TMPyP4 Distorts RNA G-Quadruplex Structures of the Disease-Associated r(GGGGCC)n Repeat of the C9orf72 Gene and Blocks Interaction of RNA-Binding Proteins.

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*Running title: TMPyP4 disrupts RNA-binding protein interaction with the C9orf72 r(GGGGCC)n RNA repeat

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**Background:** Amyotrophic lateral sclerosis and frontotemporal dementia are caused by expansion of the C9orf72 (GGGGCC)n repeat, whose RNA can form G-quadruplexes.

**Results:** r(GGGGCC)n G-quadruplex distortion by TMPyP4 ablates interaction of the hnRNPA1 and ASF/SF2 proteins.

**Conclusion:** G-quadruplexes can be modulated by TMPyP4, which can ablate protein interactions.

**Significance:** Disruption of secondary structures in the C9orf72 RNA repeats may be a potential therapeutic avenue.

**SUMMARY:** Certain DNA and RNA sequences can form G-quadruplexes, which can affect genetic instability, promoter activity, RNA splicing, RNA stability, and neurite mRNA localization. Amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) can be caused by expansion of a (GGGGCC)n repeat in the C9orf72 gene. Mutant r(GGGGCC)n- and r(GGCCCC)n-containing transcripts aggregate in nuclear foci possibly sequestering repeat-binding proteins like ASF/SF2 and hnRNPA1 – suggesting a toxic-RNA pathogenesis, as occurs in myotonic dystrophy. Furthermore, the C9orf72 repeat RNA was recently demonstrated to undergo the non-canonical repeat associated non-AUG translation (RAN-translation) into pathologic dipeptide repeats in patient brains, a process that is thought to depend upon RNA structure. We previously demonstrated that the r(GGGGCC)n RNA forms repeat tract length-dependent G-quadruplex structures that bind by the ASF/SF2 protein. Here we show that the cationic porphyrin (TMPyP4), which can bind some G-quadruplex forming sequences, can bind and distort the G-quadruplex formed by r(GGGGCC)8, and this ablates the interaction of either hnRNPA1 or ASF/SF2 with the repeat. These findings provide proof-of-concept that nucleic acid binding small molecules, like TMPyP4, can distort the secondary structure of the C9orf72 repeat, which may beneficially disrupt protein interactions, which may ablate either protein-sequestration and/or RAN-translation into potentially toxic dipeptides. Disruption of secondary structure formation of the C9orf72 RNA repeats may be a viable therapeutic avenue, as well as a means to test the role of RNA structure upon RAN-translation.

**INTRODUCTION**

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig’s disease, and frontotemporal dementia (FTD) are part of a spectrum of neurodegenerative disorders (1). Expansion of a (GGGGCC)n(GGCCCC) hexanucleotide repeat within the first intron of the C9orf72 gene is one of the most common causes of ALS and FTD (1-3). Unaffected individuals have 2-19 repeats and individuals with as few as 20-25 repeats may show symptoms of disease, while those affected can have 250-1600 repeats (2-4).

Patient cells bearing the expanded repeat contain ribonuclear foci containing the r(GGGGCC)n repeat, suggesting a toxic RNA:protein sequestration pathway similar to other repeat expansion diseases (2,5). In myotonic dystrophy type 1 (DM1), expanded r(CUG)n RNAs fold into hairpin structures that are recognized by the muscleblind (MBNL) family of alternative splicing regulators leading to their binding and sequestration. Loss of muscleblind results in the mis-splicing of its many downstream mRNA targets leading to DM1 pathogenesis (5). Several proteins have been identified that bind to the r(GGGGCC)n RNA in vitro and in vivo (6-8) which may influence ALS-FTD pathogenesis through the toxic-RNA gain-of function pathway. ASF/SF2 splicing regulator was shown to interact with the r(GGGGCC)n repeat in vitro (7) and to be present in RNA foci in cells with expanded (GGGGCC)n tracts (9). The protein, hnRNPA3 selectively binds the r(GGGGCC)n repeats in vitro and was identified in intracellular inclusions of ALS-FTD patient brains (6). Most recently, the Pur-α protein, which interacts with expanded r(CGG)n repeats associated with FXTAS and FXPOI, was demonstrated to interact with expanded r(GGGGCC)n repeats in vitro and in vivo (in Drosophila and FTD patient brain samples) forming intracellular inclusions (8). Overexpression of Pur-α in this Drosophila model reversed sequestration-associated toxicity (8). These findings support a toxic RNA-mediated protein sequestration model of ALS-FTD.
pathogenesis. Interestingly, a recent study identified point mutations in the hnRNPA1 gene resulting in protein aggregation causing ALS-FTD (10). Both hnRNPA1 and hnRNPA3 were identified as r(GGGGCC)n binding proteins, amongst others (6). The presence of hnRNPA1, hnRNPA3, or any protein at the r(GGGGCC)n-positive foci is presently unknown.

The expanded r(GGGGCC)n and r(GGCCCC)n RNAs can undergo RAN (Repeat Associated Non-AUG)-translation to form aggregating dipeptide repeat inclusions in ALS-FTD patient brains (6,11-15), a phenomenon initially demonstrated for the myotonic dystrophy type 1 and spinocerebellar ataxia type 8 repeats (16,17) and more recently by the CGG repeats of fragile X associated tremor ataxia (10,14). Some data suggest a role of RNA structures in RAN-translation (6,12,16).

The structure assumed by the r(GGGGCC)n repeats may contribute to its interaction with proteins to mediate protein-sequestration and/or RAN-translation. To this end, the toxic-RNA may serve as a potential therapeutic target to disrupt these potentially toxic downstream processes, as has been demonstrated for the myotonic dystrophy hairpin-forming r(CUG)n RNA (18). Secondary structures of the repeat tracts in the RNA appear to be the basis for both protein sequestration and RAN-translation. Our group and others recently demonstrated that r(GGGGCC)n forms extremely stable G-quadruplex structures (7,19). G-quadruplex formation by the r(GGGGCC)n repeat may affect protein binding, foci formation and RAN-translation (7).

In G-quadruplexes, four guanine residues are hydrogen bonded to one another via Hoogsteen base interactions forming a planar complex (G-quartet) (Fig. 1A). These planar G-quartets can stack upon each other in the presence of Na+ or K+ and form stable structures known as G-quadruplexes (Fig. 1C) (20-22). G-quadruplexes have been shown to exist in living cells (23,24). Functionally, G-quadruplex structures in RNA have been implicated in almost all aspects of pre-mRNA and mRNA metabolism including mRNA stability, IRES-dependent translation initiation, translational repression, alternative splicing, and alternative polyadenylation/3′ end formation, suggesting that the G-quadruplex may be an important regulatory motif. The C9orf72 gene is one of the 3000 (16%) genes in the human genome to have motifs with the potential to form G-quadruplexes present in their first intron (25). Many transcripts with G-quadruplex-forming motifs are neurologically important and their G-quadruplexes are thought to be important for transcript localization to neural synapses by the G-quadruplex-binding FMRP protein (26-31).

The cationic 5,10,15,20-tetra(N-methyl-4-pyridyl) porphyrin (TMPyP4) (Fig. 1B) has been shown to stabilize many DNA G-quadruplexes (32). In some instances TMPyP4 can destabilize and unfold RNA G-quadruplexes such as the one present in the MT3-MMP mRNA (33). Similarly, TMPyP4 destabilized the G-quadruplex of both the DNA and RNA (CGG)n repeats of FMR1, associated with premutation expansions of fragile X syndrome, fragile X associated tremor ataxia (FXTAS), and fragile X premature ovarian insufficiency (FXPOI) (34-36). Disruption of both RNA G-quadruplexes (MT3-MMP and FMR1) by TMPyP4 led to enhanced levels of translation in model systems (33,35). Here we report the binding of TMPyP4 to the ALS-FTD r(GGGGCC)8 repeat using gel mobility-shift assays, circular dichroism (CD) spectroscopy and UV spectroscopy. Additionally, we report that TMPyP4 disrupts the binding of ASF/SF2 and hnRNPA1 to the ALS-FTD-associated r(GGGGCC)8 repeat.

**EXPERIMENTAL PROCEDURES**

**RNA oligonucleotide synthesis and labeling**

The RNA oligonucleotides: r(GGGGCC)n, n=2,5,8 were purchased from Invitrogen. Oligos were end-labeled using [γ-32P]ATP. RNA concentrations were determined by recording their absorbance at 260 nm using a molar extinction coefficient of 124,280, 310,700 and 497,120 M-1cm-1 respectively (37). The RNA was dissolved in 100 mM KCl, 10 mM Tris-HCl and 0.1 mM EDTA (pH 7.5). The samples were heated to 95 °C and allowed to cool to room temperature.
CD spectroscopy

CD experiments were performed at room temperature (unless otherwise stated) using an Aviv model 62DS spectropolarimeter using 5-µM RNA samples dissolved in 100 mM KCl, 10 mM Tris-HCl and 0.1 mM EDTA (pH 7.5). An average of three CD spectra, over the wavelength range of 320 to 220 nm was recorded in a 1-mm pathlength cuvette at a scan rate of 20 nm/min. For the titrations, 1-µL aliquots of 1 M TMPyP4 in the same buffer as the RNA were added to the sample and mixed before recording the spectrum.

Native gel electrophoresis

5’-end-labeled r(GGGGCC)8 was annealed as described above. The radiolabeled RNA was incubated with the indicated amounts of TMPyP4, ASF/SF2 (Abcam) and hnRNPA1 for 30 minutes at room temperature. Each 10-µL sample contained <10 fmols [γ-32P]ATP end-labeled RNA in 100 mM KCl, 10 mM Tris-HCl (pH 7.5). For protein-binding the buffer contained 10 mM HEPES (pH 7.9), 200 mM KCl, 20 mM NaCl, 0.025% Nonidet, 1 mM DTT, 10% glycerol. After incubation the samples were loaded onto 8% polyacrylamide gel and electrophoresed at 100 V for 1 hour in 1x TBE (tris borate EDTA), at room temperature, dried and autoradiographed.

Absorption spectroscopy

Absorption spectra were measured on a Cary model 300 Bio spectrophotometer. The sample was contained in a 1-mm pathlength cuvette. Aliquots of r(GGGGCC)n, n = 2, 5, 8 in buffer were titrated into a 15-µM sample of TMPyP4 (250 µL). The titrations were terminated when the wavelength and intensity of the absorption band for TMPyP4 did not change upon further addition of RNA. All values are corrected for dilution effects.

The concentration of free porphyrin was determined using a molar extinction coefficient of 226,000 M⁻¹cm⁻¹ at 422 nm (34). The concentrations of free (Cf) and bound porphyrin (C_b) were calculated using Cf = C(1 − α) and C_b = C − Cf respectively, where C is the total TMPyP4 concentration (15 µM). The fraction of bound porphyrin (α) was calculated using the equation α = (A_f − A)/(A_f − A_b) where A_f and A_b are the absorbance of the free and fully bound TMPyP4 at the Soret maximum of the porphyrin, respectively, and A is the absorbance at the Soret maximum of porphyrin at any given point during the titration. The percent hypochromicity of the Soret band of porphyrin can be calculated using %hypochromicity = (ε_f − ε_b)/ε_f × 100 where ε_b = A_b/C_b (19, 38).

RESULTS AND DISCUSSION

TMPyP4 binds to the ALS-FTD r(GGGGCC)n repeat

To determine whether TMPyP4 can interact with the r(GGGGCC)n RNA, we performed electrophoretic mobility shift assays where radiolabeled r(GGGGCC)8 was pre-incubated in the presence of 0, 10, 25, 50 and 100 µM of TMPyP4. Multiple electrophoretic species are evident for r(GGGGCC)n representing un- and multimolecular G-quadruplex structures, as previously demonstrated (Fig. 1D, see first and last lanes) (7). Increasing the concentration of TMPyP4 led to a progressive shift in the RNA where RNA:drug complexes are retained in the well at high drug concentrations (lanes 2-5 of Fig. 1D). At concentrations higher than 25 µM TMPyP4, the intensity of the faster-migrating species decreased while the intensity of the slower migrating species did not change considerably.

To further investigate the interaction between TMPyP4 and r(GGGGCC)n, we measured the visible absorption titration spectra (Fig. 1E). This method can identify RNA:ligand interactions as previously shown for the binding of MT3-MMP mRNA to TMPyP4 (33). The r(GGGGCC)8 oligos were titrated into a solution of TMPyP4 and the Soret band was monitored as a function of RNA concentration (Fig. 1E). The hypochromicity seen upon increasing concentration of r(GGGGCC)8 was 64% with a bathochromic shift of 17 nm indicative of the binding of TMPyP4 to r(GGGGCC)8. The data also reveal an apparent isosbestic point at 439 nm. The same analysis was performed on lengths of 2 and 5 repeats with a similar effect (data not shown). The bathochromic shift for r(GGGGCC)2

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and r(GGGGCCC)5 was recorded as 15 and 16 nm (data not shown). The C-rich C9orf72 repeat strand, which does not form a quadruplex structure (7), interacted with TMPyP4 with reduced affinity relative to the G-rich strand (data not shown), consistent with a preference of TMPyP4 for G-quadruplexes. Thus, we demonstrate by two independent means that the r(GGGGCC)n repeat is able to interact with TMPyP4 in a concentration-dependent manner. A titration of increasing TMPyP4 suggests that the mode of its interaction may not be limited to external stacking at G-quadruplex ends and may involve intercalation between the G-quartets based on the ratio of the titrated TMPyP4 to the ratio of the RNA sample at the point where the binding plot plateaus (Fig. E, inset). Other reports suggest a stacking of TMPyP4 on G-quartets (39).

**TMPyP4 induces a structural change in the r(GGGGCC)n repeat**

Circular dichroism spectroscopy (CD) is useful for monitoring changes in the secondary structure of nucleic acids, including G-quadruplexes. As previously shown, the CD spectrum of the r(GGGGCC)n repeats in potassium chloride exhibits a positive peak around 260 nm and a negative peak around 240 nm (Fig. 1F-H), which are the spectral characteristics of a parallel-stranded G-quadruplex (7,40,41). Addition of TMPyP4 to r(GGGGCC)n led to the appearance of a shoulder at around 290 nm in addition to the G-quadruplex specific positive peak at 260 nm (Fig. 1F-H). An induction of a 290 nm shoulder in a G-quadruplex detected by CD spectra in the presence of TMPyP4 has previously been reported as the appearance of antiparallel conformations in another G-rich sequence (42). Thus, in the presence of increasing concentrations of TMPyP4, the r(GGGGCC)n RNA undergoes a conformational change. At low concentrations of TMPyP4, the intensity of the 260 nm peak increases and was highest at 29 μM of TMPyP4, 5 μM r(GGGGCC)₈ (data now shown). Higher concentrations of TMPyP4 led to a decrease of the intensity of the peak at 260 nm and no further change at 290 nm (Fig. 1F-H). The decrease in the intensity of the 260 nm band upon increasing the concentration of TMPyP4 may indicate possible unfolding of the r(GGGGCC)n G-quadruplex, as previously reported (33). The CD spectra in Figure 1F are consistent with the gel electrophoresis and Soret band-shift experiments in Figures 1D & 1E, respectively. It is important to note that the r(GGGGCC)n G-quadruplex is extremely thermostable and remains folded at temperatures as high as 95 °C (7). Thus, TMPyP4 can modulate the extremely stable structure assumed by the r(GGGGCC)n repeat.

To assess the stability of the r(GGGGCC)₈ G-quadruplex in the presence of TMPyP4, CD spectra were recorded at different temperatures (data now shown). The porphyrin/RNA molar ratio (20 μM TMPyP4 to 5 μM r(GGGGCC)₈) was selected where the intensity of the 260 nm band is at its highest and the shoulder around 290 nm is present. The CD spectra were measured at 0, 24 and 48 hours after addition of TMPyP4 to r(GGGGCC)₈. The spectra did not vary over this time period. Upon increasing the temperature, the intensity of the shoulder at 295 nm decreased, whereas the intensity of the positive peak at 260 nm did not change (data not shown). At 75 °C the CD spectra resembles the CD spectra of the RNA G-quadruplex in the absence of TMPyP4 (positive peak around 260 nm and negative peak around 240 nm without the shoulder at 290 nm). This is consistent with the previously observed thermostability of the r(GGGGCC)₈ G-quadruplex structure (melting temperature higher than 95°C) (7). Notably, upon lowering the temperature the CD spectrum was not different from the one recorded at 95 °C (no shoulder appeared around 290 nm) (data not shown) indicating an irreversible melting process. UV melting experiments were also performed in the presence of TMPyP4 (data not shown). The melting temperature of r(GGGGCC)₈ with TMPyP4 was 67 °C and the melting was irreversible, which was consistent between CD and UV melting experiments. The irreversibility of the melting of r(GGGGCC)₈ in the presence of TMPyP4 may be related to the molecularity of the porphyrin-induced structure, and may indicate the induction of multimeric G-quadruplexes rather than a monomeric one, as previously reported for other G-quadruplex-forming sequences (43). Thus, TMPyP4 lowered the thermal stability of the r(GGGGCC)₈.
**TMPyP4 disrupts binding of ASF/SF2 and hnRNPA1 to r(GGGGCC)n**

The interaction between TMPyP4 and the r(GGGGCC)n repeat may alter the interaction of proteins, previously shown to bind the r(GGGGCC)n repeat. To test this, we first assessed the binding of ASF/SF2 to r(GGGGCC)n in the presence of TMPyP4 (Fig. 2A). A distinct slow-migrating RNA complex was evident in the presence of purified ASF/SF2 protein (Fig. 2A, lane 2, see arrowhead). In the presence of increasing concentrations of TMPyP4, there was a disruption of ASF/SF2:RNA complexes exhibited by a loss in the band-shift complex and an increase in the protein-free RNAs (Fig. 2A). Thus, TMPyP4 can interfere with ASF/SF2:r(GGGGCC)n binding; providing proof-of-principle for disruption of potentially deleterious protein-RNA interactions.

To further test the potential of TMPyP4 to interfere with protein interaction, we assessed its effect upon the binding of hnRNPA1; an r(GGGGCC)n-binding protein that may have implications for ALS-FTD (6,10). Previously it was shown that hnRNPA1 or its proteolytic fragment UP1, could bind to various G-rich repeats, including telomeres and the fragile X associated (CGG)n repeat (44-46). To test the potential interaction between hnRNPA1 and r(GGGGCC)n, a band-shift assay was performed in the presence of increasing concentrations of purified hnRNPA1 (Fig. 2B). Binding of the r(GGGGCC)n repeat to hnRNPA1 was evidenced by retention of the RNA:protein complexes in the well (Fig. 2B, lanes 2-3, see arrowhead), a phenomenon previously observed for the interaction of MBNL1 to r(CUG)n repeats(47,48). Upon addition of increasing amounts of TMPyP4, we observed a reduction in the intensity of the autoradiographic signal in the well and a coincident increase of protein-free RNA (Fig. 2C, lanes 3-5 see arrowhead and %). This is indicative of disruption of the hnRNPA1:r(GGGGCC)n complex by TMPyP4. Thus, TMPyP4 is able to disrupt various protein:RNA complexes.

**CONCLUSION**

It has been shown that the formation of RNA foci and protein-sequestration may be pathogenic in cases of ALS-FTD caused by expansion of the C9orf72 repeat. While point mutations in hnRNPA1 were previously shown to cause aggregation of hnRNPA1 in ALS-FTD patient brains (9) and non-mutant hnRNPA1 was independently shown to bind to r(GGGGCC)n repeats (6) the role of hnRNPA1 in a toxic-RNA pathway is unknown. A potential role of ASF/SF2 in C9orf72 expansion disease pathogenesis has been suggested by the presence of ASF/SF2 proteins in expanded r(GGGGCC)n RNA foci (9). If either hnRNPA1 or ASF/SF2 are involved in a toxic-RNA pathway, the disruption of their interaction by small molecule ligands, like TMPyP4 provides-proof-of-principle that this is a viable avenue for the development of therapeutic treatments. Such an approach may complement RNA-directed antisense oligonucleotide attacks (49-51). The function of the G-quadruplex in RAN-translation, although currently unknown, could also be modulated through its interaction with TMPyP4. However, there are currently no cellular assays to test the effect of TMPyP4 on RAN-translation. The precise role of RNA structure in either pathogenesis, splicing, or RAN-translation of the C9orf72 transcript remains to be determined; if the facile and extremely stable formation of a G-quadruplex by the r(GGGGCC)n repeat is involved, the perturbation of this structure by small molecule ligands may prove useful as a tool in understanding the role of RNA structure in these processes.
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FOOTNOTES

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FIGURE LEGENDS

of RNA) All spectra were measured in 10 mM Tris–HCl (pH 7.5) and 0.1 mM EDTA at room temperature. F. CD titration of 5 µM r(GGGGCC)$_8$ with aliquots of 1 M TMPyP4 in the presence of 100 mM KCl, at 25 °C. G. CD titration of 5 µM r(GGGGCC)$_5$ with aliquots of 1 M TMPyP4 in the presence of 100 mM KCl, at 25 °C. H. CD titration of 5 µM r(GGGGCC)$_2$ with aliquots of 1 M TMPyP4 in the presence of 100 mM KCl, at 25 °C. D.E., Delta Epsilon.

**FIGURE 2.** A. Migration of [$\gamma$-$^{32}$P]-labeled r(GGGGCC)$_8$ in the presence of 20 µM ASF/SF2 and 0, 10, 25, 50 µM of TMPyP4 electrophoresed on a 8% polyacrylamide gel. The amount of RNA bound by ASF/SF2 is 69% in the absence of TMPyP4 and the amount of protein-free RNA increased with increasing amounts of TMPyP4 (see %), as determined by densitometric analysis. B. Migration of [$\gamma$-$^{32}$P]-labeled r(GGGGCC)$_8$ in the presence of 0, 7.5, 15, 20 µM hnRNPA1. The amount of RNA bound increased with increasing amounts of hnRNPA1 (see %). C. Migration of [$\gamma$-$^{32}$P]-labeled r(GGGGCC)$_8$ in the presence of 7.5 µM hnRNPA1 and 0, 10, 25, 50 µM of TMPyP4. The amount of RNA bound by hnRNPA1 is 62% in the absence of TMPyP4 and the amount of protein-free RNA increased with increasing amounts of TMPyP4 (see %), as determined by densitometric analysis. The ability of TMPyP4 to disrupt the interaction of protein-binding was reproducible and for hnRNPA1 was statistically significant (Students T, $p=0.032$).
Figure 1
Figure 2
TMPyP4 Distorts RNA G-Quadruplex Structures of the Disease-Associated r(GGGGCC)n Repeat of the C9orf72 Gene and Blocks Interaction of RNA-Binding Proteins

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