Recognition and Stabilization of Quadruplex DNA by a Potent New Telomerase Inhibitor: NMR Studies of the 2:1 Complex of a Pentacyclic Methylacridinium Cation with $d(TTAGGGT)_4$**

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Telomerase is a cellular ribonucleoprotein, consisting of a reverse transcriptase and an endogenous RNA template, that maintains telomere length at the ends of eukaryotic chromosomes.[1] Telomeres consist of double-stranded DNA composed of tandem repeats of guanine-rich sequences (5′-TTAGGG in humans) with a single-stranded 3′-end overhang necessary to ensure complete chromosomal DNA replication, while protecting the chromosome ends from fusion and degradation.[1, 2] The telomeres shorten with each round of cell division, due to incomplete lagging-strand replication, limiting the number of replicative cycles before the cell enters a state of senescence.[3] Telomere length in immortalized cells (e.g. cancer cells) is tightly regulated by the enzyme telomerase whose reverse transcriptase activity allows the addition of 5′-TTAGGG repeats to maintain telomere length through an indefinite number of cell divisions.[4] While telomerase activity is absent in human somatic cells it is detected in the majority of human tumor-derived cell lines. Consequently, telomerase has become a high-profile target for anticancer drug design.[5]

The discovery that the G-rich telomeric repeats are able to assemble into novel four-stranded quadruplex structures, consisting of guanine tetrads stabilized by monovalent cations (Na^+ and K^+) (Figure 1),[6] has focused attention for structure-specific drug design. Since telomerase reverse transcriptase activity depends on a single-stranded DNA primer,[4] small molecules that bind and stabilize the folded quadruplex form of the primer are potential inhibitors of telomerase function. A number of quadruplex-specific ligands have been reported with the common feature of an extended aromatic ring system capable of interacting through extensive π stacking with G-tetrads.[5] However, there has been a paucity of detailed structural data available on the drug–DNA complexes due to intractable NMR spectra arising from extensive drug-induced line broadening.[7] The exception is a dicationic perylene-tetracarboxylic diimide derivative (PIPER), which forms either a sandwich complex bound between the blunt ends of a quadruplex dimer formed from $d(TTAGGG)_4$, or

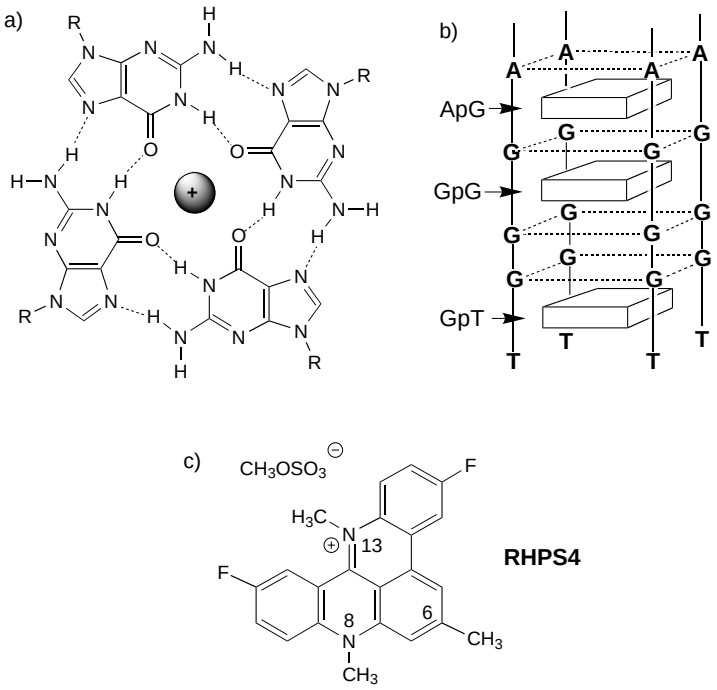

Figure 1. a) Structure of the G-tetrad showing complexation with K^+. b) Schematic of the intermolecular quadruplex showing potential sites of intercalation at ApG, GpG, and GpT sites. c) Structure of **RHPS4**.

intercalates by end-stacking at the GpT step of $d(TAGGGTTA)_4$.[8]

Here we describe the interaction and stabilization of quadruplex DNA by a novel fluorinated polycyclic methylacridinium salt (**RHPS4**; Figure 1c), which shows enhanced binding to higher ordered DNA structures (triplex/quadruplex) over duplex and single-stranded DNA as measured by differential dialysis.[9a,b] **RHPS4** has been shown to inhibit telomerase with an IC_{50} value of 0.33 μM, while decreasing tumor cell proliferation of breast 21NT cells at concentrations as low as 200 nM.[9c,d] **RHPS4** is weakly cytotoxic (mean GI_{50} value in the NCI 60 human tumor cell panel is 13.18 μM), giving a therapeutic index (GI_{50}/IC_{50}) of 40.[9a] This activity does not appear to be associated with Taq polymerase and topoisomerase II inhibition, strongly suggesting that **RHPS4** is an inhibitor of telomerase function. To investigate structural details of the drug–quadruplex interaction we have carried out ^1H NMR spectroscopic studies on the **RHPS4** complex with the intermolecular parallel-stranded quadruplex $d(TTAGGGT)_4$, formed from the human telomeric repeat (Figure 1).

1D and 2D NMR spectra of $d(TTAGGGT)_4$ are extremely well-resolved.[10] The NMR data show that the structure is highly symmetrical with all four strands equivalent.[11] Three imino proton resonances are observed between $\delta = 11-12$, each signal representing equivalent guanine bases within a given tetrad (Figure 2a). We see no resonances attributable to thymine imino protons, which precludes the formation of stable T-tetrads. The pattern and intensity of internucleotide NOEs within the G4G5G6 region is consistent with the results of previous studies of intermolecular quadruplexes, which showed strong base stacking between adjacent G-tetrads. We

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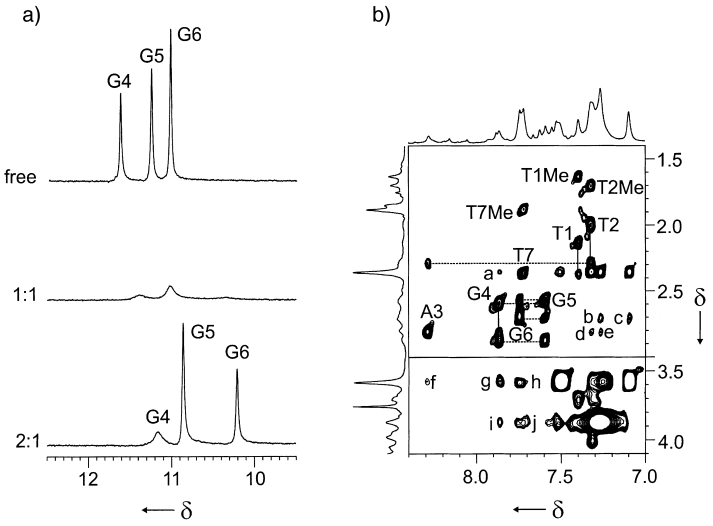

Figure 2. a) 1D ^{1}H NMR data recorded in 90% H_2O solution showing the guanine imino proton resonances for G4, G5, and G6 in d(TTAGGGT)$_4$ (free), the 1:1 complex with **RHPS4**, and the 2:1 complex all at 303 K. b) Portion of the 250 ms NOESY spectrum of the 2:1 complex recorded at 318 K highlighting a number of key NOEs. In the top panel, NOEs labeled T1 through to T7 represent intranucleotide base H6 or H8 to deoxyribose H2′/H2″. Dashed horizontal lines highlight internucleotide NOE connectivities. Thymine methyl to H6 NOEs are also assigned. Key drug–DNA NOEs are labeled as follows: a) 6-Me to G4H8, b) H7 to G6H2′/H2″, c) H5 to G6H2′/H2″, d) H1 or H12 to A3H2′/H2″, e) H7 to A3H2′/H2″, lower panel; f) 8-Me to A3H8, g) 8-Me to G4H8, h) 8-Me to G6H8, i) 13-Me to G4H8, and j) 13-Me to G6H8/T7Me.

observe a number of additional NOEs at the A3–G4 step (G4NH to A3H8 and A3H2) that indicate good stacking interactions between the two sets of purine bases. Further, the amino proton of A3 (A3-NH_2) is readily resolved at $\delta = 7.1$ and exhibits strong NOEs to G4NH and A3H2. The A3-$NH_2 \leftrightarrow$ A3H2 NOE is not expected to arise as an intranucleotide NOE but is consistent with an interaction between adenine bases within an A-tetrad. Quantitative NOE measurements involving sugar H1′ ↔ base H6/H8 confirm that all glycosidic bonds are *anti* including that of A3. This contrasts with the data of Hosur et al. for d(AGGGT)$_4$ where the adenines were shown to form an A-tetrad with the purines in the *syn* conformation, while the T and A residues of d(TAGGGT)$_4$ appeared to have a more flexible conformation resulting in poor stacking interactions.[12] Our data reveal that the A3-NH_2 signal persists at high temperature and shows similar stability to the guanine imino protons. Thus, additional thymines on the 5′-end appear to have a significant effect in stabilizing and modulating nucleotide conformation and base stacking interactions. We have calculated a high-resolution structure of d(TTAGGGT)$_4$ using an NOE-restrained MD protocol with an explicit solvation model.[13] Thus, this initial structure provides a starting point for studies of drug–quadruplex interactions.

Titration of **RHPS4** into d(TTAGGGT)$_4$ results in significant line broadening which we attribute to intermediate exchange of the drug between a number of possible bound conformations and/or a number of sites of similar affinity. The three guanine imino protons are seen to broaden considerably at a drug/quadruplex ratio of 1:1 (Figure 2a). Titrating drug beyond this point up to a 2:1 ratio produces a new set of resonances that are sharper and appreciably shifted by up to 0.8 ppm. Although the imino proton of G4 remains exchange broadened at 298 K, suggestive of drug mobility within the ligand binding site, this resonance sharpens appreciably above 318 K. The spectra were generally of superior quality at higher temperatures where the drug appears to be in fast exchange between binding sites and different bound conformations. The effect of the ligand on quadruplex stability is clearly evident from the intensity of the guanine imino proton resonances which disappear when the free quadruplex is fully melted at 333 K. However, these resonances are still visible in the 2:1 drug complex at temperatures up to 353 K. NOESY data collected at 318 K show the quadruplex structure to be fully formed with full retention of the fourfold symmetry and a largely similar pattern of sequential connectivities along the DNA backbone. Chemical shift analysis shows the thymines T1 and T2 to be unperturbed by ligand binding, however, the base H6 and CH_3 of T7 shift by 0.2–0.35 ppm. The data show that binding affects the A3G4 and G6T7 segments primarily, particularly G4 and G6. Many internucleotide NOEs between G4 and G5, and G5 and G6 indicate that the integrity of the stack of G-tetrads is preserved (Figure 2b). At the A3–G4 step the internucleotide NOEs are weaker but still evident.

Drug resonances are readily assigned from a combination of TOCSY and NOESY data and allow a number of drug–quadruplex interactions to be identified that pinpoint the drug binding sites (Figure 2b). By far the largest number of intermolecular NOEs involve G4 and G6, while none are attributed to G5 at the center of the stack of G-tetrads. NOEs are detected to both purine nucleotides at the A3–G4 site, indicating that the ApG step is a primary intercalation site. A number of NOEs to G6 show that the drug also inserts at the G6–T7 step; however, very few NOEs are observed to T7 despite the perturbations to its chemical shifts. Two very strong NOEs from the drug 8-CH_3 to G4NH and G6NH show that the drug is stacking primarily with the G-tetrads with many other drug–DNA NOEs involving base and sugar protons positioning the edges of the acridine ligand in the grooves. The general effects of ligand binding on line widths means that some weak NOEs observed in the free quadruplex are difficult to detect in the bound state. The 4-NH_2 group of A3 is still visible and stabilized against exchange, as well as being upfield shifted by 0.4 ppm, suggesting that the A-tetrad is still intact, although drug–DNA NOEs to the adenines are generally weak. We conclude that the drug complex is stabilized primarily through extensive π stacking with the G-tetrads.

Guided by 24 drug–DNA NOEs, we have modeled the complex using NOE-restrained MD simulations (100 ps) with drug molecules docked at the ApG and GpT steps. A low-energy structure that satisfies the majority of the distance restraints is shown in Figure 3a, with the partial positive charge on the acridine 13-N acting as a pseudo potassium ion positioned above the center of the G-tetrad.[14] The overlap between the π system of the drug and the complementary G-tetrad of G4 (Figure 3b) suggests several key positions around the acridine ring that may provide future points for ligand substitution to enhance quadruplex affinity and specificity through interactions in the grooves.

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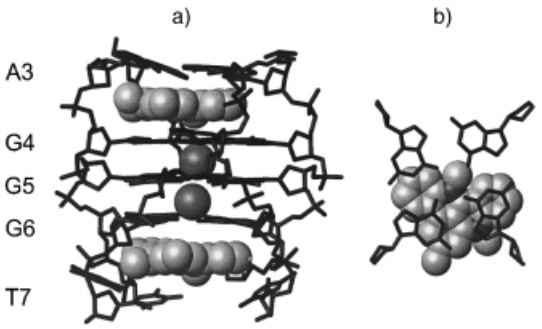

Figure 3. a) Structure of the 2:1 complex with d(TTA*GGG*T)$_4$ showing the AGGGT core with **RHSP4** intercalated at the A3–G4 and G6–T7 sites. b) π Stacking of the drug with the G4 tetrad showing the orientation with respect to the various grooves.

Recent detailed studies of cationic porphyrin–DNA quadruplex interactions have invoked both GpG intercalation and violation of the neighboring site exclusion principle.[15] In contrast, the perylenetetracarboxylic diimide intercalator (PIPER) described by Federoff et al.,[8] and the polycyclic methylacridinium cation reported here, are unable to disrupt the G-tetrad core by insertion at a GpG step and displacement of stabilizing K^+ ions. Instead the drug forms complex-stabilizing interactions by stacking on the ends of the G-quadruplex. Similar conclusions have been drawn from modeling studies of a disubstituted anthraquinone,[5e] and a number of trisubstituted acridine derivatives with the human intramolecular quadruplex where the drug is proposed to bind to the TTA cross-over loop while end-stacking with a G-tetrad.[5f] In the complex of PIPER with d(TAGGGTTA)$_4$, formation of the 1:1 complex appears to favor end-stacking at the GpT step rather than the ApG step, which the authors attribute to the difficulty of disrupting the stronger base stacking between purine tetrads.[8] In contrast, **RHPS4** appears to show relatively little discrimination between these two intercalation sites. The **RHPS4**-d(TTAGGGT)$_4$ complex represents one of a very limited number of drug–quadruplex structures so far characterized. We are currently utilizing this structural information in further rational design and targetting of these higher ordered DNA structures.

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[14] In the final energy minimized structure no restraint violations > 0.3 Å were observed for the DNA, however, three of the 24 drug–DNA NOEs were violated by > 0.3 Å. Although we have modeled a single bound drug conformation at the ApG and GpT intercalation sites, the dynamic nature of the interaction (evident from ^{1}H and ^{19}F temperature-dependent line widths) does not preclude multiple interconverting conformations, all of which will contribute to the average NOE intensity. The analysis of NOEs from a single DNA proton to several drug protons apparently remote from each other on the acridine ring can arise from the drug rotating between the four equivalent stacked conformations, though this is further complicated by the fourfold symmetry of the quadruplex which makes each strand equivalent by NMR. We are currently evaluating a more dynamic modeling approach.

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