Supporting Information For:

A Small Molecule that Targets r(CGG)^{exp} and Improves Defects in Fragile X-Associated Tremor Ataxia Syndrome

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1.) IC_{50} Curve for displacement of DGCR8Δ from $r(CGG)_{12}$ by **1a** and **1b**

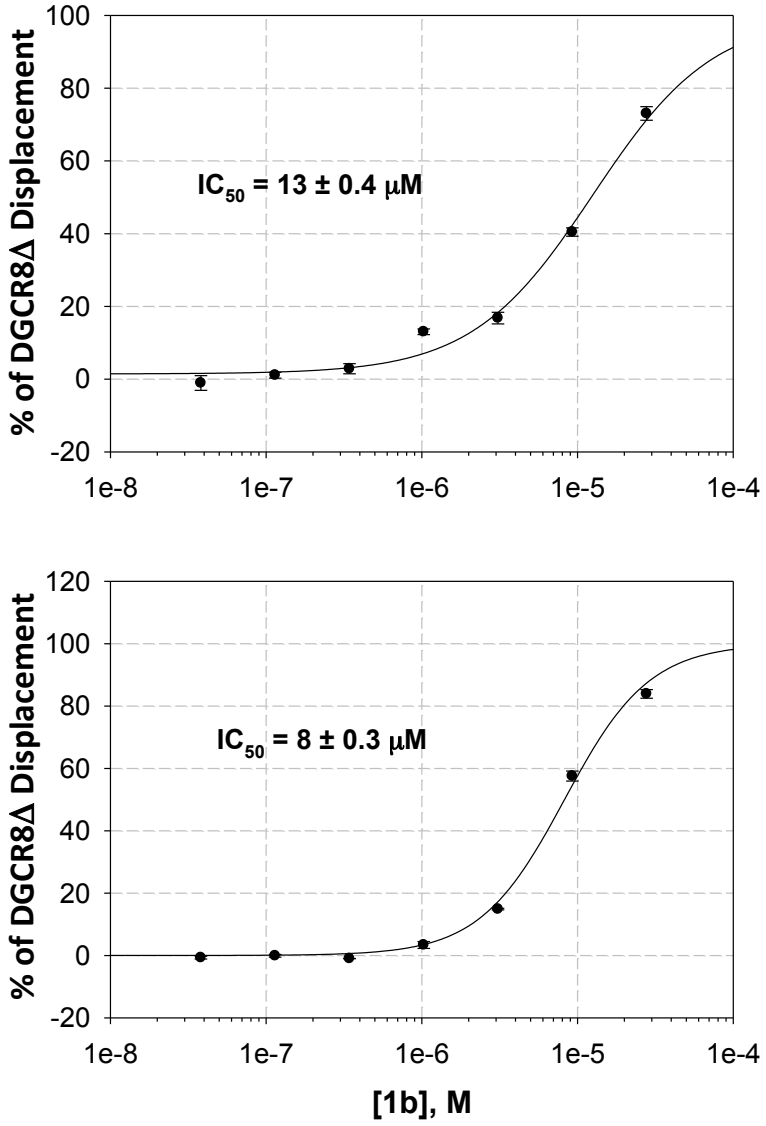

Figure S-1: Representative IC_{50} curves for the displacement of DGCR8Δ from $r(CGG)_{12}$ by **1a** and **1b** The IC_{50}'s were determined by curve fitting to the following equation:

$$y = B + \frac{A-B}{1+\left(\frac{IC50}{x}\right)^{hillslope}}$$

where y is the percentage of DGCR8Δ displaced, x is the concentration of ligand, B is the minimum percentage of protein displaced (typically 0), A is the maximum amount of protein displaced (typically 100), and the IC_{50} is the concentration of ligand where half of the protein is displaced from the RNA.

2.) Affinity of DGCR8Δ for r(CGG) repeats

Affinity of DGCR8Δ for Various RNAs Via Gel Mobility Shift Assays. Prior to screening the RNA-focused library for inhibition of the r(CGG)$_{12}$-DGCR8Δ complex, a gel mobility shift assay was used to determine the affinity of the protein for various RNAs. Briefly, the RNAs were radioactively labeled by *in vitro* transcription and [α-^{32}P] ATP as previously described (*1*). The RNAs were folded by incubating the samples at 60°C in 1X Gel Mobility Shift Buffer (50 mM Tris-HCl, pH 8.0, 75 mM NaCl, 37.5 mM KCl, 1 mM $MgCl_2$, 5.25 mM DTT, and 0.1 mg/mL yeast tRNA) excluding the 1 mM $MgCl_2$ followed by slow cooling on the bench top. Then, $MgCl_2$ was added to a final concentration of 1 mM and increasing amounts of DGCR8Δ were added in a total volume of 10 µL. The samples were incubated at room temperature for 30 min, and then 2 µL of 6× Loading Buffer (40% glycerol, 0.125% Bromophenol Blue, and 0.125% Xylene Cyanol) was added. A 10 µL aliquot of the solution was loaded on a 8 % polyacrylamide (80:1 mono/bis) gel pre-chilled in ice water. The gel was run in 1× TBE for 30 min at 10 V/cm at 0 °C, and subsequently dried and exposed to a phosphorimager screen. The gel was imaged using a Typhoon phosphorimager. Protein-RNA binding curves were fit to the following equation:

$$y = \frac{x\, B_{max}}{k_d - x} \qquad (1)$$

where y is percentage of bounded DGCR8Δ, x is the concentration of protein, B_{max} is maximum percentage of protein bound (restrained to equal 100%), and k_d is dissociation constant, which is approximately equal to protein concentration where 50% of maximum binding is achieved.

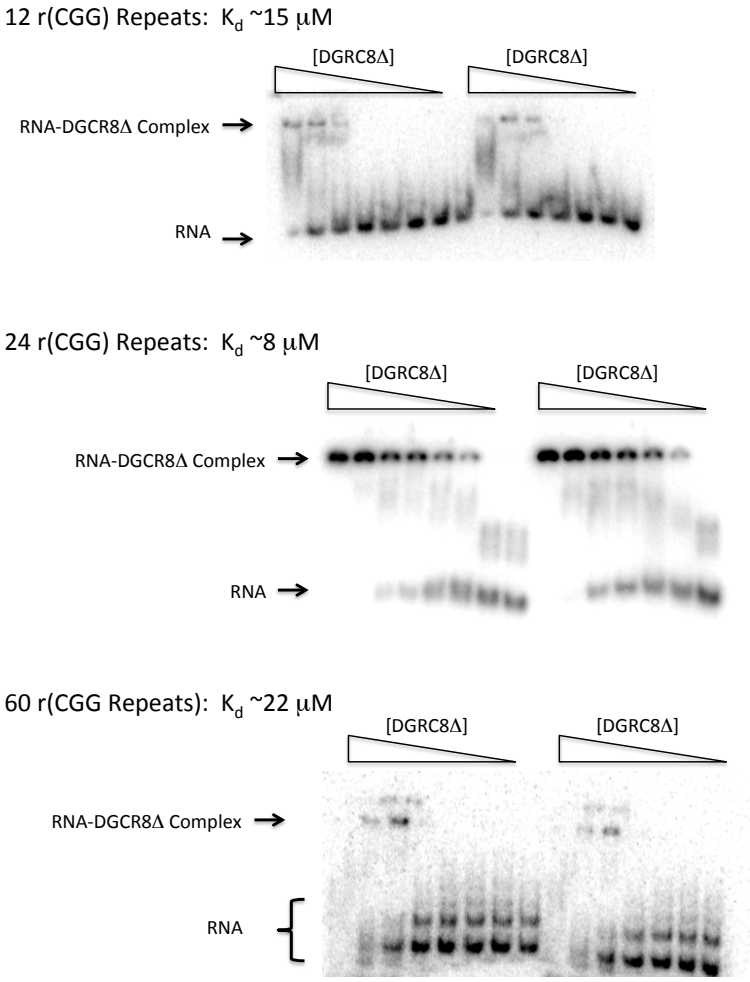

Figure S-2: DGCR8Δ binds to RNAs with different numbers of r(CGG) repeats similarly as determined by a gel mobility shift assay.

3.) Affinities of **1a** and **1b** for an RNA containing one 5'CGG/3'GGC motif

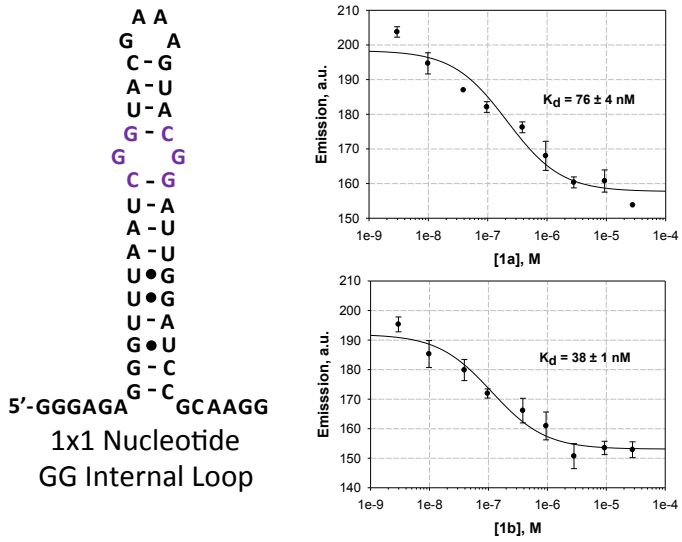

Figure S-3: Ligands **1a** - **1d** (curves for **1a** and **1b** shown) were studied for binding to an RNA with a 1x1 nucleotide GG internal loop via a competitive binding assay. Hoechst 33258 was used as the fluorescent indicator, as it has been previously shown to bind to a variety of RNA 1x1 nucleotide internal loops, including GG internal loops (*2*). The 1x1 nucleotide GG internal loop RNA (1 μM) was complexed with an equimolar amount of Hoechst 33258 (1 μM) and aliquots of a small molecule (**1a**-**1d**) were added and allowed to equilibrate for 5 min. Then, the reduction in fluorescence of Hoechst 33258 was measured using a Bio-Tek FLX-800 plate reader as a function of ligand concentration (**1a – 1d**) and was fit to the following equation (*3*):

$$\Theta = \frac{1}{2[Ht]_0}\left[K_t + \frac{K_t}{K_d}[C_t]_0 + [RNA]_0 + [Ht]_0 - \sqrt{\left(K_t + \frac{K_t}{K_d}[C_t]_0 + [RNA]_0 + [Ht]_0\right)^2 - 4[Ht]_0[RNA]_0}\right] + A \quad (eq.4)$$

where Θ is the fraction bound of Hoechst 33258, K_t is the dissociation constant for Hoechst 33258, K_d is the dissociation constant of the competing ligand, $[Ht]_0$ is the total concentration of the Hoechst 33258, $[C_t]_0$ is the total concentration of the competing ligand, A is the fraction bound of Hoechst 33258 at infinite concentration of the competing ligand, and $[RNA]_0$ is the total concentration of RNA.

4.) Table of RT-PCR Primers

Table S-1: Primer sets used for RT-PCR analysis of alternative splicing.

Gene	Forward Primer	Reverse Primer
SMN2 mini-gene	5’ GGT GTC CAC TCC CAG TTC AA	5’ GCC TCA CCA CCG TGC TGG
Bcl-x mini-gene	5’ GGA GCT GGT GGT TGA CTT TCT	5’ TAG AAG GCA CAG TCG AGG
cTNT mini-gene	5’ GTT CAC AAC CAT CTA AAG CAA GAT G	5’ GTT GCA TGG CTG GTG CAG G
PLEKHH2 mini-gene	5’ CGG GGT ACC AAA TGC TGC AGT TGA CTC TCC	5’ CCG CTC GAG CCA TTC ATG AAG TGC ACA GG
INSR mini-gene	5’ GTA CAA GCT TGA ATG CTG CTC CTG TCC AAG ACA G	5’ GCC CTC GAG CGT GGG CAC GCT GGT C

5.) Representative RT-PCR Gels of the *PLEKHH2* and cTNT mini-genes and the corresponding plots of data

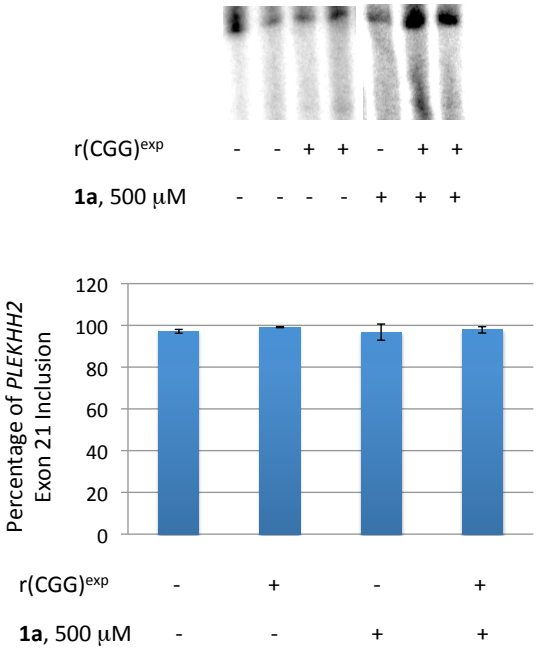

Figure S-4: **1a** does not affect the alternative splicing of a *PLEKHH2* mini-gene. Briefly, COS7 cells were co-transfected with (i) a plasmid containing no r(CGG) repeats and the *PLEKHH2* mini-gene or (ii) a plasmid containing 60 rCGG repeats and a *PLEKHH2* minigene Top, a representative gel autoradiogram. Bottom, plot of the corresponding data.

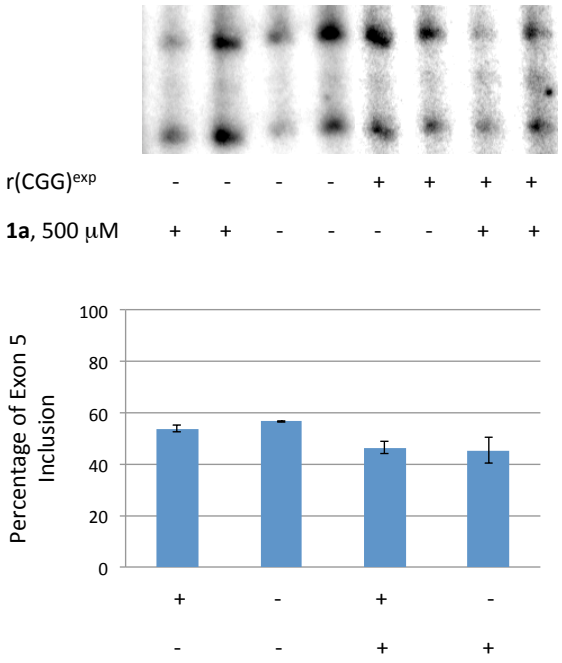

Figure S-5: **1a** does not affect the alternative splicing of a cTNT mini-gene. Briefly, COS7 cells were co-transfected with (i) a plasmid containing no r(CGG) repeats and the cTNT mini-gene or (ii) a plasmid containing 60 rCGG repeats and a cTNT minigene Top, a representative gel autoradiogram. Bottom, plot of the corresponding data.

6.) Characterization of Compounds

Mass spectra were collected on a Varian 500 MS spectrometer equipped with Varian Prostar Autosampler 410.

The purities of compounds were determined by analytical HPLC using a Waters 1525 Binary HPLC Pump equipped with Waters 2487 Dual λ Absorbance Detector system and the following conditions: a Waters Symmetry® C8 5µm 4.6×150 mm column, room temperature, a flow rate of 2.4 mL/min, and a linear gradient of 0% to 100% B in A for 60 min. A is water while B is methanol.

Table S-2: Characterization of 1a and derivatives thereof including HPLC retention times, and calculated and observed masses.

Compound	Molecular Formula	HPLC Retention Time (min)	MS (Calculated)	ESI(+)-MS (Found)
1a	$C_{24}H_{28}N_3O^+$	21	374.2 (M)	
1b	$C_{23}H_{28}N_3O^+$	14	362.2 (M)	362.3 (M)
1c	$C_{19}H_{19}N_2O^+$	18	291.2 (M)	291.2 (M)
1d	$C_{18}H_{17}N_2O^+$	17	277.1 (M)	277.1 (M)
1e	$C_{17}H_{14}N_2O$	29	263.1 $(M+H)^+$	263.1 $(M+H)^+$
1f	$C_{17}H_{14}N_2$	32	247.1 $(M+H)^+$	247.1 $(M+H)^+$

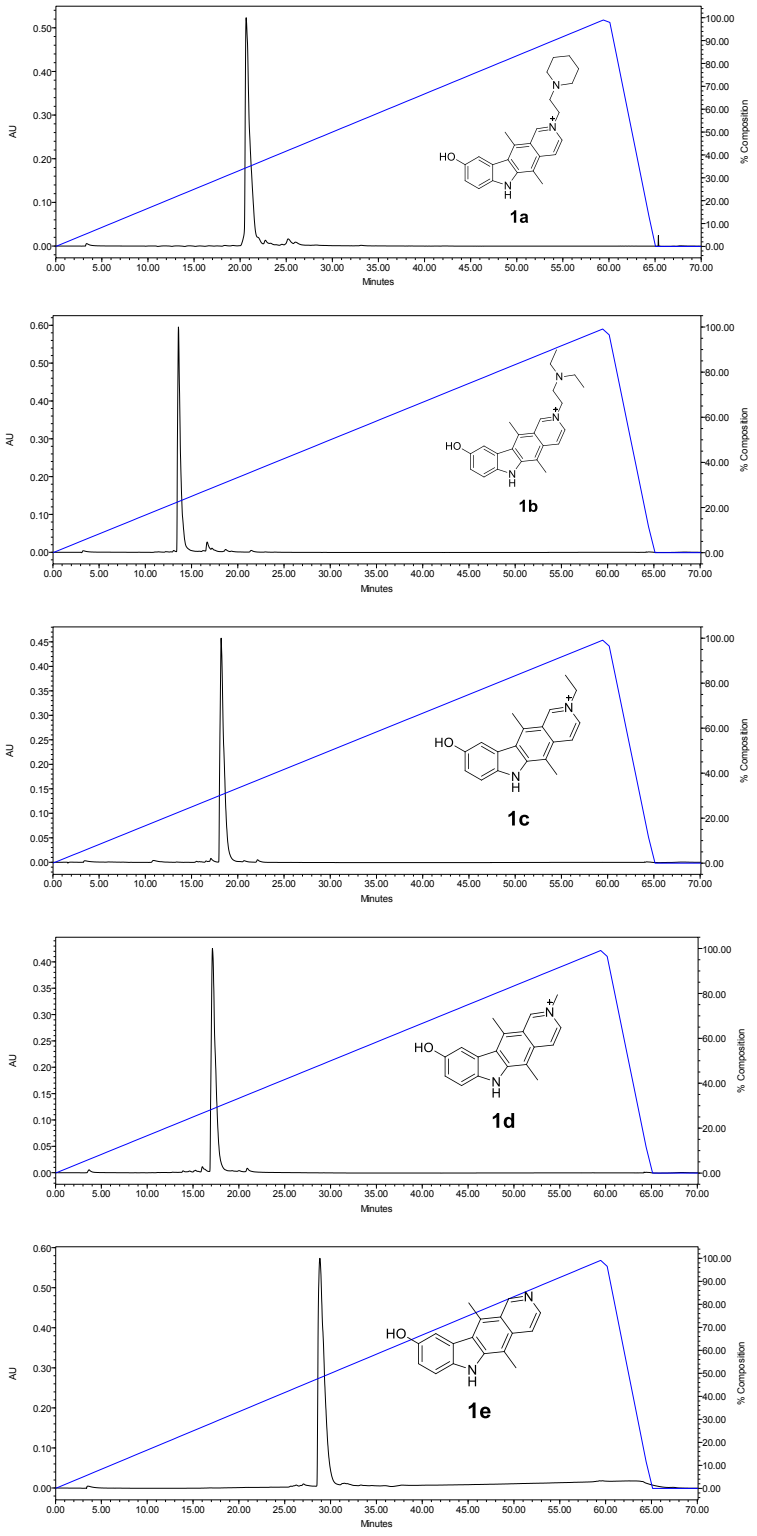

Figure S-6: HPLC chromatograms of compounds (blue: linear gradient of MeOH).

REFERENCES

1. Tran, T., and Disney, M. D. (2010) Two-dimensional combinatorial screening of a bacterial rRNA A-site-like motif library: defining privileged asymmetric internal loops that bind aminoglycosides, *Biochemistry 49*, 1833-1842.
2. Cho, J., and Rando, R. R. (2000) Specific binding of Hoechst 33258 to site 1 thymidylate synthase mRNA, *Nucleic Acids Res 28*, 2158-2163.
3. Childs-Disney, J. L., Wu, M., Pushechnikov, A., Aminova, O., and Disney, M. D. (2007) A small molecule microarray platform to select RNA internal loop-ligand interactions, *ACS Chem Biol 2*, 745-754.