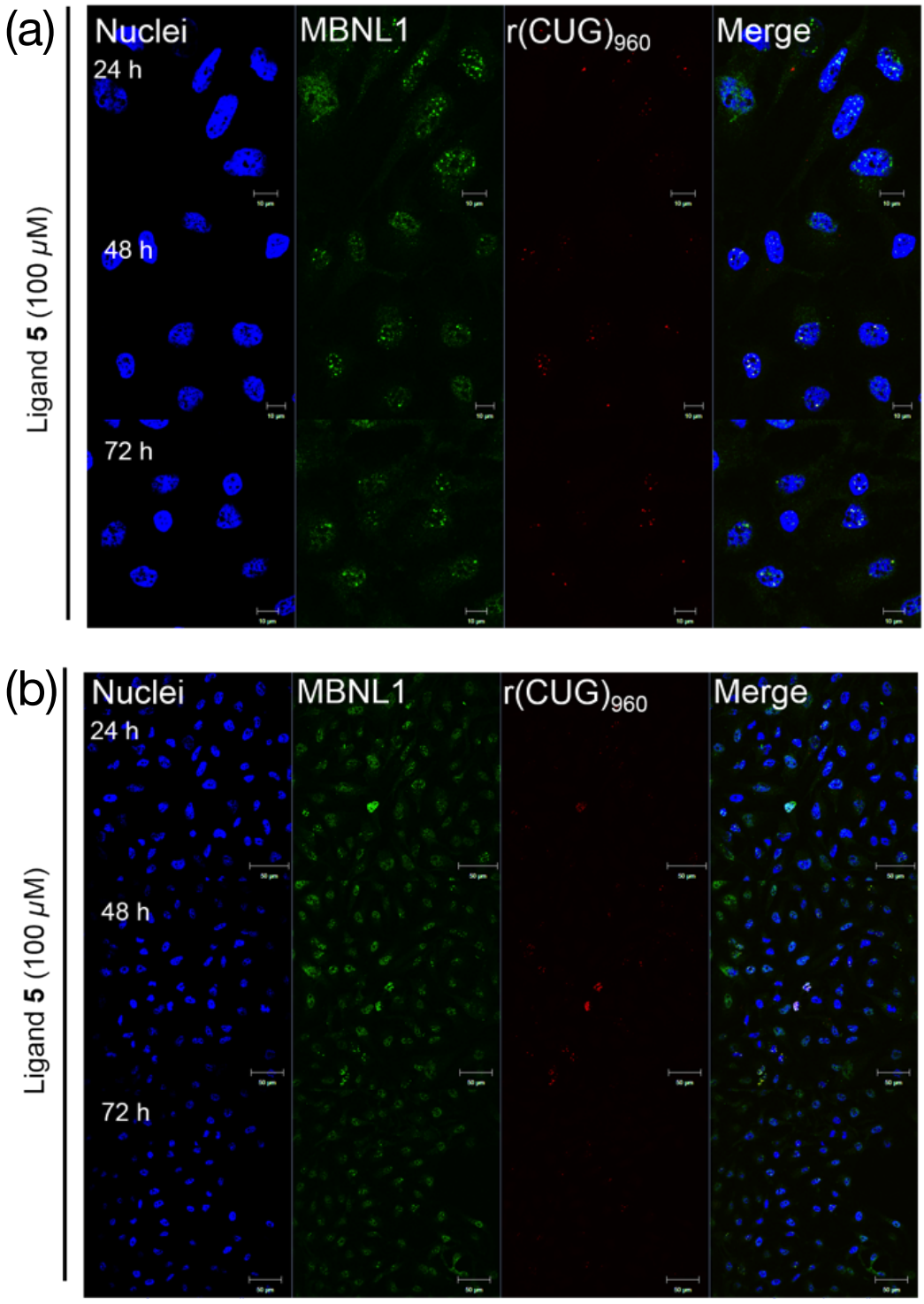


Figure S6. Ligand **5** does not disrupt ribonuclear foci in DT960-transfected HeLa cells following treatment at up to 72 h. Images are acquired at (a) 40× magnification. Scale bar = 10 μm. (b) 20× magnification. MBNL1 was visualized using mouse anti-MBNL1 and goat anti-mouse AlaxaFluor488 antibodies; $r(CUG)_{960}$ was imaged using FISH with 1 ng/μL Cy3-$(CAG)_{10}$. Nuclei were stained with 10 μg/mL Hoechst 33342. Scale bar = 50 μm.

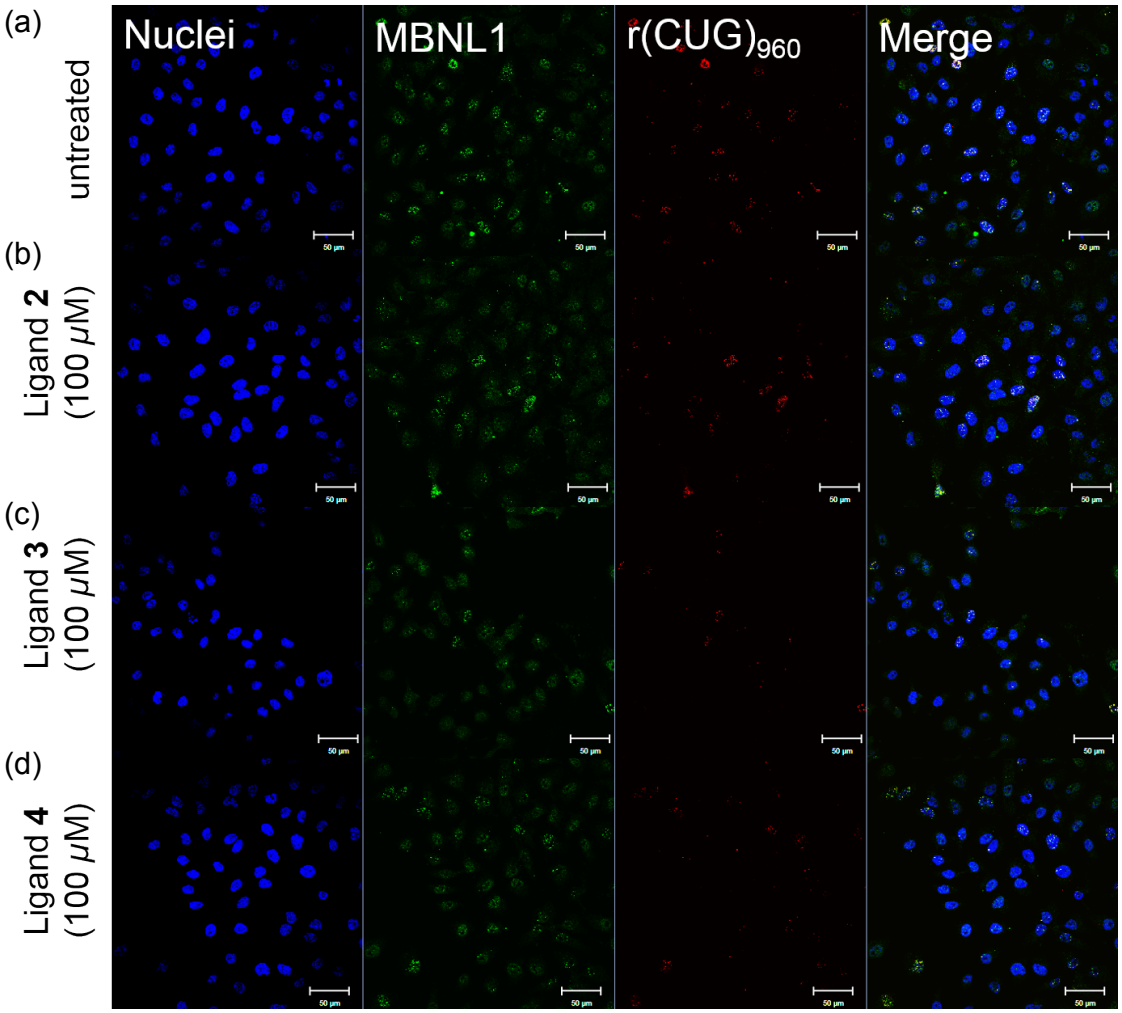


Figure S7. Head-to-head comparison of ligands **2**–**4**. Confocal images of (a) untreated DT960-transfected HeLa cells. Treatment of DT960-transfected HeLa cells with (b) 100 μM of ligand **2**, (c) ligand **3** and (d) ligand **4** for 48 h. MBNL1 was visualized using mouse anti-MBNL1 and goat anti-mouse AlaxaFluor488 antibodies; r(CUG)$_{960}$ was imaged using FISH with 1 ng/μL Cy3-(CAG)$_{10}$. Nuclei were stained with 10 μg/mL Hoechst 33342. Images acquired at 20X magnification. Scale bar = 50 μm.

Splicing assays

Approximately 120,000 HeLa cells were seeded in each well of a 6-well plate in complete growth media the day before transfection. In assaying the cTNT pre-mRNA, cells were transfected the following day at 70–80% confluence with 500 ng each of wild type cTNT and DT960 or DT0 minigenes with Lipofectamine 2000 (Invitrogen, Life Technologies) according to the manufacturer's protocol. For testing the IR pre-mRNA, cells were similarly transfected the following day at 70–80% confluence with 500 ng each of IR and DT960 or DT0 minigenes with Lipofectamine 2000 (Invitrogen, Life Technologies) according to the manufacturer's protocol.

After 4 h, the transfection media was removed and **3**, **5**, or pentamidine was added to the complete growth media if being assayed. Cells treated with 75 μM of pentamidine were harvested 16–20 h post-treatment because of observed toxicity. For time course experiments, cells were treated with 100 μM of **3** or **5**, harvested after 24, 48, or 72 h for **3** and after 72 h for **5**. For dose dependence experiments with cTNT minigene, cells were treated with **4** at concentrations of 25, 50, 75 and 100 μM, harvested after 72 h. For the IR minigene, cells were treated with **3** at concentrations of 20, 50, 75 and 100 μM, harvested after 72 h.

To harvest the cells, cells were washed once with 1X PBS and detached using trypsin with 0.05% EDTA (Fisher Mediatech). Following harvesting, RNA was immediately isolated using Total RNA kit I (Omega Bio-Tek). 900 ng of isolated RNA was reverse transcribed with iScript cDNA synthesis kit (Bio-Rad) according the manufacturer's protocol. The reverse transcription reaction was cleaned up using QIAquick PCR purification kit (Qiagen).

For the cTNT minigene, 50 ng of cDNA was subjected to 30 cycles of PCR amplification using gene specific primers. The forward primer was 5'-GTT CAC AAC CAT CTA AAG CAA GAT, and the reverse primer was 5'-GTT GCA TGG CTG GTG CA. For the IR minigene, 70 ng of cDNA was subjected to 31–35 cycles of PCR amplification using gene specific primers. The forward primer was 5'-GTA CCA GCT TGA ATG CTG CTC CT, and the reverse primer was 5'-CTC GAG CGT GGG CAC GCT. The linear range for PCR for the cTNT and IR constructs was found to be between 25–35 cycles. The PCR products were ran on an 8% polyacrylamide gel with 1X TBE (National Diagnostics) at 120 V for 55 min. The gel was post stained with ethidium bromide and subsequently imaged using Gel Doc XR+ system (Bio-Rad). The bands were quantified using ImageJ (NIH).

Drosophila Genetics

Flies were raised at 25°C on standard corn meal medium supplemented with dry yeast. Fly lines bearing *UAS-(CTG)$_{60}$* and *UAS-(CTG)$_{480}$* (9) were kind gift of Prof. Rubén Artero Allepuz (Universitat de València, Estudi General, Spain). *UAS-DsRed-CAG$_{100}$* (10) and *UAS-EGFP-CGG$_{90}$* (11) fly lines were obtained from Profs. Nancy Bonini (University of Pennsylvania, USA) and Stephen Warren (Emory University, USA), respectively.

Drug Treatment in Drosophila

Ligands 2 and 4 were dissolved in water and mixed with fly food. Final concentrations of the compounds were either at 200, 400, and 800 μM. Genetic crosses were set up in drug-containing fly food, and progeny flies of the correct genotypes were analyzed by light and scanning electron microscope analyses.

Examination of Adult Fly Eyes by Microscopies

Light microscopic examination was performed on an Olympus SZX-12 stereomicroscope (12). Eye images were captured using a SPOT Insight CCD camera (Diagnostic Instruments). Scanning electron microscopy (SEM) was performed according to a previously reported procedure (13). In brief, 2–3-day-old adult fly heads were fixed in 2.5% glutaraldehyde (EM grade, Electron Microscopy Sciences) in phosphate buffer (pH 7.4) for 4 h, then post-fixed with 1% osmium tetroxide (Electron Microscopy Sciences), dehydrated to 100% ethanol and critical-point dried with liquid CO_2. Gold–palladium-coated specimens were examined with a JEOL JSM-6301FE microscope operated at 5 kV.

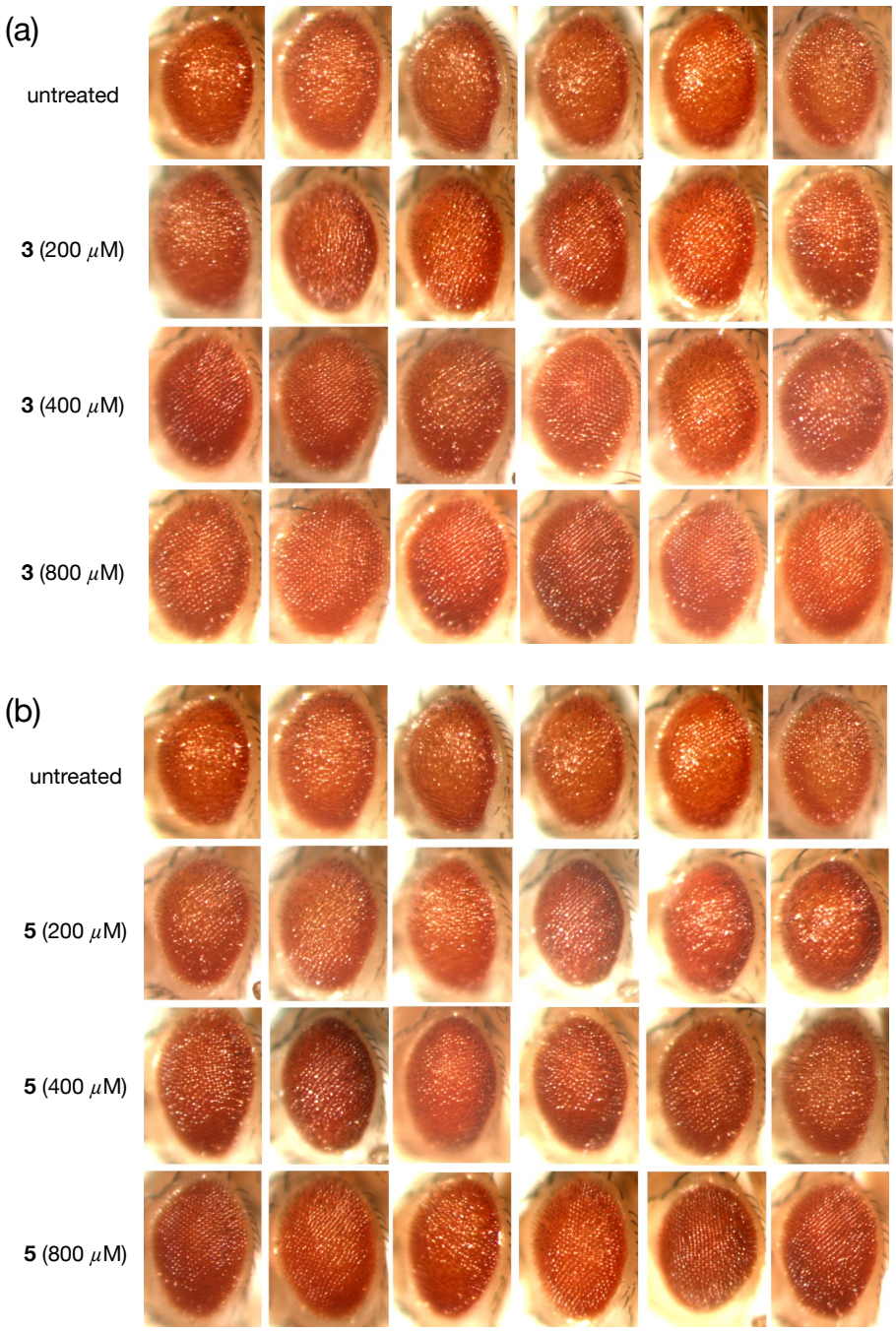


Figure S8. Additional light microscope images of *Drosophila* expressing *i*$(CUG)_{480}$ treated with (a) ligand **3** and (b) ligand **5**.

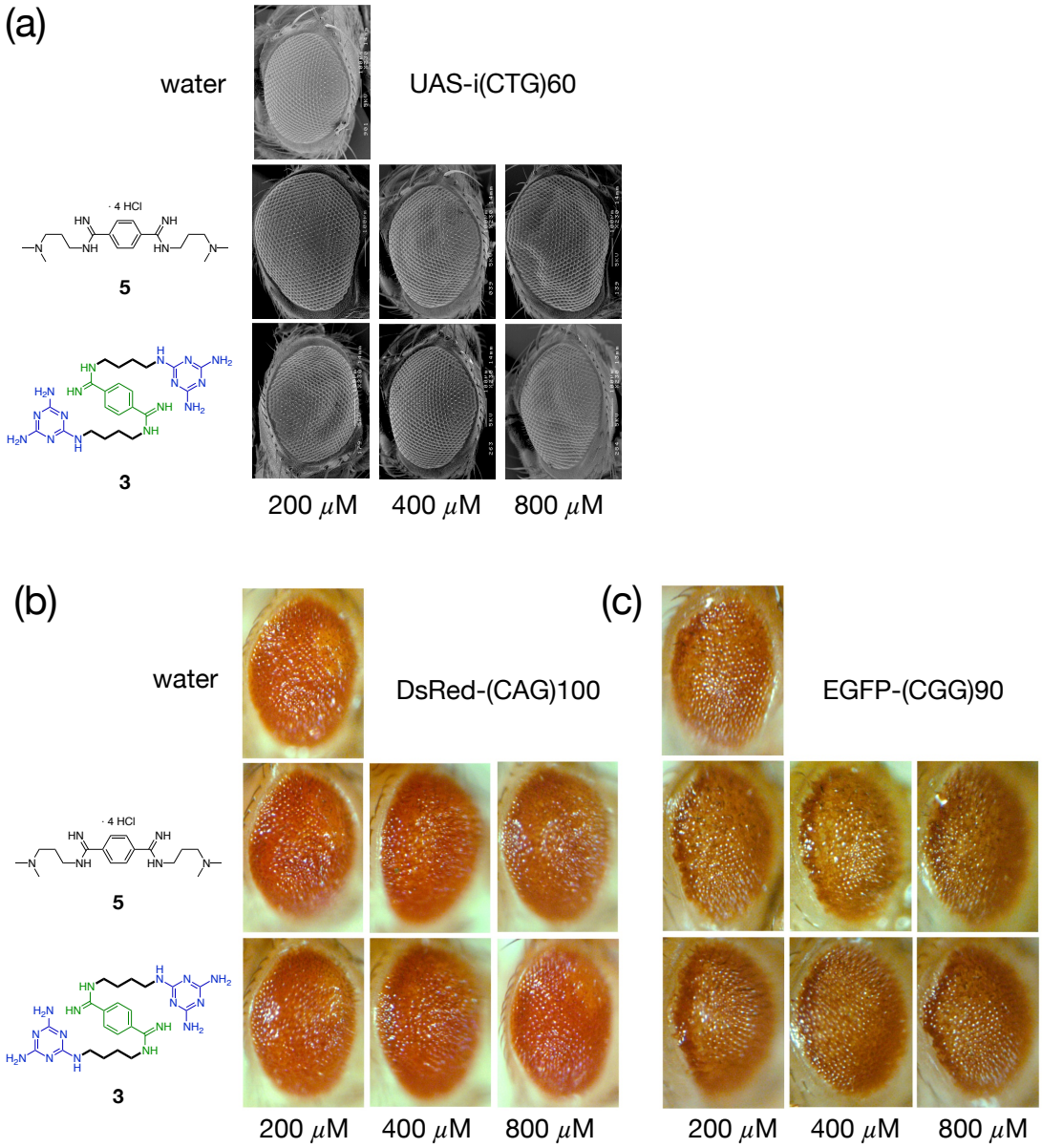


Figure S9. Effect of ligands **3** and **5** on *i*(CUG)$_{60}$ flies and non-DM1 flies. (a) SEM images of *Drosophila* expressing *i*(CUG)$_{60}$ treated with ligands **3** and **5**. Ligands **3** and **5** did not show deleterious effects in the control flies expressing shorter CUG repeats. Light microscope images of *Drosophila* expressing (b) *DsRed-(CAG)$_{100}$* or (a) *EGFP-(CGG)$_{90}$* treated with ligands **3** and **5**.

SRB Toxicity Assay

The SRB assay was performed according to the method of Vichai and Kirtikara (8). DMEM supplemented with 10% FBS (98 μL) was placed in a 96-well plate. Ligand solution (2 μL) was added to give final concentrations from 5 nM to 100 μM, five repeats for each concentration. HeLa cells (10,000 cells/well), DM1 fibroblast cells (GM03132, ATCC) (10,000 cells/well), 3T3 cells (ATCC) (10,000 cells/well) were then plated in the 96-well plate. The cells were incubated at 37 °C. After 72 h, 100 μL of cold 10% (w/v) trichloroacetic acid were added in each well, followed by incubation at 4 °C for 1 h. The cells were washed twice with tap water and then air-dried. The cells were stained with 100 μL of 0.0057% (w/v) sulforhodamine B in 1% acetic acid at room temperature, 30 min. The plate was rinsed twice with 1% (v/v) acetic acid to remove the unbound stain. The bound protein stain was solubilized in 200 μL of 10 mM Tris base, pH 10.5 and left for 30 min. The optical intensity was measured at 510 nm using microplate reader (SPECTRAmax PLUS). Percentage of dead cells was calculated using the following formula:

$$\%\ \text{cell dead} = 100\% - \frac{\text{Mean}_{\text{OD sample}} - \text{Mean}_{\text{OD dead control}}}{\text{Mean}_{\text{OD live control}} - \text{Mean}_{\text{OD dead control}}} \times 100\%$$

The data was plotted using Excel and curves were fitted using Table Curve (Systat) to estimate the IC_{50} values. The cytotoxicity experiment was triplicated for HeLa cells.

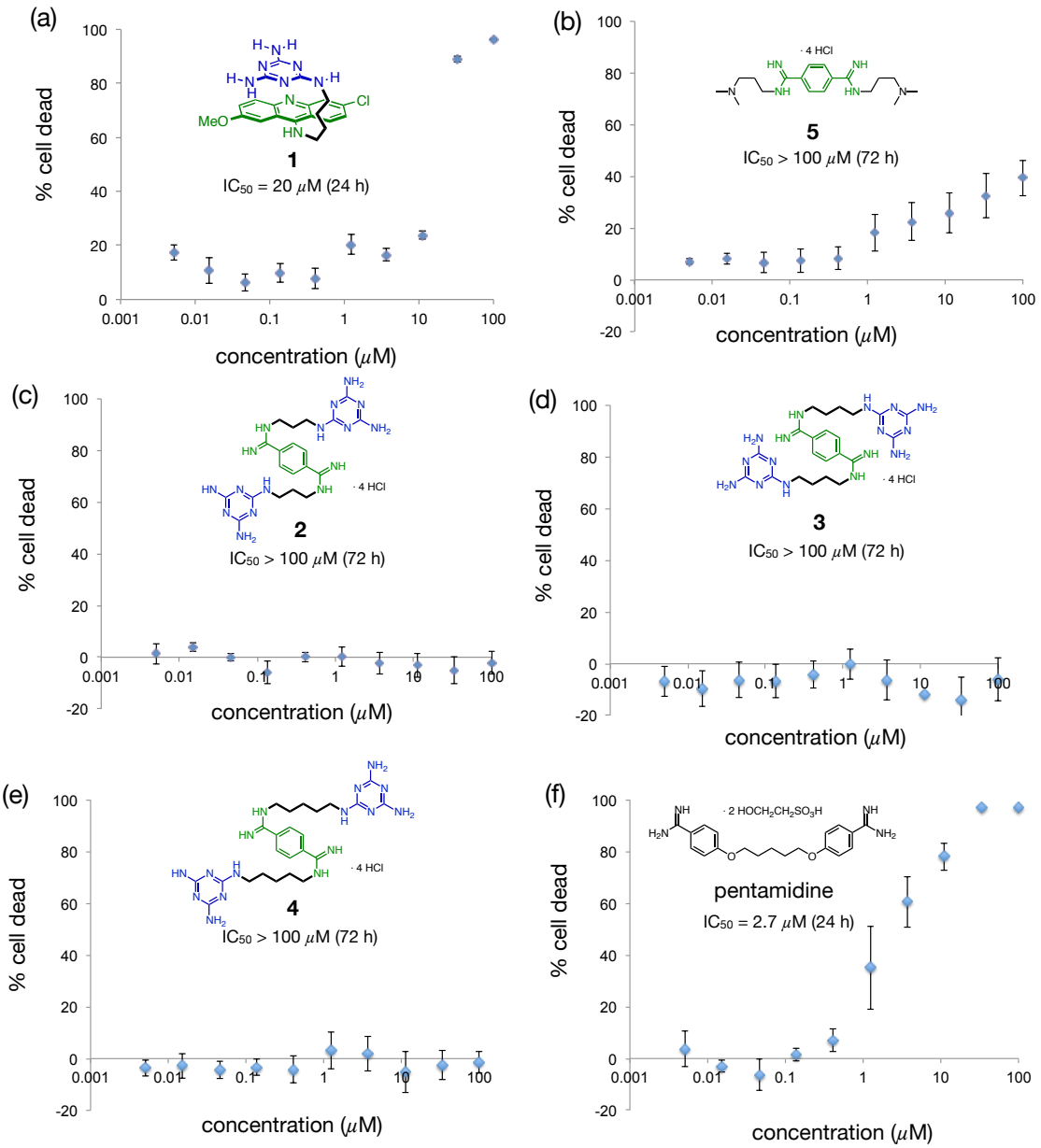


Figure S10. Evaluation of the toxicity of various compounds to HeLa cells. a) Ligand **1** was incubated with cells for 24 h, (b-e) ligands **2-5** were incubated with HeLa cells for 72 h, and f) pentamidine was incubate with HeLa cells for 24 h. Cell death was assessed using sulforhodamine B (SRB) (8), error bars represent standard deviations of three independent experiments

Supporting References

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