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G-Quadruplex Formation Interferes with P1 Helix Formation in the RNA Component of Telomerase hTERC

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Guanine-rich nucleic acids can adopt unusual structures called guanine quadruplexes (G4) that are based on stacked guanine quartets (Figure 1 A, bottom right). DNA sequences that are prone to adopt such structures are found in telomeric repeats, in several promoters, in ribosomal DNA, and in the immunoglobulin switch region; these structures appear to be biologically relevant.[1] Formation of G4 has been demonstrated in vivo in ciliate telomeres,[2] and during G-rich sequence transcription[3] (G-loop). More recently, G4 formation in RNA sequences has been investigated.[4,5]

Telomerase, the nucleoprotein complex that is responsible for telomere maintenance and homeostasis, is a key component of cell proliferation and its reactivation is often associated with tumorigenesis. Inhibitors of telomerase function,[6] including antisense oligonucleotides that target the telomerase RNA component[7] (hTERC) or G-quadruplex ligands that target the telomeric 3′-overhang substrate for telomerase elongation and binding,[8] offer therapeutic promise. The telomerase RNA is structurally conserved among many organisms, but its primary sequence and size are not.[9] In most vertebrates, the predicted structures possess a helical domain called the P1 helix that is located 5′ to the template sequence. This helix is thought to serve as a template boundary element to limit reverse transcription to the 6-nucleotide template.[10] In many mammals, a 5′ tail precedes this helix, but no precise function has been assigned to this tail. Analysis of these sequences shows a skewed base composition with several well-conserved runs of guanines in this region (Figure 1 B). Such G-rich RNA sequences have the potential to form G quadruplexes that may interfere with P1 helix formation and its template boundary function (Figure 1 A).

In this work, we demonstrate that RNA fragments with the sequence of the 41 5′-most nucleotides of hTERC form a quadruplex. This G4 formation hinders the formation of a helix that corresponds to P1. We further demonstrate that this guanine quadruplex interacts with a specific guanine-quadruplex ligand, **360A**;[11] the presence of **360A** enhanced inhibition of P1 helix assembly.

We synthesized an oligoribonucleotide with the sequence of the first 41 nucleotides of hTERC, **41R** (Table 1). We also designed and synthesized the **41m** control sequence (changes are underlined in Table 1), in which guanine runs have been disrupted by G-to-A changes. UV absorbance as a function of temperature offers a convenient method for characterizing nucleic acids structures. With **41R**, we observed a typical cooperative reversible transition around 295 nm; the temperature of mid-transition (T_m) depended upon the nature and the concentration of the monovalent cation present in the solution. T_m values ranged from 38 °C in 100 mM LiCl to more than 80 °C in 100 mM KCl (Figure 2 A). Results are presented in detail in Table 2. The structure that is formed by this sequence was surprisingly stable even in the absence of added potassium and sodium ($T_m = 39$ °C, see Table 2). The addition of as little as 0.1 mM KCl in the absence of any other added monovalent cation was sufficient to stabilize the structure. The temperature range in which the transition occurred was independent of the concentration of oligonucleotide in the 0.5 to 25 μM range; this demonstrates that folding was intramolecular (data not shown). No transition was observed at 295 nm for the **41m** control. These finding suggest that **41R** adopts an intramolecular G-quadruplex structure.[13]

Thermal difference spectra (TDS) for **41R** in Li^+, Na^+ or K^+ conditions were also typical of G quadruplexes,[14] with a negative peak around 297 nm and two positives peaks around 274 nm and 242 nm (Figure 2 B). Circular dichroism (CD) spectra of **41R** had a positive peak at 263 nm and a negative peak at 240 nm (Figure 2 C), as is typical of G4 formation. Although CD spectra with these characteristics have been attributed to parallel G quadruplexes in the light of recent publications,[15,16] we prefer to describe these as type I spectra. These spectra are characteristic of quadruplexes with all stacked quartets with the same polarity,[16] likely to be in the present case with all guanine glyocosidic bonds in the *anti* conformation and thus with parallel strands. CD and TDS spectra for **41m** do not correspond to those typical of G quadruplexes (not shown).

Finally, enzymatic probing with RNase T1 confirmed quadruplex formation. In the wild-type **41R** sequence, several Gs were protected in buffer that contained K^+, but not in buffer that contained only Li^+ as a cation (see Figure S1, left in the Supporting Information). These results confirm the formation of a cation-dependent folded structure by **41R**: In K^+, five protected clusters of G were observed; this suggests either a complex intramolecular folding or the coexistence of at least two foldings that share three runs of Gs. In contrast, digestion of **41m** showed only a slight protection of the central part of the sequence (Figure S1, right).

Having demonstrated that the 5′ fragment of hTERC 5′ forms a stable quadruplex, we then investigated whether this structure could interfere with secondary structure formation. Some of the guanines that are involved in the intramolecular quadruplex are base-paired in the P1 helix, thus making these

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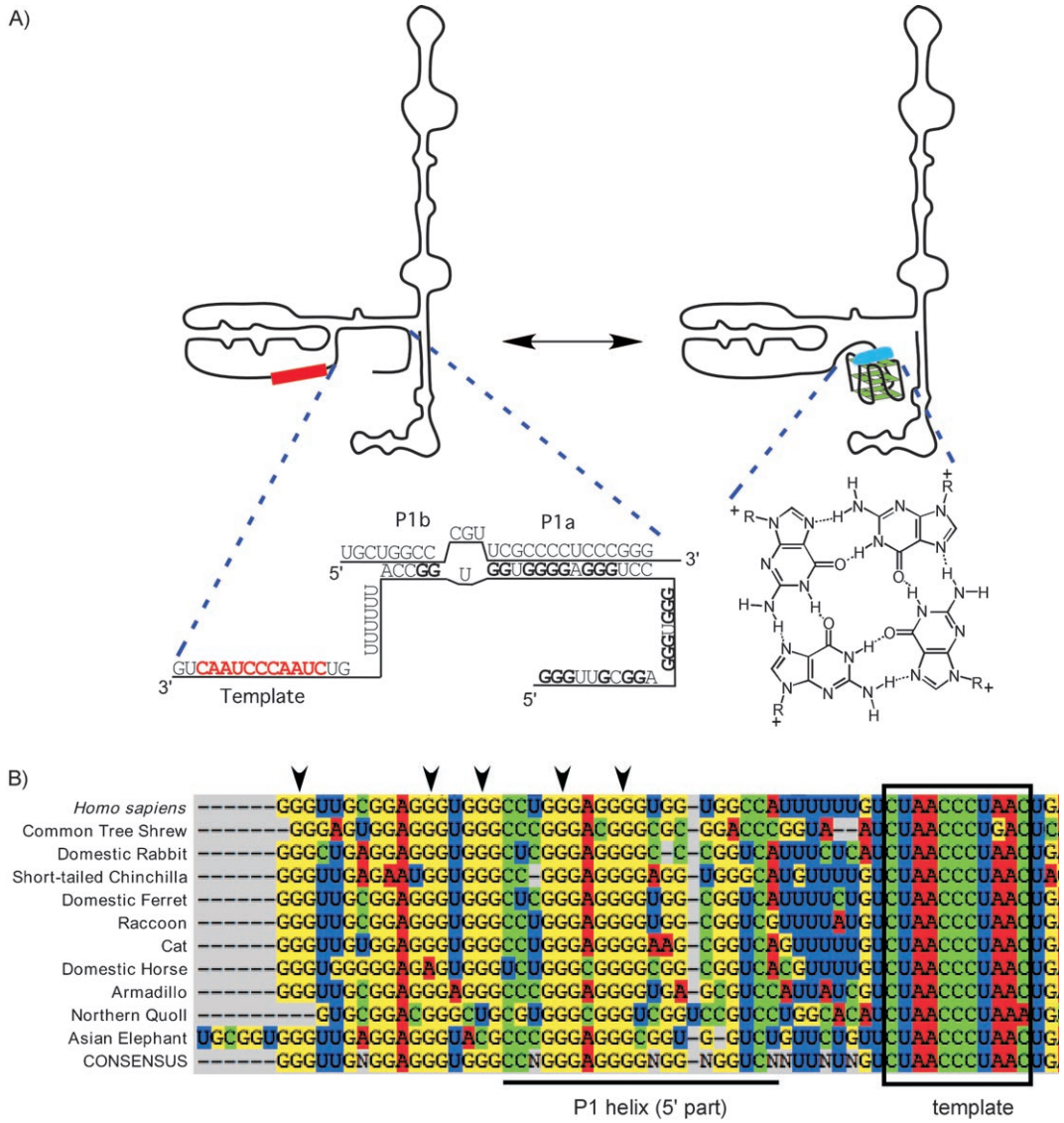

Figure 1. A) Schematic representation of a folded hTERC (left) in equilibrium with a partially unfolded one (right). The lower portion of the figure shows the sequence of the 5′ region of hTERC with the P1a and P1b helices and the template region indicated. In this partially unfolded hTERC shown on the right, a G-quadruplex is formed between the 5′ tail and a region that is part of the P1a helix. A G4 ligand stabilizes this structure (blue ellipse). The lower portion of this Figure shows a G-quartet. B) Sequence alignment of various mammalian TERCs. The regions 5′ and through the template are represented. The template and P1-helix regions are noted and arrows indicate conserved runs of Gs. A recent publication[12] suggested that G4 formation occurs within the first 17 nucleotides of hTERC. Li et al. argue for a telomerase inhibitory effect of this G-rich tail that is unrelated to G4 formation.

Table 1. Oligonucleotide sequences.

Name	Sequence (5′–3′)	ε [L mol^{-1} cm^{-1}]
41R	GGGUUGCGGAGGGUGGGCCUGGGAGG-GGUGGUGGCCAUUUU	401 300
41m	GAGUUGCGAAGAGUGAGCCUGAGAGA-AGUGAUGGCCAUUUU	423 100
25rP1	UGCUGGCCCGUUCGCCCCUCCCGGG	210 900
25rP1m	UGCUGGCCCGUUCGCUUCUCUCGGG	218 200

two structures mutually exclusive (Figure 1 A). Formation of the duplex was first tested by using spectroscopic methods (Table 2). Under conditions that are unfavorable to quadruplex formation (100 mM LiCl) no transition was observed at 295 nm as a function of temperature when **41R** was mixed with an equimolar concentration of the complementary strand **25P1** (Table 1), but a clear duplex transition with a T_m of 75 °C was observed at both 240 and 260 nm. Under low K^+ conditions (10 mM) but with the same total monovalent cation concentration, the duplex transition was observed with a comparable T_m but a transition at 295 nm suggested a coexistence of both duplex and quadruplex. In 100 mM K^+, the T_m of the transition that is assigned to the duplex was slightly decreased (73 °C) and partial quadruplex formation was observed (data not shown). From these experiments, it is clear that the duplex was formed at physiological temperature but it was not possi-

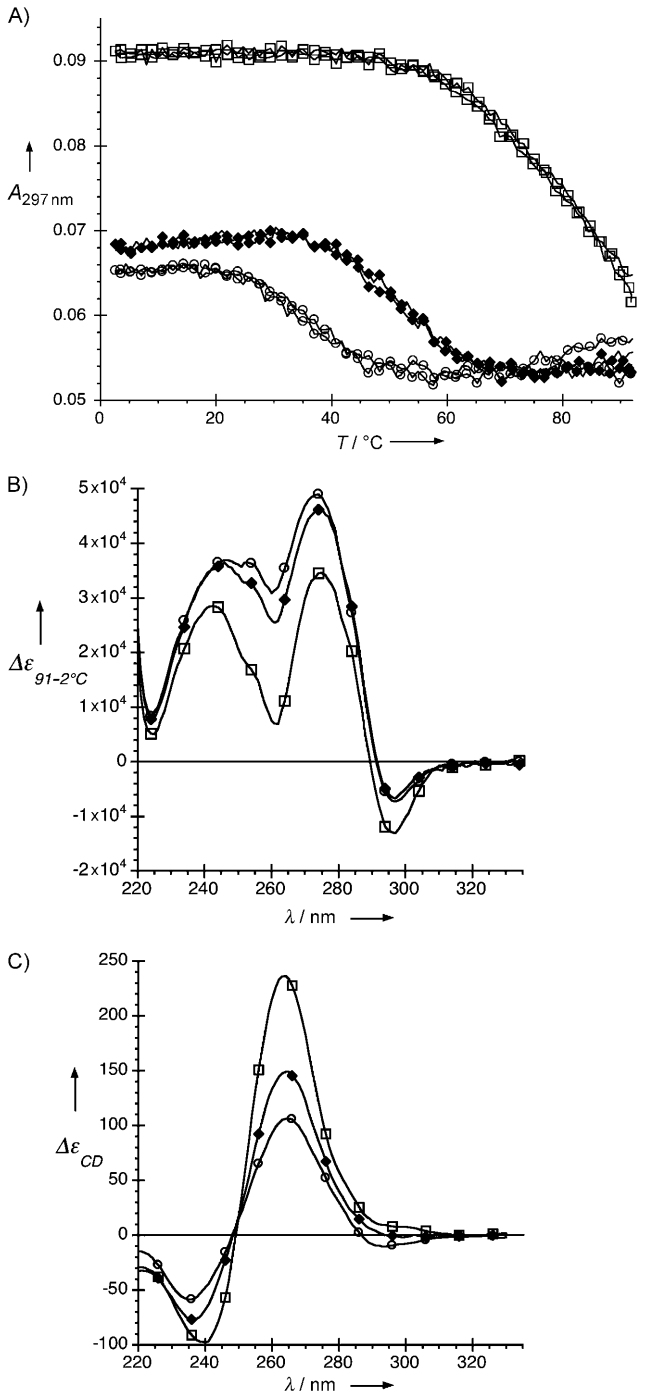

Figure 2. A) UV melting (and annealing) followed at 297 nm of **41R** (2 μM) in 20 mM lithium cacodylate buffer and 100 mM LiCl (○), 100 mM NaCl (◆) or 100 mM KCl (□). B) Thermal difference spectra and C) Circular dichroism spectra at 25 °C of **41R** (2 μM) in 20 mM lithium cacodylate buffer and 10 mM LiCl (○), 10 mM NaCl (◆) or 10 mM KCl (□). $\Delta\varepsilon$ are expressed in $L\,mol^{-1}\,cm^{-1}$.

ble to conclude whether quadruplex formation interfered with this duplex formation. With sequences **41m** and **25rP1m**, a slightly less thermostable duplex was formed that had a T_m of 63 °C; this was expected because this duplex had a lower GC content than that formed by **41R** and **25rP1** (58% vs. 74%).

Table 2. Mid points of absorbance melting transitions of quadruplex and duplexes formed by wild-type and mutant hTERC sequences.

Oligonucleotide	Conditions	T_m [°C]
41R	no added cation[a]	39[b]
41R	100 mM LiCl	38[b]
41R	100 mM NaCl	51[b]
41R	0.1 mM KCl	43[b]
41R	1 mM KCl	54[b]
41R	10 mM KCl	68[b]
41R	100 mM KCl	> 80[b]
41R	0.1 mM KCl, 99.9 mM LiCl	38.5[b]
41R	1 mM KCl, 99 mM LiCl	50[b]
41R	10 mM KCl, 90 mM LiCl	66[b]
41R + **25rP1**	100 mM LiCl	75.5[c]
41R + **25rP1**	10 mM KCl, 90 mM LiCl	75[c]
41R + **25rP1**	100 mM KCl	73[c]
41m + **25rP1m**	100 mM LiCl	63[c]
41m + **25rP1m**	10 mM KCl 90 mM LiCl	63.5[c]
41m + **25rP1m**	100 mM KCl	62.5[c]

[a] The buffer contains 18 mM Li^+. [b] Quadruplex T_m was determined on the 295 nm melting profiles. [c] Duplex T_m was determined on the 240 nm melting profiles, no T_m could be determined on the 295 nm melting profiles.

Gel-shift experiments by using radiolabeled **25rP1** or **25rP1m** clearly demonstrated a specific and quasi-stoichiometric interaction between these oligonucleotides and the corresponding 41-mers under Li^+ conditions (Figure 3, left). In K^+ (KCl 10 mM/LiCl 90 mM or KCl 100 mM), much higher concentrations of **41R** (50 and 500 nM, respectively) were required to give rise to the band that is assigned to the duplex (Figure 3A); under these conditions, duplex formation was quasi-stoichiometric with **41m** (Figure 3B). These experiments demonstrate that G4 formation hindered the formation of a duplex that corresponds to the P1 helix.

The data that were obtained from the above experiments prompted us to study the effect a quadruplex ligand, the pyridine dicarboxamide **360A** (Figure 4A), on duplex formation. This molecule was previously shown to bind to[17] and induce the formation[18] of DNA quadruplexes, and we first established that this molecule recognizes RNA quadruplexes. We characterized the direct interaction of **360A** with different RNA strands by using fluorescence quenching of **360A** to demonstrate a very tight binding to the G4-forming sequence **41R**, and a much lower-affinity binding to **41m**, **25rP1** and **25rP1m** (Figures 4B and S2). A specific interaction between **360A** and **41R** was confirmed by CD titration and FRET melting (see Figures S3 and S4).

We then analyzed the effect of the ligand on the duplex–quadruplex competition. For **41R** in K^+, gel-shift experiments with increasing concentrations of **360A** that were added prior formation of the duplex demonstrated that this ligand prevented duplex formation with an IC_{50} of approximately 0.5 μM, whereas no inhibition of duplex formation was observed for **41m** and **25rP1m** (Figure 4C), or for either system in 100 mM LiCl (Figure S5). A control experiment showed that there was no interaction between the ligand and either of the 25-mer-labeled strands (data not shown).

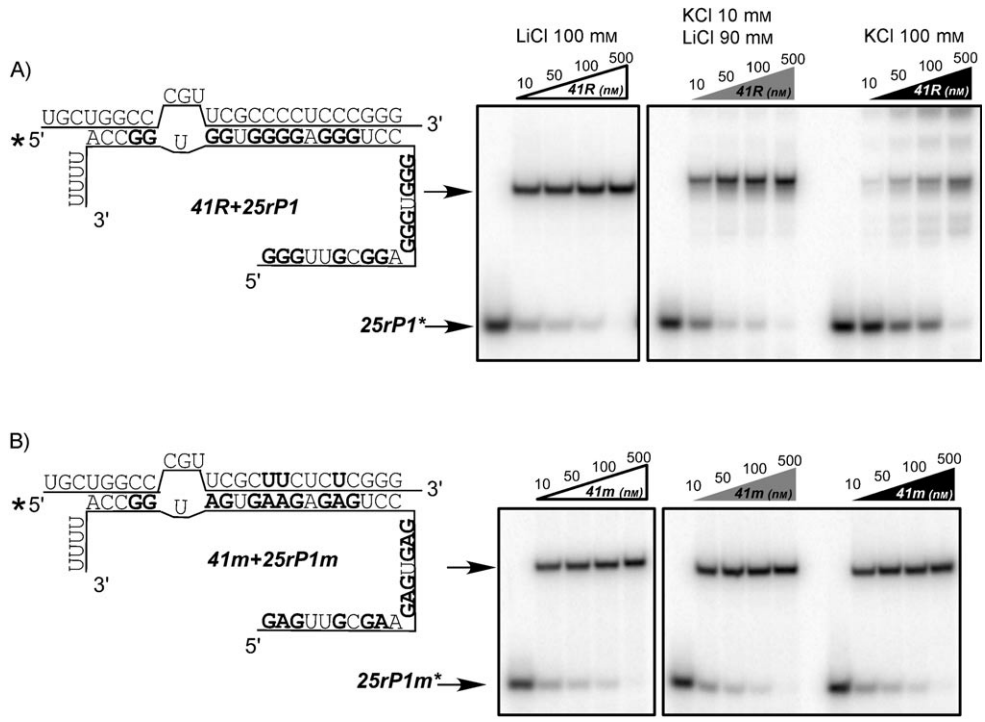

Figure 3. A) Gel-shift analysis of duplex formation between **41R** and radioactively labeled **25rP1**. Left panel: Duplex formation in 50 mM HEPES and 100 mM LiCl (left), 10 mM KCl, 90 mM LiCl (center) or 100 mM KCl (right) with **41R**. Arrows indicate migration of **25rP1*** as single strand (lower part of the gel) and as part of a duplex with **41R** (upper part of the gel). B) Gel-shift analysis of duplex formation between **41m** and **25rP1m*** under the same conditions that are described in panel A.

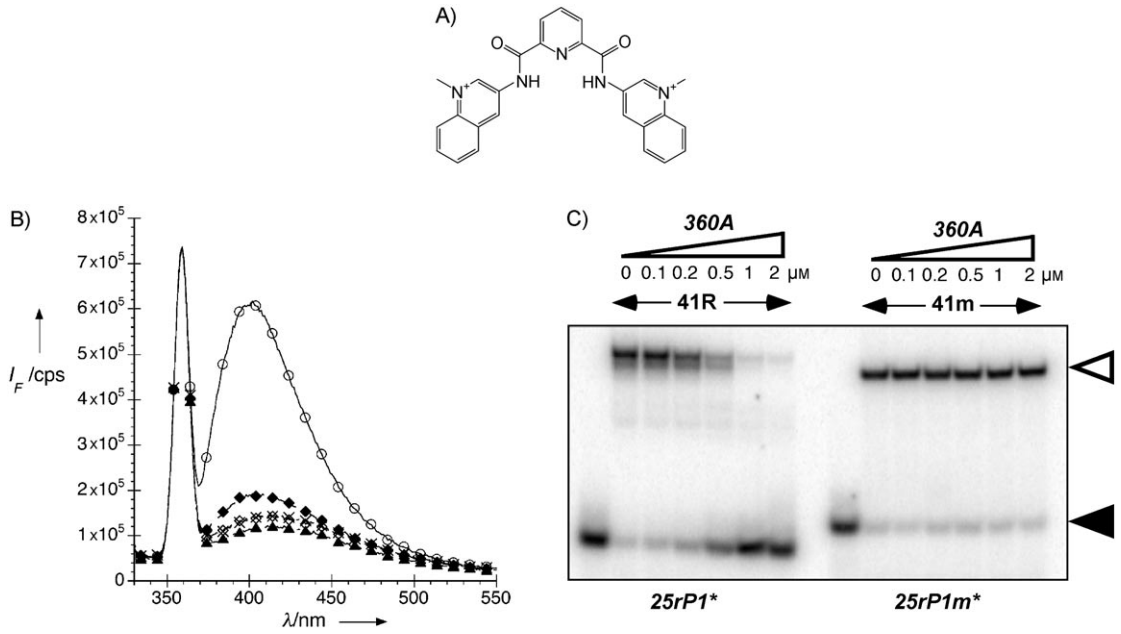

Figure 4. A) Formula of **360A**. B) Fluorescence titration of **360A** (0.4 μM, open circles) with increasing concentrations of **41R** (0.1 μM, ◆; 0.2 μM, ◇; 0.4 μM, ×). The fluorescence of the buffer is represented in triangles. Buffer: 20 mM lithium cacodylate, 10 mM KCl, 90 mM LiCl. Excitation: 320 nm (Raman at 359 nm). C) Inhibition of P1-helix assembly by **360A**. Radiolabeled **25rP1*** (left) or **25rP1m*** (right) was mixed with an excess of the complementary 41-mer (50 nM of **41R** or **41m**, respectively) and increasing concentrations of **360A** (100 nM to 2 μM) in 50 mM HEPES, 10 mM KCl, 90 mM LiCl. Migration of the 25-mer alone and of P1 helices are indicated by filled and open triangles, respectively.

We have demonstrated that the sequence of the 5′-end of hTERC forms a quadruplex and that this structure interferes with P1 helix formation. A known quadruplex ligand shifted this competition toward the quadruplex. This is, to our knowledge, one of the first reports of the effects of a RNA quadruplex ligand on a physiologically relevant RNA. Wieland and col-

leagues demonstrated that a cationic porphyrin could modulate the activity of a ribozyme with a G4-prone sequence,[19] but this ligand has a relatively modest selectivity for quadruplexes.[20] Because **360A** interacted with the G4 structure that was formed by the telomerase RNA, researchers should use caution when accessing the effect of G4 ligands on telomeres or in cellular systems in general.

Our work, as previously suggested by Kumari et al.,[5] shows that G4 RNA might constitute a relevant therapeutic target for G4 ligands. The possibility that hTERC forms a quadruplex in vivo suggests that stabilization of this G4 structure by a small ligand might change the overall conformation of the full-length hTERC and prevent formation of the P1 helix. Without this helix, the telomerase template boundary is not defined, and function might be impaired.[10] Complex biochemical experiments will be required to demonstrate a hTERC-mediated effect of this ligand on telomerase, because a direct assay must be used to differentiate the effects of the ligand on the DNA substrate from those on the telomerase RNA.[21] Ideally, by comparing the effects of two quadruplex ligands, one highly selective either for the telomeric DNA quadruplex, the other for the hTERC RNA quadruplex would simplify the analysis. Unfortunately, these molecules remain to be discovered, as all the ligands that we have tested so far bind to both types of quadruplexes (data not shown). The identification by DCC of a ligand that presents a modest but encouraging selectivity (2.6 fold) between two DNA quadruplexes and still a low affinity ($K_d = 9\ \mu M$) has been recently reported.[22] Such an approach might be suited to identify RNA versus DNA quadruplexes ligands.

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