DOI: 10.1002/cbic.200700667

Ligand-Stabilized Hairpin Structures Interfere with Elongation of Human Telomere

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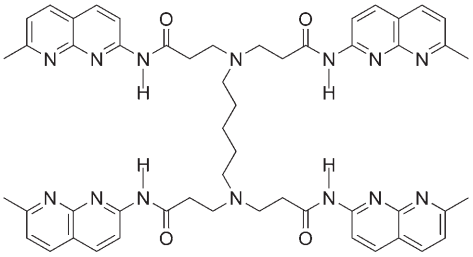
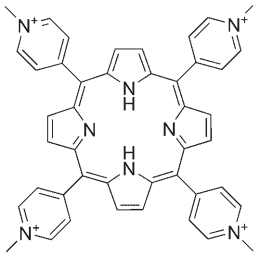
Small-molecule ligands that bind to the single-stranded overhangs of human telomeric repeats $d(TTAGGG)_n$ and interfere with telomere elongation by telomerase have been anticipated as potential anticancer agents.[1–3] The characteristic G-quadruplex structures[4–6] produced in telomeric overhangs are the target of these molecules. Ligand-stabilized G quadruplexes on the template have been shown to act as obstacles for DNA synthesis by polymerase and telomerase-mediated telomere elongation.[7–13] We have studied molecular ligands that bind to the nonquadruplex structure of the telomeric repeat. Recently we reported that 1) naphthyridine tetramer (**NT**) can cause denaturation of interstrand quadruplex structures produced by the human telomere model sequence telo15 (5′-TTAGGGTTAGGGTTA-3′), 2) **NT** can bind to telo15 with a 1:1 stoichiometry, and 3) **NT**-binding to telo15 does not involve hydrogen bonding to guanine N7, but binds to the Watson–Crick face. Results obtained with mutant sequences of telo15 suggested that **NT** binds to G–G mismatches produced by the pairing of two GGG units in the hairpin secondary structure,[14,15] which is one of possible intermediates during the folding of telomeres into G quadruplexes.[5,16] Here, we report that **NT**-stabilized structures in telomeres can effectively interfere with DNA synthesis by Taq DNA polymerase. Using systematic studies with different number of telomeric repeats in the polymerase-stop assay,[17] we have clarified that **NT** in fact



naphthyridine tetramer (**NT**)



TMPyP4

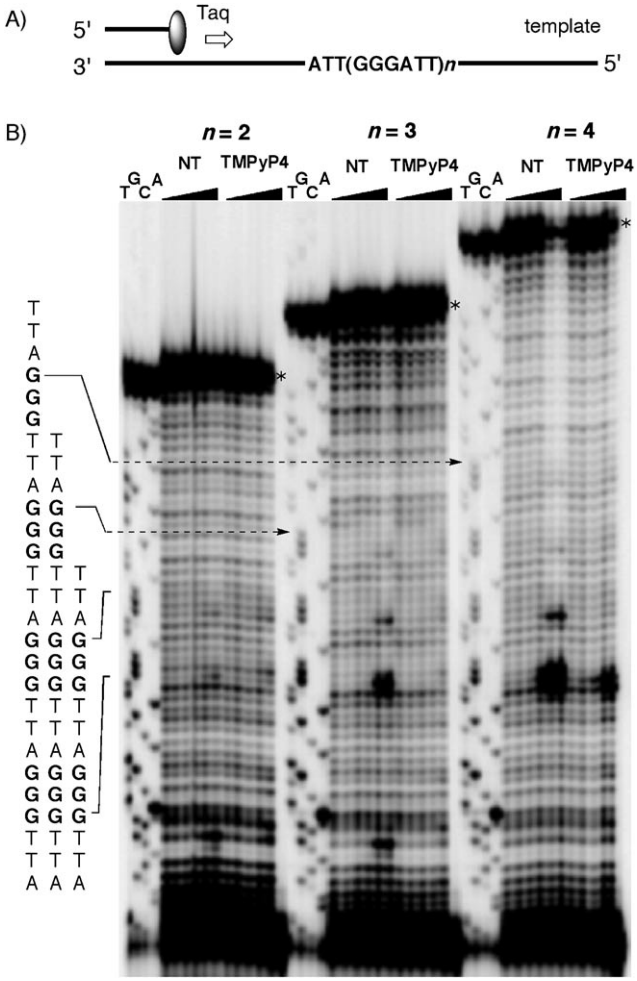


Figure 1. A) Schematic illustration of polymerase-stop assay with various numbers of telomeric repeats. B) Concentration-dependent interruption of Taq polymerase-mediated DNA synthesis with **NT** (0, 0.3, 1, 3, 10, 30 μM) and TMPyP4 (0, 0.03, 0.1, 0.3, 1, 3 μM) with DNA templates (0.1 μM) that contained (left to right) two, three, and four human telomeric repeats. The lane markers T, G, C, and A indicate the bases on the template strand. The lanes with asterisks indicate the lack of elongation of the primer. Guanines in the major arrested regions are emphasized in bold.

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stabilizes the hairpin secondary structure of telomeric repeats. With this unique sequence preference, **NT** would be an additional class of ligand that could be used to gain a deeper insight into the biological consequences of ligand–telomere interactions.

In order to determine whether **NT**-stabilized structures can interfere with the elongation of the telomere sequence, the polymerase-stop assay[17] was performed with templates that contained two- to four-repeat units of the d(TTAGGG) sequence as the model telomeric overhang in the presence of **NT** and a reference ligand TMPyP4[7] (Figure 1). In separate experiments, it was confirmed by CD spectroscopy that the **NT**-bound structure is in equilibrium with the G-quadruplex structures regardless of their folding (see Figure S1 in the Supporting Information). In the absence of ligand, the 20-mer primer that was hybridized to the 3′ end of the templates was fully elongated by Taq DNA polymerase regardless of the length of telomeric repeats. For the template that contained two repeats, **NT** interfered with the Taq polymerase reactions very weakly to produce faint paused bands at the first GGG site from the 3′ end of the repeat (Figure 1 B).

For the three-repeat template, distinct paused bands were observed predominantly at the first GGG site in the presence of **NT**, and weak paused bands were observed at the second GGG site. The G-quadruplex binding ligand, TMPyP4, did not produce any paused bands with the two- and three-repeat templates, but totally suppressed the polymerase reaction at 3 μM; this resulted in the lack of elongation of the primer. In marked contrast, both **NT** and TMPyP4 interfered with the Taq polymerase reaction at the first GGG site of the four-repeat template. Minor paused bands were observed for **NT** at the second GGG site, but not for TMPyP4.

When using two- and three-repeat templates (Figure 1 B), a high concentration of **NT** produced the paused bands that were observed at the bottom of the gel. It is likely that **NT** bound to structure(s) that were formed between guanines from the sequence 5′-GCGC-3′ in the template and guanines from the telomeric repeats, which then interfered with the Taq polymerase reaction. In the case of the four-repeat template **NT**-bound structures formed in the telomeric repeats would be thermally dominant; thus the paused bands caused by minor **NT**-bound structures disappeared.

These results clearly show that the sequence requirements necessary to form a stable ligand-bound structure and to interfere with the polymerase reaction are quite different for **NT** and TMPyP4. Four-repeat units were necessary to form TMPyP4-stabilized intrastrand G quadruplexes and interfere with the polymerase reaction.[17] Paused bands were not detected on the three-repeat template with TMPyP4; this indicates that neither inter- nor intrastrand G quadruplexes were produced on this template with this ligand. Therefore, the formation of paused bands on the three-repeat template with **NT** indicates that **NT**-bound nonquadruplex structures were stable enough to interfere with the polymerase reaction. Since three repeats was the minimum sequence required for **NT** to efficiently interfere with Taq polymerase, the paused bands were due to interference in the polymerase reactions by **NT**-bound hairpin structures on the template. Although **NT** bound to the two-repeat template,[14] the resulting **NT**-bound hairpin was most likely not sufficiently stable to interrupt the polymerase, probably due to a short hairpin loop.

The telomeric overhang in human telomeres is 130–210 nucleotides in length.[18,19] It was conceivable that increasing the telomeric repeats in the human telomeric overhang would result in the formation of multiple **NT**-stabilized hairpin structures. The effects of repeat length on **NT** binding and interference in the polymerase reaction were therefore investigated with the polymerase-stop assay by using templates that contained up to eight telomeric repeats (Figure 2). With the five-repeat template, the paused bands were observed at the first and the second GGG sites from the 3′ end with an almost

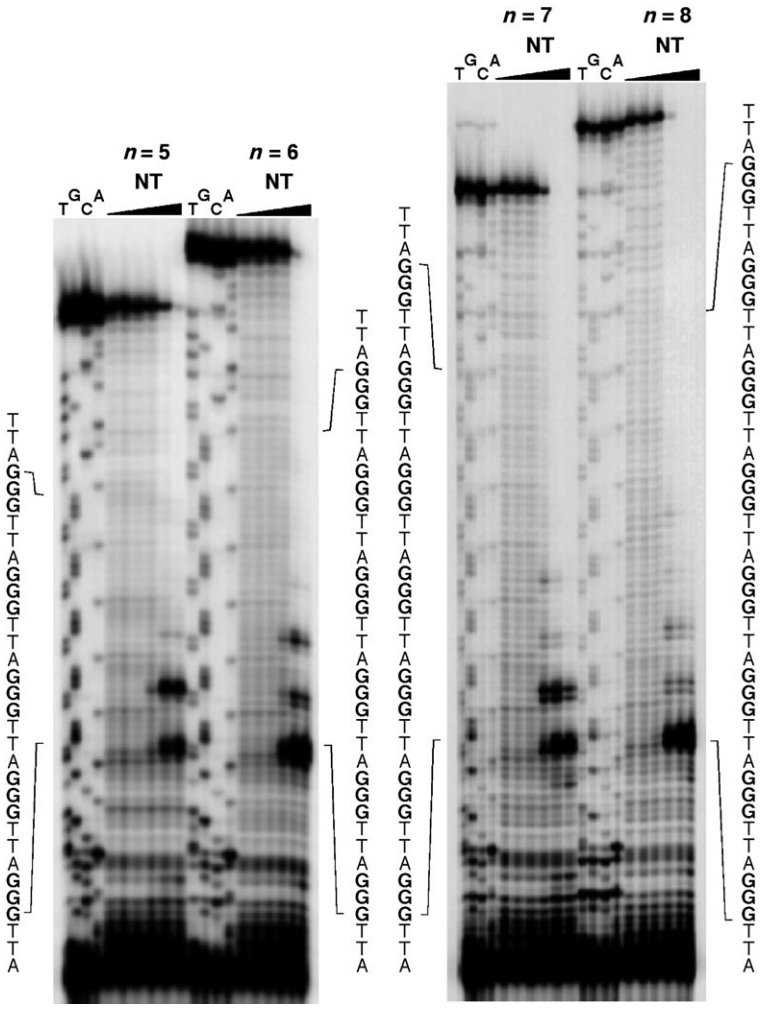

Figure 2. Concentration-dependent inhibition of Taq polymerase-mediated DNA synthesis with **NT** (0, 0.3, 1, 3, 10, 30 μM) and DNA templates (0.1 μM) that contained (left to right) five, six, seven, and eight human telomeric repeats. Guanines in the major arrested regions are emphasized in bold.

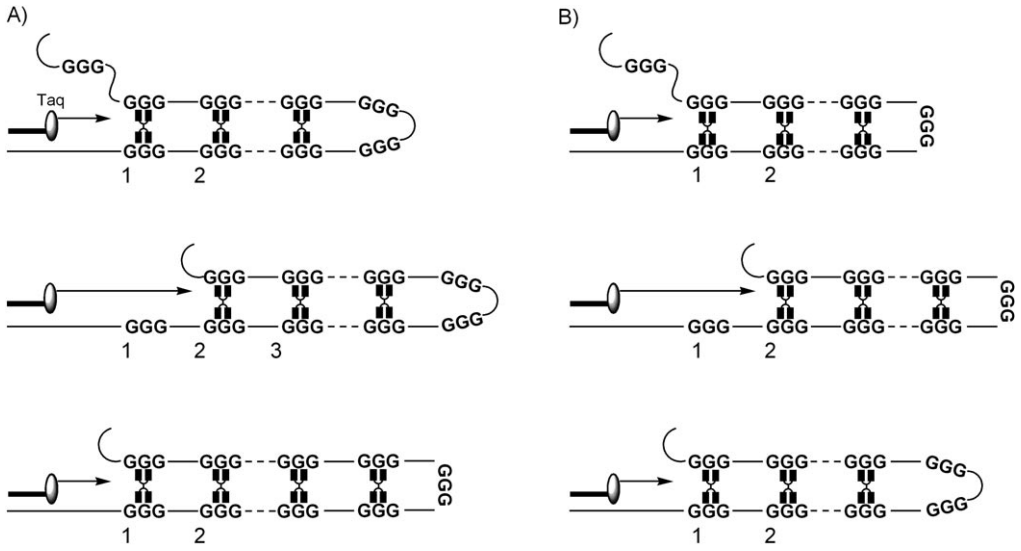

Figure 3. Illustration to rationalize the interruption of polymerase by **NT**-stabilized hairpin structures for A) odd-numbered telomere repeats, and B) even-numbered telomere repeats. The numbers (1, 2, 3) below the GGG units show the position of the GGG from the 3′ end of the sequence.

equal intensity. In contrast, predominant paused bands at the first GGG site were observed for the six-repeat template. Minor paused bands were detected at the second and third GGG, but not at other GGG sites.

With the seven-repeat template, two major paused bands were detected at the first and second GGG sites. The bands at the second GGG site, however, became less intense as **NT** concentration was increased; this coincided with an increase in band intensity at the first GGG site. With the eight-repeat template, the polymerase reaction was exclusively interrupted at the first GGG site. A threshold concentration for effective interference became critical with the eight-repeat template. Polymerase reaction was totally suppressed at 3 μM **NT**, whereas paused bands were not detected at 1 μM.

The results of the experiments with the five- to eight-repeat templates as models for telomeric overhangs confirmed that **NT** effectively interferes with the polymerase reaction. The site of interruption was repeat dependent—mostly the first GGG site on the template. These observations can be well rationalized by the formation of the most stable **NT**-bound hairpin structure. For odd-numbered telomeric repeats (Figure 3 A), **NT**-stabilized hairpin structures can contain two GGG units at the hairpin loop and one extra GGG at either end of the hairpin. By increasing **NT** concentration, the extra GGG unit could become involved in the hairpin stem to leave one GGG in the loop. Thus stabilization of GGG–GGG by **NT** could move the equilibrium of the hairpin structure from a two-GGG loop to a one-GGG loop. For even-numbered telomere repeats, the most thermally stable hairpin structure would be formed with two GGG units in the loop. Thus, Taq polymerase was interrupted selectively at the first GGG regardless of **NT** concentration.

In summary, **NT**-stabilized hairpin structures in telomeric overhang sequences effectively interfered with DNA synthesis by Taq polymerase. Considering its unique sequence preference for binding, **NT** could be a useful addition to the G-quadruplex binding ligands for studying the biological consequence of ligand binding to telomeres.

Experimental Section

Polymerase-stop assay: A reaction mixture of template DNA (0.1 μM; four-repeat template for example; 5′-dTCCAACTATGTATACTTAGGGTTAGGGTTAGGGTTAGGGTTAGTCATGTCTAGCGCACGCAATTGCCCCTATAGTGAGTCGTATTA-3′; the human telomere sequence is underlined and its length was changed as necessary) and 5′ Texas Red-labeled primer (0.1 μM; 5′-dTAATACGACTCACTATAGGG-3′) was heated to 95 °C for 3 min in Taq (TaKaRa) reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), and cooled to ambient temperature for 30 min. The requisite amount of ligand was added to the reaction mixture and incubated for 30 min at room temperature prior to the addition of polymerase. Taq DNA polymerase and dNTPs were then added to the mixture and the reaction was performed at 42 °C for 30 min. The reaction products were analyzed by using an Hitachi SQ5500E automated sequencer.

Keywords: DNA recognition · DNA structures · hairpins · inhibitors · telomeric overhangs

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Received: November 7, 2007
Published online on January 25, 2008

