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Letter

Pathogenic SNPs Affect Both RNA and DNA G-Quadruplexes' Responses to Ligands

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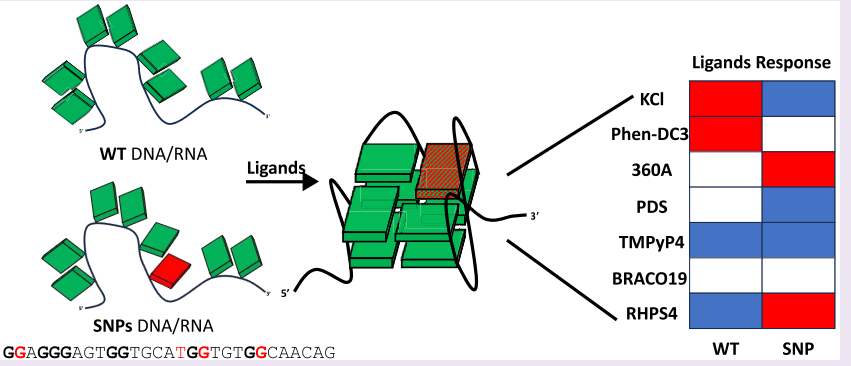

ABSTRACT: Single nucleotide polymorphisms (SNPs) are common genetic variations that are present in over 1% of the population and can significantly modify the structures of both DNA and RNA. G-quadruplex structures (G4) are formed by the superposition of tetrads of guanines. To date, the impact of SNPs on both G4 ligands' binding efficacies and specificities has not been investigated. Here, using a bioinformatically predicted G4 and SNPs found in the α-synuclein gene as a proof-of-concept, it was demonstrated that SNPs can modulate both DNA and RNA G4s' responses to ligands. Specifically, six widely recognized ligands (Phen-DC3, PDS, 360A, RHPS4, BRACO19, and TMPyP4) were shown to differentially affect both the structure and the polymerase stalling of the different SNPs. This work highlights the importance of choosing the appropriate G4 ligand when dealing with an SNP identified in a G-rich gene.

G-quadruplexes (G4s) are noncanonical secondary structures found in both DNA and RNA that are composed of guanine tetrads.[1] G4s are associated with many cellular processes including, but not limited to, replication, transcription, and translation.[2] In recent years, thousands of small molecules have been designed and synthesized to both stabilize and induce the folding of G4 structures.[3] However, the efficiency and selectivity of each of these are not only unpredictable but controlled by many factors, including the number of G-tetrads, the competition with Watson−Crick base pairing, and the type of nucleic acid targeted (DNA or RNA).[4,5] As a consequence, the G-quadruplex/ligand interaction can be modulated by modifying only one nucleotide.[6]

Single nucleotide polymorphisms (SNPs) are nucleotide variations present in more than 1% of the population. Over 600 000 SNPs have been reported in the human genome, with roughly 30 000 having been associated with various diseases.[7] Few studies have explored the influence of SNPs on G-quadruplex formation, and those that did primarily focused on DNA.[8] For instance, an SNP in the promoter of HSPB2 has recently been shown to destabilize a G4 and increase the expression of HSPB2.[9] However, it is important to note that SNPs can also modulate the folding of RNA G4s and thus open the way to identifying specific targeting strategies at both the DNA and the RNA levels. Understanding how these mutations could affect potential G4 ligand treatments is crucial for developing targeted therapeutic strategies. Herein, the ability of SNPs to modulate both DNA and RNA G-quadruplexes' responses to some of the most often used ligands was explored.

In order to study the relationship between SNPs, G4s, and ligands, the sequence of the α-synuclein gene was used as a proof-of-concept. The resulting SNCA protein is found at the neurons' presynaptic terminals and regulates the reserve pool of synaptic vesicles for neurotransmitter release.[10] However, in the case of Parkinson's disease, this protein is either overexpressed or mutated, causing protein aggregation which leads to, among other things, both proteasomal and

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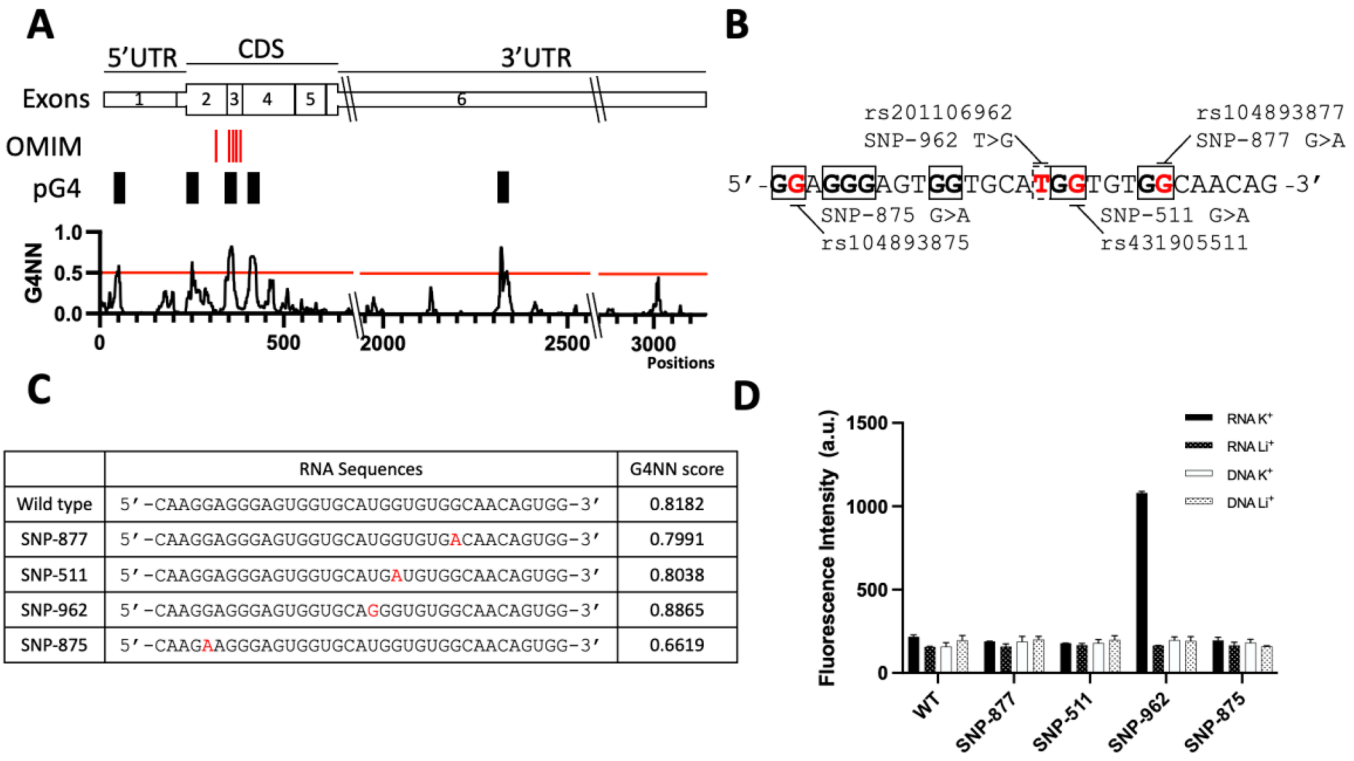

	RNA Sequences	G4NN score
Wild type	5′-CAAGGAGGGAGUGGUGCAUGGUGUGGCAACAGUGG-3′	0.8182
SNP-877	5′-CAAGGAGGGAGUGGUGCAUGGUGUGACAACAGUGG-3′	0.7991
SNP-511	5′-CAAGGAGGGAGUGGUGCAUGAUGUGGCAACAGUGG-3′	0.8038
SNP-962	5′-CAAGGAGGGAGUGGUGCAGGUGUGGCAACAGUGG-3′	0.8865
SNP-875	5′-CAAGAAGGGAGUGGUGCAUGGUGUGGCAACAGUGG-3′	0.6619

Figure 1. Evaluation of pG4 formation in both the α-synuclein gene and transcript. (A) Representation of the pG4s found in the α-synuclein transcript and in the OMIM allelic variant phenotype (i.e., the SNPs). The G4NN score for every window along the α-synuclein transcript is shown. CDS stands for coding sequence and UTR for untranslated region. (B) The four SNPs located in the predicted G4 regions. (C) G4NN score and sequence of the WT and of each SNP. (D) NMM fluorescence at 605 nm of the different RNA and DNA SNP sequences in 20 mM Li-cacodylate buffer in the presence of 100 mM of either potassium chloride or lithium chloride (KCl or LiCl). Results are shown by arbitrary units of fluorescence (a.u.).

mitochondrial dysfunction and to an increase in oxidative stress.[10] Using the University of California Santa Cruz (USCS) database and a neuronal network trained RNA G4 predictor (i.e., RNA G4 Screener with the default 0.5 threshold for the resulting G4 Neuronal Network (G4NN) score), an RNA potential G-quadruplex (pG4) site located in the coding region of the α-synuclein transcript was found to intercept with four SNPs known to be associated with both Parkinson's disease and with the Lewy body dementia gene (Figure 1A).[7,11] The G4NN score, and two other scores of this G4 predictor (cGcG and G4 Hunter (G4H)), were also evaluated with different sliding window sizes in order to improve the pG4 confidence (Figure S1).[12,13] While G4H did not predict the presence of any G4s, no matter what the tested window size used was, the same G4 "hit" as was found for the G4NN prediction was also found with two smaller windows when using cGcG. Based on these results, the transcriptomic flanking nucleotides should not affect G-quadruplex formation. Of note, smaller windows are usually associated with a higher number of hits, but with lower precision.[12] Three of these SNPs are guanine to adenine (G > A; SNP-877, SNP-511, and SNP875), while one of them, SNP-962, is uracil to guanine (U > G; Figure 1B). Despite the disruption of the guanine doublet by the mutation to adenine, all three SNPs still exhibited a G4NN score higher than the threshold (Figure 1C), confirming the prediction of a G4 despite the nucleotide change (G > A or U > G). As expected, the introduction of a guanine (U > G) had a positive effect on the score and revealed a high potential for G4 formation as compared to the wild type (WT). While the scores remained relatively similar (even when using an older predictor, G-rich sequences (QGRS) Mapper[14]), the presence of the SNPs significantly influenced the number of potential ways that the RNA G4 could fold, ranging from 11 to 53 possibilities. (Supplementary Table 1). Overall, when compared to the WT, SNP-962 seems to be more suitable for G4 formation, while the three other SNPs seem to impair the folding.

Next, the ability of the WT and SNP sequences to fold in G4s *in vitro* was evaluated using the *N*-methylmesoporphyrin IX molecule (NMM), a "turn-on" G4-specific fluorescent ligand.[15] The experiments were performed with both DNA and RNA molecules because SNPs can differently affect their G4 formation. An enhanced G4 fluorescence emission at 605 nm was observed exclusively under potassium (K^+) conditions with SNP-962 in RNA. The differences in the NMM emissions between the lithium (Li^+) and the K^+ conditions, which were up to 6.5-fold, provided evidence supporting the formation of G4 structures with only SNP-962 in RNA, contradicting the predictions made by the G4 RNA screener, with a given set of parameters (Figures 1D and S2). In order to understand why the other sequences do not fold into G4s *in vitro*, the minimal free energies (MFE) of the DNA and RNA sequences were predicted using RNAfold.[16] All the SNPs had predicted MFEs that were similar to that of the WT, specifically −13 kcal/mol for the RNA and −6 to −7 kcal/mol for the DNA sequences (Figure S3). The absence of G4s in most structures can be expected since two G-tetrad sequences are usually less stable than the predicted Watson−Crick structures.[17−19] These results make the α-synuclein gene an ideal candidate with which to evaluate the ability of different ligands to fold the

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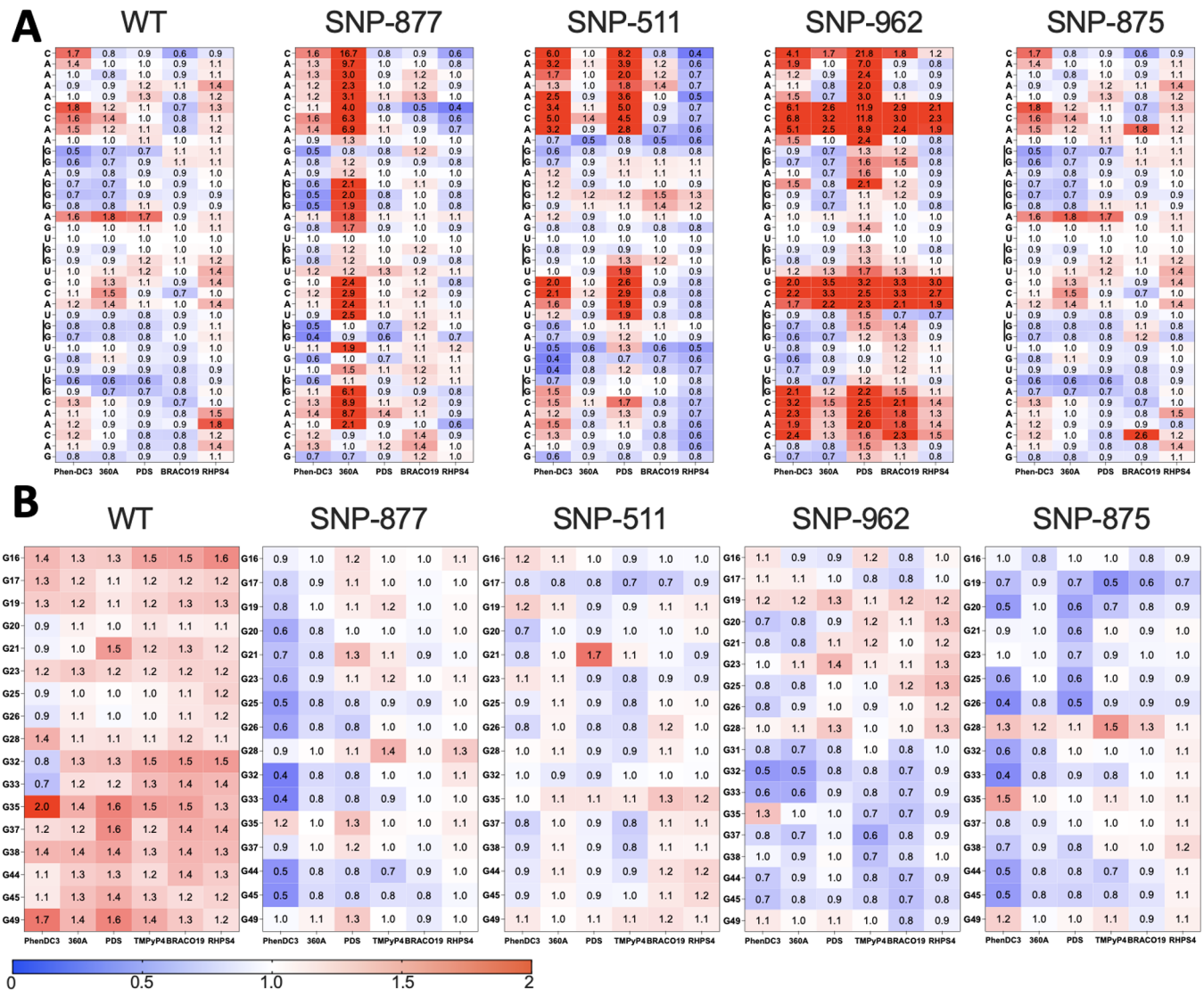

Figure 2. Single-nucleotide accessibility of the RNA and DNA sequences incubated with G4 ligands. (A) Heat maps illustrating the single-nucleotide accessibilities of the WT and the SNP sequences in *in-line* probing experiments in the presence of the different ligands. All nucleotides are shown. (B) Heat maps of the WT and the SNP sequences DMS footprinting experiments performed in the presence of the different ligands. The guanines are shown. For both experiments, the peaks were quantified by densitometry and then normalized to the KCl condition. The legend shows the fold change color palette over the KCl condition.

SNP sequences into G4s since most sequence variants do not seem to fold into G4 structures in both DNA and RNA.

In order to evaluate the impact of the ligands on the secondary structure, the accessibility of individual nucleotides to each sequence incubated with G4 ligands was monitored. This was done by either *in-line* probing for RNA (Figure 1A) or by dimethyl sulfate (DMS) footprinting for DNA (Figure 1B), respectively. *In-line* probing is based on spontaneous hydrolysis of RNA in the presence of a high concentration of magnesium, while DMS footprinting is based on guanine nitrogen 7 (N7) methylation and piperidine strand break.[20,21] In the in-line probing, the nucleotide located in a duplex or the G4 core should be protected from cleavage, in opposition to the ones located in the loops or flanking the G4. In the case of DMS footprinting, the guanines implicated in a Hoogsteen base pairing (e.g., in the G4 core) are expected to be less cleaved than the one in the loops or in the flanking sequences. The reactions were analyzed by electrophoresis on denaturing polyacrylamide gels, then were quantified by densitometry (Figures S4 and S5) and the results normalized to the KCl condition (Figure 2). The experiments have been performed with three families of ligands: pyridodicarboxamide (PhenDC3, 360A and PDS) and acridine (BRACO19 and RHPS4).[3,22−24] These ligands were selected because of their high selectivity for G4 and their popularity within the G4 research community.[3] Additionally, these ligands have been reported to both stabilize and induce the formation of G4 structures within two G-quartet motifs in long noncoding RNA.[25] The popular porphyrin TMPyP4 was also added into the analysis, even though some suggest that it could be also a G4 destabilizing ligand.[26,27] These two methods showed nucleotide accessibility modifications depending on both the ligands and the SNPs used. Global changes were also noticed between RNA and DNA accessibilities depending on the ligand used. For the RNA sequences, in SNP-962 (U > G), the nucleotides located upstream of the first stretch of guanines, those located between the third and the fourth stretches of guanines, and those located downstream of the last stretch of

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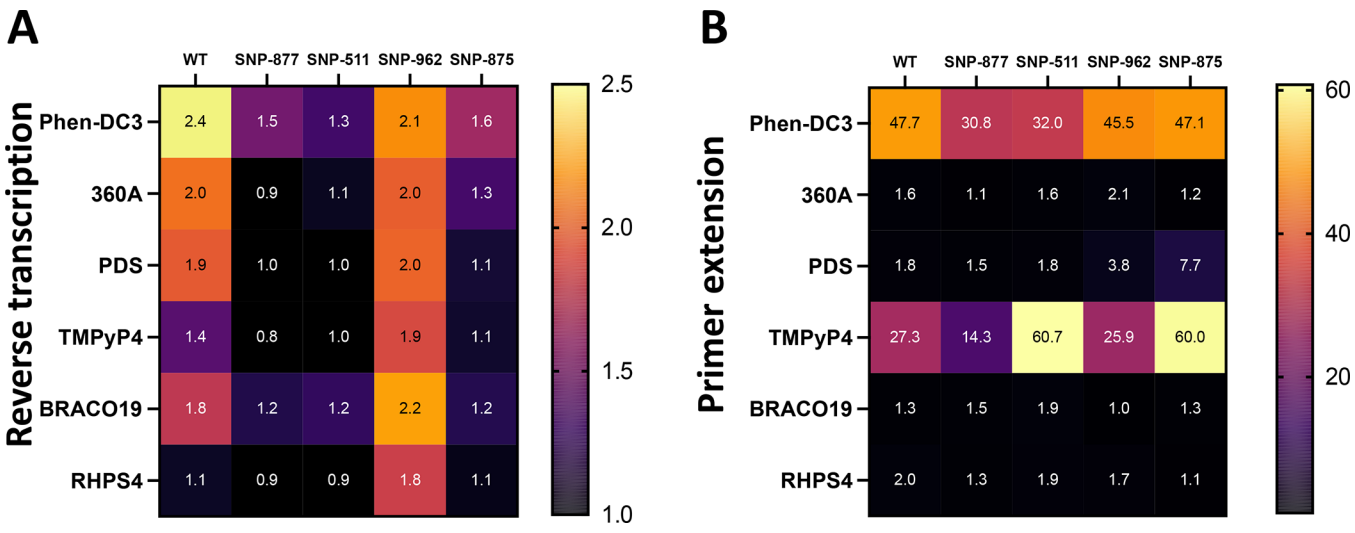

Figure 3. Evaluation of the stalling sites induced by the ligands. Data were plotted as heat plots. (A) Results from the RTS assays in the presence of 10 equiv of the ligands. The stalling sites were quantified for each ligand, and then were normalized to the KCl condition. (B) Results from the PE assays in the presence of 9 equiv of the ligands. The stalling sites were quantified for each ligand and then were normalized to the KCl condition. The legend shows the fold change color palette over the KCl condition. Together, the results presented in this Letter show that the SNPs present in α-synuclein have a strong impact on G4 ligand response. Three SNPs were shown to reduce this response, and one was shown to be beneficial for G4 folding and to inhibit polymerase progression. Although the G4 ligands are quite similar chemically (i.e., they are rich in planar, aromatic cyclic moieties), the observed differences in stabilization efficiencies have been well documented over the past 20 years.[5] However, their differential impacts on SNPs, especially on RNA G4s, are very clear here. To our knowledge, this is the first report describing the effect of SNPs on G4 ligand binding efficacy and specificity. While this study used α-synuclein as a proof-of-concept, SNPs are highly prevalent in the human genome and the interception of those with G4s must be considered for future G4 targeting. This concept is of importance for personalized medicine, where a weaker ligand could be selected to stabilize a desired SNP-G4 patient, without stabilizing every G4 and deregulating signaling pathways in cells.

guanines become, in general, at least 2 times more accessible to all ligands. This is characteristic of G4s.[20] The accessibility in SNP-511 seems to be highly modified only when it is incubated with both Phen-DC3 and 360A, where a 6- to 8-fold change was observed. All other ligands were observed to have a mild effect. Most of the SNP-511 structure is less accessible when incubated with RHPS4, a phenomenon observed only for this candidate. One possible way of explaining this observation is that a different type of G4 is folded in the presence of RHPS4. BRACO19 is the only ligand that induces a modification in base accessibility for SNP-875, while 360A is the only one that does so for SNP-877, ranging from 2- to 3- and 2- to 16-fold, respectively. The accessibilities of the WT and SNP-875 are less affected than those of the other three SNPs. In the case of the DNA sequences, the WT guanine accessibilities seem to increase with the presence of the ligands, up to a maximum change of 2-fold, except for Phen-DC3 where a mixture of accessibility changes was observed. The guanines in SNP-877, SNP-926, and SNP-875 were all around 2-fold less accessible when incubated with Phen-DC3 as compared to those with the other ligands. Again, this decrease is characteristic of G4 formation. PDS seems to only be able to stabilize SNP-875. All ligands produced a clear cleavage pattern in *in-line* probing experiments (Figure S4), except for TMPyP4 where a smear was observed. Consequently, it is hard to conclude and be quantitative in the case of TMPyP4. TMPyP4 has been described as a promising G4 ligand in many studies, but recently, doubts have been raised about its efficacy.[28] Dynamic light scattering experiments have revealed that the G4 stabilization/destabilization capacity of TMPyP4 is often misinterpreted because of its capacity to induce precipitation. The smear results mentioned above could confirm the occurrence of precipitation.[28] Globally, these results show that, depending on the SNP, the ligands induced differential folding on the different nucleic acid sequences.

Next, it was attempted to see if these nucleotide accessibility differences would lead to a difference in polymerase progression through the newly folded structure. With this aim, the sequences were incubated with the ligands and then were analyzed by a reverse transcriptase stalling assay (RTS) for RNA and by primer extension (PE) assay for DNA. For both techniques, a hairpin was added at the end of each sequence for primer hybridization, as described previously.[29] Importantly, these hairpins were not able to induce, nor able to impair, the natural structure of the α-synuclein sequences (Figures S6 and S7). All of the reactions were analyzed by electrophoresis on denaturing polyacrylamide gels and then were quantified by densitometry (Figures S8 and S9). The stalling assays were initially performed in the presence of either LiCl or KCl without any ligands to see if G4s could naturally impact the enzyme's progression (Figures S8 and S9, lanes 2 and 3). These results showed a differential response to the cation used in both RTS and PE, with the LiCl condition showing slightly more stalling sites than did the KCl one. These differential efficiencies of the enzyme's progression between LiCl and KCl were not expected, since G4s are generally considered to be absent in the presence of LiCl and would therefore not induce any more. Thus, the processivity of the enzyme seems to be affected by the cation used. Even though SNP-962 RNA was shown to fold into a G4 by fluorescence assay (Figure 1), the structure formed in the presence of KCl is not stable enough to induce stalling (Figure S8, see SNP-962 in lane 3). Subsequently, the RTS and PE were performed in the presence of 9 to 10 equiv of ligand in the presence of KCl in order to both promote G4 folding and to mimic intracellular conditions.[30] Data were normalized to

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the KCl condition so as to accommodate for cation processivity. For the RTS assays, both the WT and the SNP-962 RNA sequences, when incubated with the ligands, were shown to induce more stalling sites than did the three other SNPs (except for the WT in the presence of RHPS4; Figure 3A). These two candidates were the ones with the highest G4 scores. Therefore, all guanine doublets seem to be of some importance for ligand stabilization, and the conversion of a doublet into a triplet highly increases the response. Based on both the fluorescent and the RTS results, the absence of a signal with the NMM ligands and the strong stalling site for the WT points toward the presence of new ligand-induced G4s, while ligand-stabilizing effects are most likely what happened in the case of SNP-962, as evidenced by the initial fluorescence observed with NMM and by the strong stalling site. The Phen-DC3 ligand was able to induce stalling sites when incubated with each RNA candidate. This had previously been demonstrated, more specifically that Phen-DC3 acts as a chaperone for RNA G4 folding with long-non coding RNA.[25] Of note, two weak stalling sites that are not located in front of any stretch of guanines seem to be present in each construction with BRACO19, i.e., at positions 58 and 59. This could be attributed to the nonspecific binding interaction of BRACO19, previously reported with duplex and triplex structures.[31] For the PE assays, the stop differences between the KCl condition and the best ligands (Phen-DC3 and TMPyP4) were much stronger (on average around 40-fold versus 2-fold). Of note, while the PE assays were performed with Taq polymerase at 37 °C, the RTS assays were performed with reverse transcriptase (MMuLV) at 45 °C, which can have the effect of reducing G4 propensity as compared to what is seen at 37 °C. This could explain the stalling difference between the two experiments. Phen-DC3 was also found to be highly efficient in increasing the number of stalling sites of each DNA candidate. A reduction in the complete TMPyP4 product was also noted. However, as was observed in the *in-line* experiments, it seems like TMPyP4 induced precipitation since no apparent stop can be identified prior to the final one (as seen previously[28]). Contrary to the RTS results, DNA WT and SNP-962 did not seem to inhibit the polymerase's progression at the same level with each ligand. A particular polymerase-impairing interaction between SNP-875 and PDS seems to be specific for this pair (7.7-fold). Once again, these results point toward a differential binding of the ligands for the different sequences and a different response between DNA and RNA G4s to the ligands.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.4c00104.

Material and Methods and additional experimental figures and tables; G4RNA Screener scores by position; NMM fluorescence; structure prediction; gel analysis (PDF)

QGRS analysis (XLSX)

Oligonucleotides used in this study (XLSX)

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Author Contributions

M.A.T. and J.P.P. conceptualized this study. M.A.T. performed the experiments. M.A.T. and J.P.P. analyzed the data. MAT and JPP wrote and revised the manuscript.

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Notes

The authors declare no competing financial interest.

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