# ChemComm

## COMMUNICATION

# Ligand selectivity in stabilising tandem parallel folded G-quadruplex motifs in human telomeric DNA sequences

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**Biophysical studies of ligand interactions with three human telomeric repeat sequences (d(AGGG(TTAGGG)$_n$, $n$ = 3, 7 and 11)) show that an oxazole-based 'click' ligand, which induces parallel folded quadruplexes, preferentially stabilises longer telomeric repeats providing evidence for selectivity in binding at the interface between tandem quadruplex motifs.**

The prevalence of guanine-rich sequences throughout the human genome, coupled with their ability *in vitro* to adopt stable folded quadruplex structures in the presence of physiological concentrations of monovalent cations, suggests that this structural motif serves a wide functional role in regulating gene expression,[1–5] making quadruplexes potential therapeutic targets.[6–8] More specifically, telomeres, which are found at the ends of chromosomes, are composed of 150–250 nucleotides of d(TTAGGG) repeats and play a critical role in limiting cell proliferation by shortening with each round of replication.[9] The up-regulation of the enzyme telomerase (hTERT) in the majority of cancer cell lines serves to stabilise the length of the telomere and extend the replicative potential. The activity of hTERT is dependent upon a single-stranded template for sequence extension, making the stabilisation of compact telomeric quadruplex structures fertile ground for the design of small molecule telomerase inhibitors.[6–8]

The single-stranded telomere has the potential to adopt multiple quadruplex structures in the manner of a series of 'beads on a string'. The extent to which interactions between these adjacent or tandem quadruplex 'beads' can result in the formation of higher-order capping structures may have some relevance in protecting DNA double-strand ends from being recognised as strand breaks, and in activating DNA damage-repair mechanisms. Evidence suggests that extensive quadruplex formation within the telomeric

sequence induces strong exonuclease resistance, constituting an effective protection mechanism against hydrolysis.[10]

Although interfacial interactions between proximal quadruplex motifs have been proposed on the basis of spectroscopic analysis and modelling studies,[11,12] the stability of folded telomeres appears to be inversely correlated with their length, indicating that steric and electrostatic repulsions between folded motifs result in destabilising interactions.[11–14] Model building from X-ray structures has suggested the possibility of ligand-binding pockets at these interfacial sites employing both G-tetrad stacking and contacts with A/T nucleotides within the conformationally flexible loops.[15] However, the extent to which these 'loose' interfaces could provide novel targets for ligand design and binding has remained largely unexplored, and experimental evidence that ligands can bind selectively at the interface between quadruplex motifs, and target telomeric DNA sequences, is still lacking.

We have characterised the folding and stability of the human telomeric repeat sequence 5'-AGGG(TTAGGG)$_n$, for $n$ = 3 (HT), 7 (HT$^2$) and 11 (HT$^3$), in detail using circular dichroism (CD) spectroscopy in buffered K$^+$ solution at pH 7.0.[14] The three sequences are capable of forming either a single quadruplex motif, or two or three in tandem. In agreement with previous studies,[12,13] the thermal stabilities decrease across the series (solid lines in Fig. 2) with a depression of the $T_m$ of the trimer (HT$^3$) by ~8–9 °C below that of the single monomer (HT) (see Table 1). The CD spectral features, backed-up by NMR structural analysis, are consistent with the formation of (3 + 1) hybrid anti-parallel motifs, which appear to be sterically destabilised by

**Table 1** Quadruplex stability measurements from CD melting curves with and without bound ligands

| Sequence | $T_m$ (K$^+$)$^a$ no ligand | $T_m$ (K$^+$)$^a$ + **RHPS4** | $T_m$ (K$^+$)$^a$ + **DR4-47** | $T_m$ (Na$^+$)$^b$ + **DR4-47** |
|---|---|---|---|---|
| HT | 69.3 ± 0.1 | 86.6 ± 0.1 | 83.5 ± 0.1 | 72.0 ± 0.5 |
| HT$^2$ | 61.5 ± 0.1 | 82.0 ± 0.1 | >90 | 84.0 ± 0.5 |
| HT$^3$ | 60.9 ± 0.1 | 74.7 ± 0.1 | >95 | 82.0 ± 0.5 |

$^a$ 100 mM KCl, 10 mM K$_2$HPO$_4$/KH$_2$PO$_4$, pH 7. $^b$ 100 mM NaCl, 10 mM Na$_2$HPO$_4$/NaH$_2$PO$_4$, pH 7.

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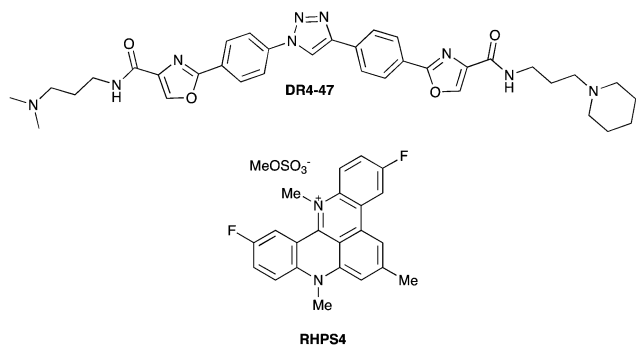$^c$ School of Pharmacy, University Park, University of Nottingham,
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Fig. 1 Structure of G-quadruplex ligands **RHPS4** and **DR4-47**.



Fig. 3 Normalised CD spectra of HT, HT$^2$ and HT$^3$ unbound (solid lines colour coded) in 100 mM KCl, 10 mM K$_2$HPO$_4$/KH$_2$PO$_4$, pH 7 and in the presence of **DR4-47** (dashed lines) at a 1 : 5 ratio, showing a large ligand-induced increase in ellipticity at 265 nm (arrow).

nearest neighbour interactions.[12] These studies conclude that the stabilities, but not the structures, show sequence-dependent changes which we investigate further in the context of two quadruplex binding ligands with quite different structural architectures and hence binding selectivities.

We have previously shown that the fluoroquinolinoacridinium cation **RHPS4** (Fig. 1) binds to AGGG(TTAGGG)$_3$ and induces changes in the CD spectrum[16] which are consistent with the features reported for the basket-type (2 + 2) anti-parallel structure observed in Na$^+$ solution, and characterised structurally by Wang and co-workers.[17] The dimer and trimer quadruplex sequences HT$^2$ and HT$^3$ showed analogous ligand induced interconversion to the (2 + 2) basket-type fold. Sigmoidal thermal unfolding curves, recorded for solutions containing two equivalents of ligand per quadruplex (Fig. 2), resulted in large stabilising effects of between 14–21 °C (Table 1). The order of stability of the bound complexes mirrors the unbound telomeres (HT > HT$^2$ > HT$^3$), suggesting that steric interactions between tandem quadruplex motifs continue to be destabilising in the ligand-bound state, despite the ligand-induced conformational switch.

We subsequently investigated the hybrid oxazole–triazole ligand **DR4-47** (Fig. 1), designed as a quadruplex-selective binder.[18] In contrast to **RHPS4**, CD analysis of **DR4-47** showed ligand-induced

enhancement of the ellipticity at 262 nm in the spectrum of the HT sequence and a reduction at 290 nm, both of which are indicative of a significant population of parallel stranded structures at equilibrium (Fig. 3). The same characteristic spectral changes were evident in **DR4-47** titration studies with HT$^2$ and HT$^3$, with a high proportion of parallel-folded structure evident at a quadruplex : ligand ratio of 1 : 5 (Fig. 3).

Thermal unfolding experiments (monitored at 260 nm) revealed a quite different trend in relative stabilities compared to the unbound telomeric sequences (Fig. 4). While the sigmoidal melting profile for the complex with HT is well defined, the profiles for HT$^2$ and HT$^3$ are incomplete at $\sim$100 °C, indicating that the longer telomeres are still partially folded at this temperature showing preferential stabilisation of the ligand-induced arrangement of tandem parallel folded quadruplexes.[19]

We tested these observations further by changing to buffered Na$^+$ solutions (100 mM NaCl, 10 mM Na$_2$HPO$_4$/NaH$_2$PO$_4$, pH 7) where quadruplex stabilities are lower. All three telomeric sequences showed clear evidence of a conformational shift to the (2 + 2) anti-parallel basket structure characterised by a strong negative ellipticity at 265 nm in the CD spectra (Fig. 5). Ligand titration studies
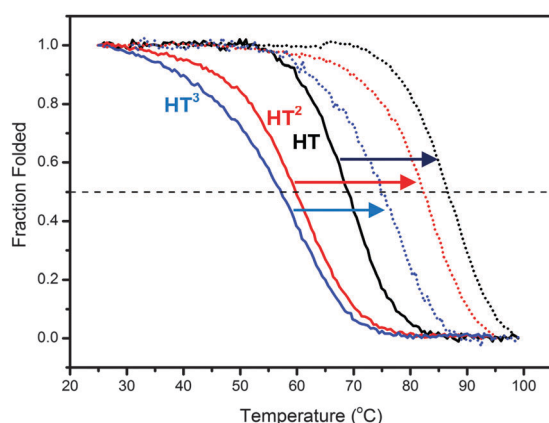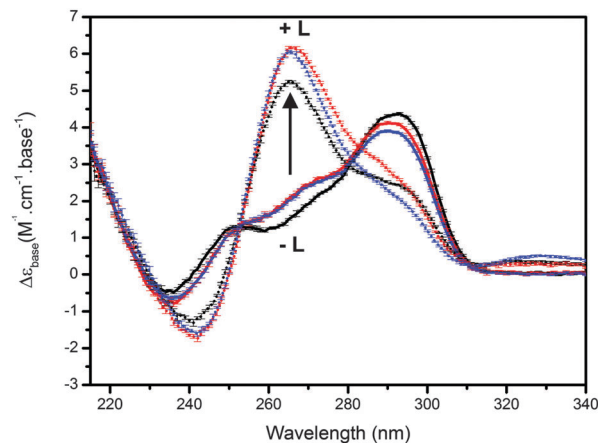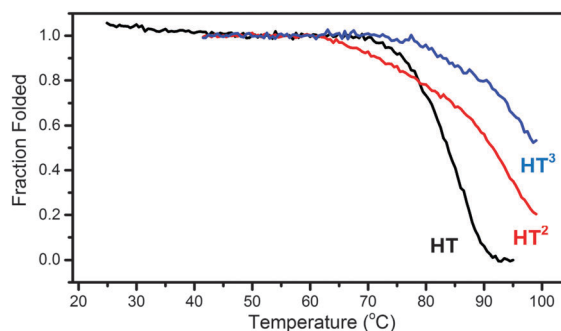


Fig. 2 CD thermal unfolding profiles (at 290 nm) for HT, HT$^2$ and HT$^3$ (solid lines colour coded) and shifts (shown by arrows) when bound to **RHPS4** (dotted lines) in 100 mM KCl, 10 mM K$_2$HPO$_4$/KH$_2$PO$_4$, pH 7 with two equivalents of ligand per quadruplex (HT = 3.83 μM, HT$^2$ = 1.53 μM, HT$^3$ = 0.61 μM).



Fig. 4 CD thermal stability profiles for HT, HT$^2$ and HT$^3$ at a fixed ratio of 1 : 3.4 of quadruplex motif to **DR4-47** in 100 mM KCl, 10 mM K$_2$HPO$_4$/KH$_2$PO$_4$, pH 7.
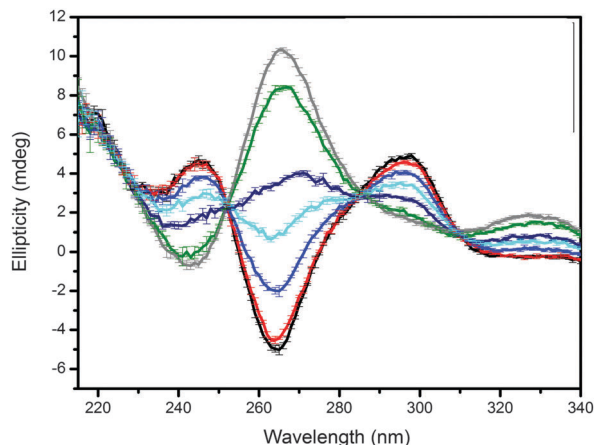
**Fig. 5** CD studies showing the titration of HT (in 100 mM NaCl, 10 mM Na$_2$HPO$_4$/NaH$_2$PO$_4$, pH 7 solution) with **DR4-47** and the conformational shift to the parallel folded structure associated with an increase in positive ellipticity at 265 nm.

under identical conditions revealed the same shift towards parallel folded structures observed in K$^+$ solutions, with the negative band at 265 nm becoming increasingly positive at increasing ligand : quadruplex ratios (Fig. 5). Isodichroic points at 252, 285 and 310 nm are consistent with interconversion between two well-defined quadruplex topologies.

The changes in the spectral features during ligand titrations in both K$^+$ and Na$^+$ buffered solutions show very similar conformational transitions, supporting the notion that ligand binding strongly favours the parallel folded structure irrespective of any intrinsic structural preference of the sequence under the prevailing ionic conditions. The overall decrease in stability in Na$^+$ solution brings all three CD melting curves into an accessible temperature range (Fig. 6), and confirms the observations in K$^+$ buffered solutions that the longer telomeric repeat sequences are preferentially stabilised over the monomeric quadruplex motif (HT).

We used molecular dynamics (MD) simulations[11,12] to investigate different modes of interaction that could account for the
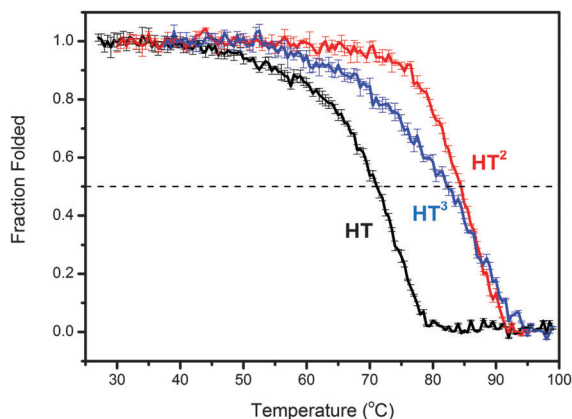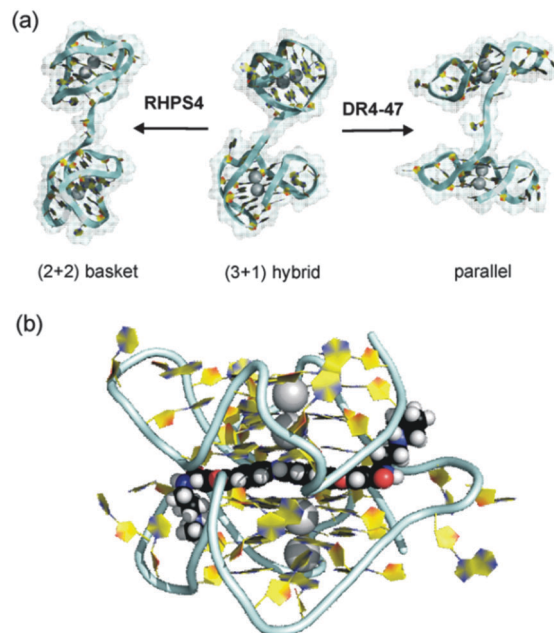


**Fig. 7** (a) Structure of HT$^2$ from MD simulations showing the (3 + 1) hybrid structure and ligand-induced (2 + 2) basket (**RHPS4**) and parallel (**DR4-47**) structures. (b) Model of the **DR4-47** complex with HT$^2$ showing a sandwich π-stacking complex of the ligand with flexible arms extending into the grooves of both quadruplexes simultaneously.

preferential stabilisation of tandem parallel folded quadruplex motifs. Structures of **DR4-47** were subjected to geometry optimisation with Gaussian 03 and the ligand manually docked into various plausible orientations that allowed for π-stacking interactions at the ends of the quadruplex.[20] These included a sandwich complex between the two quadruplexes of HT$^2$ with the formation of stacking interactions with G-tetrads at the ends of both motifs simultaneously. This complex, and other 'open' conformations, was stable during short 2 ns MD simulations. In the sandwich complex (Fig. 7), the mobile divergent 'arms' of the ligand were able to interact with the grooves of the two quadruplex motifs simultaneously in a number of different conformations via dynamic contacts. This sterically plausible model, which remains to be experimentally verified, suggests stabilising contacts between the different quadruplex loop sequences which are offset to minimise steric repulsions.

In conclusion, telomeric DNA, consisting of multiple repeats of d(TTAGGG)$_n$, is a validated drug target for the inhibition of telomerase through the stabilisation of quadruplex motifs.[6–8] To date, drug design has focused on the targeting of single quadruplexes, primarily through π-stacking on the terminal G-tetrads.[6–8] Telomeric sequences are known to be highly polymorphic and quadruplex binding ligands have been shown to induce different conformations within these individual structural motifs.[16]

Here, we report biophysical studies of two quadruplex-selective ligands binding to telomeric sequences which form multiple (two or three) quadruplex motifs in series. We have demonstrated that a hybrid oxazole–triazole ligand[16,18] induces parallel-folded structure which leads to preferential stabilisation of longer telomeric



**Fig. 6** CD melting profiles for HT, HT$^2$ and HT$^3$ in the presence of **DR4-47** (100 mM NaCl, 10 mM Na$_2$HPO$_4$/NaH$_2$PO$_4$ at pH 7.0) showing preferential stabilisation of the dimer and trimer over the single quadruplex motif.

sequences where quadruplex motifs are formed in tandem. In contrast, the quinoacridinium cation (**RHPS4**) enforces a largely anti-parallel fold which, although significantly stabilising at the level of the individual quadruplex motif ($\Delta T_m \sim$ 15–20 °C), shows no evidence for preferential stabilisation of the longer sequences. MD simulations suggest a plausible structural model for the **DR4-47** complex in which the ligand is sandwiched between tandem quadruplexes through π-stacking with the terminal G-tetrads, with additional groove contacts involving electrostatic interactions (Fig. 7). Alternative models have suggested that a TATA tetrad formed between loop residues could also provide the basis for ligand stacking interactions at the interface.[15] We have now shown evidence amongst at least one group of ligands for selectivity in binding at this unique site through induction of a parallel folded conformation in the highly polymorphic human telomeric DNA sequence.

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19 Ligand and oligonucleotide concentrations, and the ratio of the two, were varied and showed that thermal stability of the complexes was dependent on ligand concentration, but not on oligonucleotide concentration and hence intermolecular associations.

20 MD simulations and modelling used a 4 CPU workstation with Fedora 10 running the AMBER 8 suite of programs. Models for the sequences HT, HT² and HT³ were constructed using published PDB quadruplex structures with the closest correspondence to the topologies of interest: for the parallel HT structure 2LD8, basket (2 + 2) 2KF8 and 143D, (3 + 1) hybrid HT structures 2HY9 (hyb1), 2JPZ (hyb2).