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# Investigating Pharmacological Targeting of G-Quadruplexes in the Human Malaria Parasite

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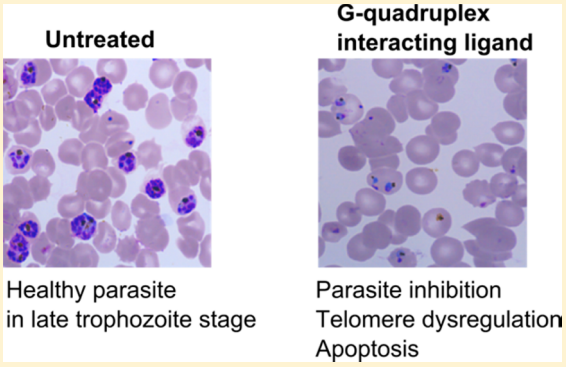
Supporting Information

**ABSTRACT:** The unique occurrence of G-quadruplexes in the AT-rich genome of human malaria parasite *Plasmodium falciparum* provides hints about their critical roles in parasite survival, pathogenesis, and host immune evasion. An intriguing question is whether these noncanonical structures can serve as molecular targets for small molecule-based interventions against malaria. In this study, we have investigated the pharmacological targeting of G-quadruplexes for parasite inhibition. We observed that bisquinolinium derivatives of 1,8-naphthyridine and pyridine affected the stability and molecular recognition properties of G-quadruplexes in telomeric and subtelomeric regions in *P. falciparum*. Parasite inhibition and cytotoxicity assays revealed that these ligands effectively inhibit parasite growth with minimal toxic effects in human cells. G-quadruplex interacting ligands caused degeneration and shortening of parasite telomeres. Ligand-induced perturbations in telomere homeostasis also affected transcriptional state of the subtelomeric region harboring antigenic variation genes. Taken together, our results suggest that quadruplex−ligand interaction disturbs telomeric/subtelomeric chromatin organization and induces DNA damage that consequently leads to parasite death. Our findings also draw attention to the striking differences in telomere dynamics in the protozoan parasite and human host that can be exploited for selective targeting of the telomeric quadruplex of the parasite as a potential antimalarial strategy.




Noncanonical nucleic acid structures are suggested to play important roles in transcriptional and translational regulation in both prokaryotic and eukaryotic genomes.[1,2] One of the widely studied noncanonical nucleic acid secondary structures is G-quadruplex, containing planar array of Hoogsteen bonded guanine quartets assembled to form intramolecular or intermolecular structures.[3,4] In humans, these structures are enriched in recombination hot spots, promoter region of proto-oncogenes, untranslated regions, and telomeric ends.[5,6] In addition, the recent identification of potential G-quadruplex-forming sequences in the human noncoding transcriptome has expanded the repertoire of their biological roles.[7−9] Experimental evidences suggest that quadruplexes play diverse roles that are critical for cell survival, proliferation, and senescence.[6,10] Interestingly, these cellular functions are dictated by molecular recognition properties of quadruplex motifs and their interaction with specific proteins that drive biological roles (activator or repressor) in a context-dependent manner.[11−13] Immense research efforts are being invested to exploit these structures as potential drug targets to inhibit uncontrolled proliferation of human cancers and to understand the molecular mechanisms for quadruplex-associated diseases.[14−16]

Besides their roles in humans, G-quadruplexes are gaining attention in pathogenic microbes because of their potential role in antigenic variation to escape from host immune surveillance.[17] In viruses, the quadruplex is suggested to play a role in transcriptional and/or translational regulation that has likely implications in viral latency and reactivation.[18,19] In pathogenic microbes, quadruplex motifs identified in the pilin gene (in *Neisseria*) and antigenic variation gene cluster (in *Borrelia* and *Trypanosoma*) are suggested to facilitate antigenic switching in surface antigen proteins that contribute to persistence, superinfection, and transmission of infection.[20−22] Similarly, in human malaria-causing parasite *Plasmodium falciparum*, the occurrence of secondary structures in subtelomeric multigene families may play a role in the diversity of surface antigens and their cytoadherence properties.[23−26] An

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important feature of this protozoan parasite is that its uniquely regulated stage-specific gene expression allows it to withstand intense environmental fluctuations and immune threats during its life cycle in the human host (asexual phase) and mosquito vector (sexual phase).[27] It is therefore conceivable that the ability of the parasite to survive in hostile cellular environments is likely to be regulated by noncanonical secondary structures.[25,28] Interestingly, unlike other disease-causing microorganisms, *P. falciparum* has a very unique AT-rich genome (>80%)[29] wherein quadruplex-forming potential is restricted to telomeric, subtelomeric, and a few biologically important nontelomeric regions.[23,25,26] Because of the lack of sufficient experimental evidences, the role of these non-canonical regulatory motifs in the *P. falciparum* genome remains underappreciated.

High predominance of quadruplexes in the parasite's telomeric and subtelomeric regions provides hints about their critical roles in telomere architecture and function. *P. falciparum* telomeric DNA adopts alternative secondary structures and interacts with non-nucleosomal proteins to form a multi-protein−telomeric complex.[30,31] This organization protects telomeric ends from degradation, anchors them to the nuclear periphery, and reversibly represses the transcription of subtelomeric genes through recruitment of epigenetic modifier proteins.[30−32] Disturbing telomere function through small molecules could be explored as the available antimalarial treatments are becoming increasingly ineffective because of emerging drug resistance problems. Generally, the prevalent antimalarial drugs inhibit protein targets that are critical for heme detoxification in the digestive vacuole, folate or pyrimidine biosynthesis in the cytosol, electron transport in the mitochondrion, or translation in the apicoplast,[33] but the parasite has progressively acquired point mutations in these protein drug targets so that it is no longer inhibited by existing antimalarial drugs.[33] Targeting noncanonical structures in the parasite's telomeres may serve as an alternative approach to prevent or delay drug resistance because acquiring random mutations to disrupt these secondary structures in telomeric repeats will incur enormous biological fitness cost.

The telomeric quadruplex of *P. falciparum* is suggested to be a target for small molecule-mediated parasite inhibition,[34−36] but this facet has not been extensively explored. In this study, we have attempted to understand quadruplex−ligand interaction mediated parasite inhibition. We observed that naphthyridine- and pyridine-based scaffolds with quinolinium side chains[37] affected the conformation and stability of quadruplexes in telomeric and subtelomeric regions in *P. falciparum*. This interaction drastically affected the telomere homeostasis and heterochromatin state of subtelomeric regions, consequently resulting in parasite death. The length and complexity of telomeric and subtelomeric regions of both the human host and the protozoan parasite have significantly diverged. Hence, their contrasting features can be exploited for quadruplex−small molecule interaction-mediated telomere dysregulation in the parasite.

## MATERIALS AND METHODS

**Biophysical Experiments.** High-performance liquid chromatography-purified DNA oligonucleotides (Table S1) were procured from Sigma. G-quadruplex interacting ligands used in this study were prepared as reported previously.[37] Oligonucleotides (quadruplex or duplex, 5 μM each) were heated and gradually cooled overnight (in the absence and presence of 15 μM ligands) in 10 mM sodium cacodylate buffer, 10 mM NaCl, and 90 mM LiCl (pH 7.0). For melting studies, CD spectra were recorded in a Jasco-815 spectrometer at different temperatures with a heating rate of 0.3 °C/min. In all the experiments, the CD spectrum of the blank (buffer containing ligand) was subtracted from the spectra recorded for quadruplex−ligand samples.

***P. falciparum* Culturing and Inhibition Assay.** *Determination of $IC_{50}$ and $CC_{50}$.* 3D7 (chloroquine-sensitive) and K1 (chloroquine-resistant) strains of *P. falciparum* were maintained at 8−10% parasitemia and 2% hematocrit in RPMI complete medium (RPMI 1640, 0.2% sodium bicarbonate, 0.5% albumax, 45 mg/L hypoxanthine, and 50 mg/L gentamycin) at 37 °C in a humidified $CO_2$ incubator. The culture was synchronized with 5% sorbitol (20 min at 37 °C) followed by three washes by 1× phosphate-buffered saline (PBS). The antimalarial activity ($IC_{50}$) of bisquinolinium derivatives of 1,8-naphthyridine and pyridine compounds was evaluated against both 3D7 and K1 strains of *P. falciparum* using the SYBR Green I-based fluorescence assay (MSF).[38] The stock concentrations of G-quadruplex interacting compounds were prepared in dimethyl sulfoxide (DMSO) and subsequently diluted in incomplete RPMI (without albumax) medium. Chloroquine diphosphate (CQ) (C-6628, Sigma) was used as the reference compound for both 3D7 and K1 strains. Parasite inhibition experiments were conducted at 0.8% parasitemia maintained at 1% hematocrit in RPMI complete medium. Ring-stage parasites were treated with different dilutions of G-quadruplex interacting ligands (3AQN, 6AQN, and 360A) for 72 h, and cells were lysed with lysis buffer [20 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.008% saponin, and 0.08% Triton X-100] containing 1× SYBR Green dye (S7585). The fluorescence was recorded in an FLX800, BIOTEK instrument (excitation at 480 nm, emission at 520 nm). The parasite culture (8−10% parasitemia) was treated with the ligand (5 μM, 24 h) at different stages (ring, early trophozoite, and schizont stage) followed by microscopic examination of the giemsa-stained smear. In another set of experiments, the parasite was treated with the ligand (5 μM) and the ligand was washed out with incomplete RPMI medium after a 24 h treatment. Subsequently, the parasite culture was maintained without ligand pressure for the next 48 h. Thin blood smears were prepared from treated and untreated cultures and fixed with methanol prior to staining with giemsa. The slides were observed under a bright-field optical microscope (Q imaging, Olympus).

The cytotoxic effect of G-quadruplex interacting ligands was evaluated in human embryonic kidney cells (HEK293T) cultured in DMEM (D5648). Briefly, 10000 HEK293T cells per well were seeded in a 96-well plate. The next day, cells were treated with different dilutions of G-quadruplex interacting ligands. After 72 h, 10 μL of MTT (M1415.0001, Duchefa Biochemies) was added to the cells at a working concentration of 0.5 mg/mL and the mixture incubated for 2−4 h until the purple precipitate was visible. After incubation, MTT was replaced with 100 μL of DMSO to dissolve the purple precipitate and the absorbance was measured at 540 nm.

*JC1 Staining for Mitochondrial Potential.* Ligand treatment (5 μM, 24 h) was given to the 3D7 strain at the ring stage maintained in RPMI complete medium (3−5% parasitemia and 2% hematocrit). After the treatment (24 h), the culture (1 mL) was collected and washed twice with 1× PBS. Red blood cells (RBCs) were pelleted (2000*g* for 5 min), and the pellet was


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subsequently diluted in 1× staining buffer in PBS to achieve 5% hematocrit. This sample was incubated with 2 μM JC1 dye (J3645, Sigma) for 30 min (in the dark) at 37 °C. After incubation, the sample was pelleted and washed twice with 1× PBS. This sample was also incubated with Hoechst (30 μg/mL) in PBS for 3 min (room temperature, in the dark). The samples were washed again with 1× PBS and diluted to 50% hematocrit to prepare a thin smear. The slides were then examined under a Nikon fluorescence microscope (100× magnification).

***P. falciparum* Telomeric Length Estimation.** *Polymerase Chain Reaction (PCR) Based Telomeric Length Estimation.* Ligand treatment (5 μM, 24 h) was given to 3D7 strain at the ring stage maintained in RPMI complete medium (8−10% parasitemia and 2% hematocrit). After treatment (24 h), RBCs were collected and washed with 1× PBS followed by 0.1% saponin lysis. Genomic DNA (gDNA) was isolated for control and compound-treated samples using the Reliaprep Blood gDNA Miniprep System Kit (Promega A501). PCR was performed using 10 ng of gDNA/50 μL reaction mixture supplemented with 3% DMSO in *Taq* polymerase reaction buffer. The forward primer was designed from the PF3D7_ 1480100 gene that is proximal to the telomeric end, while the reverse primers were complementary to the G-rich overhang [PfCx1 and PfCx2 (Table S2)]. The PCR products were resolved via Native-polyacrylamide gel electrophoresis (6 to 10% gradient) with 0.5× Tris-borate EDTA (TBE) as the running buffer. The gels were visualized using Molecular ImagerR Chemi DocTM XRS (Bio-Rad).

*Southern Hybridization.* The 3D7 strain was treated with ligands (5 μM) at the ring stage maintained in RPMI complete medium (8−10% parasitemia and 2% hematocrit). After treatment (24 h), RBCs were collected and washed with 1× PBS followed by 0.1% saponin lysis. Genomic DNA (gDNA) was isolated from untreated and ligand-treated samples using the Reliaprep Blood gDNA Miniprep System Kit (Promega A5081). Three micrograms of gDNA was digested with AluI (10 units) and MboII (5 units) for 5 h at 37 °C. The digested samples were resolved on 0.8% agarose and subsequently denatured using 1.5 M NaCl and 0.5 M NaOH for 30 min. The gel was rinsed with Milli-Q (MQ) water and neutralized with buffer containing 3 M NaCl and 1 M Tris-HCl (pH 7.0) for 30 min. This gel was transferred to a nylon membrane (N3656, Sigma) overnight through capillary-based transfer in 10× saline sodium citrate (SSC buffer, 1.5 M NaCl and 150 mM sodium citrate tribasic dihydrate). This was followed by ultraviolet (UV) cross-linking (250 × 100 μJ/cm$^2$, 60 s). After this, the membrane was incubated in hybridization buffer (5× SSC, 0.1% *N*-lauryl sarcosine, 0.02% sodium dodecyl sulfate, and 1% blocking reagent from Roche) for 2 h. The probe for detection was prepared by amplifying the region of the PF3D7_1480100 gene using primers mentioned in Table S2. PCR was performed using the DIG (digoxigenin) labeling kit (11585550910, Roche). The probe (700−1000 ng) was denatured at 100 °C for 10 min, and hybridization was performed at 65 °C overnight in hybridization buffer. The membrane was blocked in 1× blocking buffer [0.1 M maleic acid, 0.15 M NaCl (pH 7.5), and 1× blocking reagent] for 3 h. The anti-digoxigenin antibody (1:10000) was prepared in 1× blocking buffer and incubated for 2 h. The membrane was then washed (three times for 15 min) with washing buffer [0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl, and 0.2% Tween 20]. The membrane was developed using detection buffer [0.1 M Tris (pH 9.5) and 0.1 M NaCl containing 1% BCIP/NBT substrate]. As the smear starts to appear, the reaction is quenched with MQ.

**Real-Time PCR Experiments.** Ligand treatment (5 μM, 24 h) was used for the 3D7 strain at the ring stage maintained in RPMI complete medium (8−10% parasitemia and 2% hematocrit). After treatment, RBCs were collected and washed with 1× PBS followed by 0.1% saponin lysis. The parasite pellet obtained was immediately resuspended in Trizol (Invitrogen), and RNA was isolated by following the manufacturer's protocol. One μg of RNA (1 μg) was used to prepare cDNA (Verso cDNA synthesis AB1453/B), and SYBR Premix Ex TaqTM (Tli RNaseH Plus, RR420A) was used in the experiments. cDNA was diluted 5-fold to be used as a template in real-time experiments. Real-time PCR experiments were performed for subtelomeric genes (with and without potential quadruplex-forming sequence), genes involved in DNA damage response, and histone deacetylase. Actin was used as an internal control (Table S3). Real-time data analysis was performed through $2^{\Delta\Delta Ct}$ method.

## RESULTS

**Biophysical Characterization of Quadruplex−Ligand Interaction.** Potential quadruplex-forming sequences present in the telomeric and subtelomeric region (*Var* and *Rifin* multigene families) of the *P. falciparum* genome were selected from a previous study[23] (Table S1). Depending upon the loop length and its composition, investigated sequences adopted predominant parallel (sequences Q4−Q6), antiparallel (sequences Q2 and Q3 and Pf−TELQ), and a mixture of both conformations (sequence Q1) in 10 mM sodium cacodylate buffer containing 90 mM LiCl and 10 mM NaCl (Figure 1 and Figure S1). Li$^+$ ions have the weakest effect on induction or stabilization of quadruplexes, whereas Na$^+$ or K$^+$ ions remarkably stabilize these structures in a concentration-dependent manner.[39] To maintain the required ionic strength (110 mM monovalent cations)[39] for biophysical characterization of G-quadruplex interacting ligands, we used a buffer containing Na$^+$ (20 mM) supplemented with Li$^+$ (90 mM). G-quadruplex interacting ligands investigated in this study have a naphthyridine or pyridine central core and quinolinium side chains[37] (Scheme 1A). We observed that ligands (3AQN, 6AQN, and 360A) induced conformational changes and

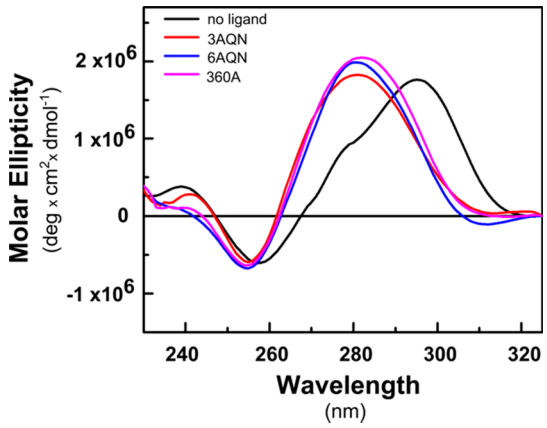


**Figure 1.** CD spectra of the Pf−telomeric quadruplex [5 μM, $(G_3TTCA)_3G_3$] in the absence and presence of ligands (15 μM) in 10 mM sodium cacodylate buffer, 90 mM LiCl, and 10 mM NaCl (pH 7.0).



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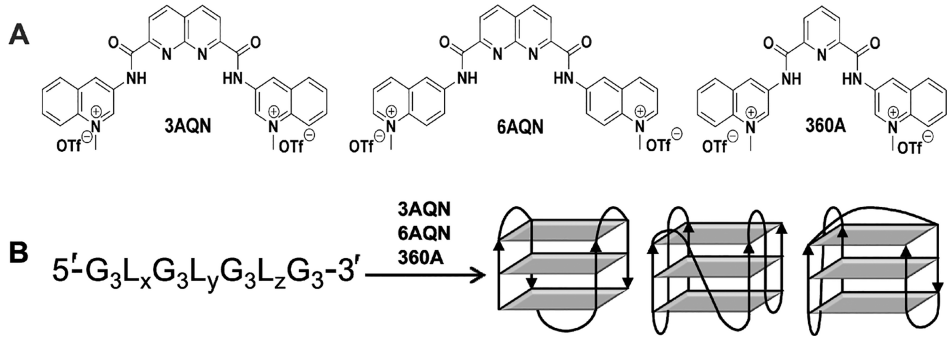
**Scheme 1. (A) Structures of G-Quadruplex Interacting Ligands (bisquinolinium derivatives of 1,8-naphthyridine and pyridine) Investigated in This Study and (B) Schematic Representation of Ligand-Induced Topologies in Potential Quadruplex-Forming Sequences in *P. falciparum***




significantly enhanced the stability of quadruplex motifs (Figure S1 and Table S1). For instance, the Pf–telomeric quadruplex (Pf–TELQ) adopts a predominant antiparallel conformation with a positive peak at 295 nm and a negative peak at 260 nm. Ligands induced a structural transition in the Pf–TELQ towards mixed topology (both parallel and antiparallel strand orientations), leading to a notable shift in the positive peak from 295 to 282 nm and a moderate shift in the negative peak from 260 to 255 nm (Figure 1). These ligands also induced a structural transition in the human telomeric quadruplex, leading to a shift in the positive peak from 297 to 293 nm with no significant change in the negative peak at 265 nm (Figure S2A). It is noteworthy that there is only modest variation in the loop length and composition of human and *P. falciparum* telomeric quadruplex-forming sequences, but they exhibit clear differences in ligand-induced structural transitions in their respective telomeric quadruplexes (Figure 1 and Figure S2A). Ligand-driven structural transition and stabilization were also observed for quadruplex-forming sequences in subtelomeric genes (Figure S1 and Table S1). For these sequences, ligand-induced conformational switching toward mixed topologies was evident from the shift of their respective CD signature toward 280 nm (Figure S1 and Scheme 1B). Another important observation about these ligands is that they display preferential binding to the quadruplex and do not induce conformational changes or increase the thermal stability of duplex DNA (Figure S2B and Table S1).

**Quadruplex Interacting Ligand-Induced Parasite Inhibition.** To understand whether quadruplex–ligand interaction translates into a biological response in the parasite, we performed a parasite inhibition assay for 3AQN, 6AQN, and 360A. Antimalarial activity was determined through the SYBR Green I-based drug sensitivity assay.[38] These ligands exhibited promising antimalarial activity in both chloroquine-sensitive (3D7) and -resistant (K1) strains with minimal toxicity in the mammalian host (Table 1). Microscopic examination revealed that these ligands affected parasite growth and their inhibitory effect was stage-specific (Figure 2A and Figure S3). When the treatment was performed at the ring stage, ligand-treated parasites progressed into the trophozoite stage but displayed a distorted morphology and did not advance to the next stage. In contrast, untreated (control) parasites progressed into the late trophozoite/early schizont stage (Figure 2A and Figure S3). However, when the ligand treatment was performed at the trophozoite stage, all the parasites were completely arrested in that stage. These parasites were extremely stressed and displayed a loss of chromatin integrity. Similar observations

**Table 1. Parasite Inhibition ($IC_{50}$) and Cytotoxicity ($CC_{50}$) of Bisquinolinium Derivatives of 1,8-Naphthyridine and Pyridine[a]**

| | inhibitory concentration ($IC_{50}$) ($\mu$M) | | |
|---|---|---|---|
| compound | 3D7 strain | K1 strain | cytotoxic concentration ($CC_{50}$) ($\mu$M) |
| 3AQN | 1.8 ± 0.2 | 2.5 ± 0.2 | 170 ± 25 |
| 6AQN | 1.5 ± 0.2 | 2.7 ± 0.3 | 170 ± 25 |
| 360A | 0.9 ± 0.1 | 1.2 ± 0.2 | 120 ± 15 |

[a]Parasite inhibition was performed in 3D7 (Chloroquine-sensitive) and K1 (Chloroquine-resistant) strains. Cytotoxicity experiments were performed in HEK293T cells. $IC_{50}$ and $CC_{50}$ are the half-maximal inhibitory and cytotoxic concentrations, respectively. $IC_{50}$ and $CC_{50}$ values were determined 72 h after ligand treatment. Data are presented as average values obtained from three biologically independent experiments with their standard deviations. Chloroquine (CQ), a 4-aminoquinoline derivative, was used as a reference compound in the parasite inhibition assay. $IC_{50}$ values obtained for CQ were 6 ± 1.5 and 300 ± 50 nM in CQ-sensitive and CQ-resistant parasites, respectively.

were made when the ligand treatment was performed at the schizont stage (Figure S3). Parasite inhibition was most severe at the trophozoite stage, wherein multiple rounds of DNA replication take place, which may elicit enormous ligand-induced DNA damage. Potent parasite inhibition was observed for 360A in comparison to 3AQN and 6AQN at all stages of the parasite's asexual life cycle (Figure 2A). We also performed experiments in which the ligand pressure was removed after treatment for 24 h, but the parasite did not recover from ligand-induced effects (Figure S4). Additional experiments were performed to check the induction of apoptosis in ligand-treated parasites. A distinctive feature of the induction of apoptosis is alternations in mitochondrial potential. We used a membrane permeant JC1 dye, widely used as an indicator of mitochondrial activity. At the physiological mitochondrial membrane potential, this lipophilic dye forms J-aggregates, which leads to pronounced red fluorescence. However, in cells with an altered mitochondrial membrane potential, the JC1 dye remains in its monomeric state and exhibits green fluorescence.[40] In our experiments, untreated control parasites showed prominent red fluorescence and very mild green fluorescence signals from the JC1 monomer (Figure 2B), whereas all the ligand-treated samples exhibited mainly green fluorescence, indicating a severe disturbance in the mitochondrial membrane potential (Figure 2B).



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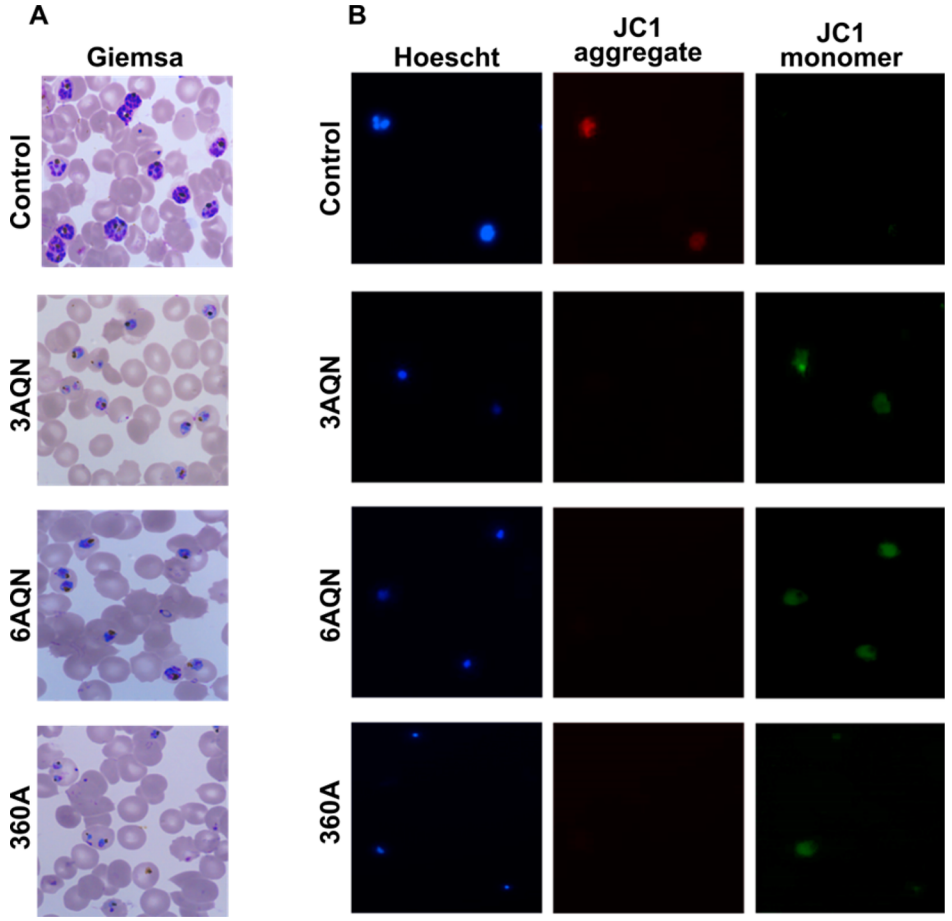


**Figure 2.** Microscopic examination of 3D7 parasite-infected RBCs upon exposure to G-quadruplex interacting ligands. Parasites were treated with 5 $\mu$M ligands for 24 h and stained with (A) Giemsa and (B) Hoescht or JC1 dye.

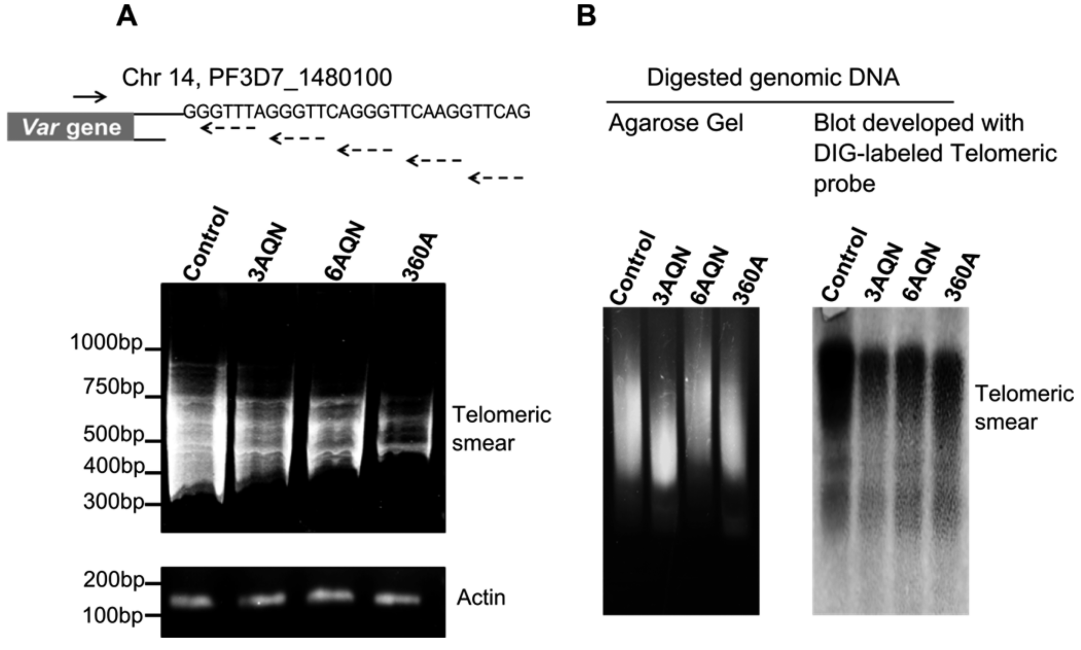


**Figure 3.** Telomeric length estimation upon ligand treatment. (A) PCR-based estimation of telomere length using the forward primer from the PF3D7_ 1480100 gene and the C-rich primer complementary to the telomere. gDNA isolated from untreated and ligand-treated parasites (5 $\mu$M, 24 h) was used as the template for PCR. (B) Southern hybridization based detection of the telomere using the DIG-labeled probe, prepared by PCR amplification of the PF3D7_ 1480100 gene.



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**Effect of the Quadruplex Interacting Ligand on Telomere Homeostasis.** The promising antimalarial activity of G-quadruplex interacting ligands (3AQN, 6AQN, and 360A) suggests that these ligands may affect critical cellular targets or pathways in the parasite. Although quadruplex-forming sequences are present in different important regions of the *P. falciparum* genome,[25] except the telomeric quadruplex, other biologically active quadruplexes have a C-rich complementary strand that drives structural competition toward the formation of the thermodynamically stable duplex state.[41] To understand the underlying molecular cascades in G-quadruplex interacting ligand mediated parasite inhibition, we focused on the telomeric quadruplex. Biophysical and parasite inhibition studies hint toward ligand-induced irreversible damage probably caused by the disturbance of telomere integrity (Figures 1, 2, S3, and S4). To confirm this, a PCR-based telomeric length estimation (Table S2) was performed for the genomic DNA isolated from the ligand-treated parasite. The observed shrinkage of the telomeric smear suggests telomere erosion in ligand-treated samples (Figure 3A). This observation was also validated through southern hybridization experiments, wherein a similar shrinkage in the parasite telomere smear was observed upon exposure to quadruplex-interacting ligands (Figure 3B).

To understand whether telomere degeneration induced by quadruplex-interacting ligands is signaled to the subtelomeric region, real-time PCR for subtelomeric genes was performed (Table S3). In *P. falciparum*, the subtelomeric region encodes multigene families (*Var*, *Rifin*, and *Stevor*) that undergo unique transcriptional switching in which only one gene is expressed, while the rest remain transcriptionally silent.[32,42−44] The exposure to 3AQN, 6AQN, and 360A resulted in transcriptional activation of subtelomeric genes (*Var*, *Rifin*, and *Stevor*) (Figure 4 and Figure S5). Furthermore, disruption of telomere homeostasis by G-quadruplex interacting ligands also caused transcriptional upregulation of important genes (*Mre11*, *H2A*, and *RPA1*)[45−47] involved in DNA repair and recombination to ameliorate the effect of DNA damage induced by telomere malfunction (Figure S6). To rule out the possibility of nonspecific effects of G-quadruplex interacting ligands on cellular homeostasis, the changes in transcript levels of histone deacetylase *Sir2a* were also monitored. Sir2a localizes to telomeres and the nucleolus to regulate expression of subtelomeric genes and rRNA genes, respectively.[32,42,44,48] Ligand treatment did not significantly affect the transcriptional state of *Sir2a*, thus confirming that the observed transcriptional activation of genes involved in DNA repair is due to ligand-induced telomere degeneration (Figure S6).

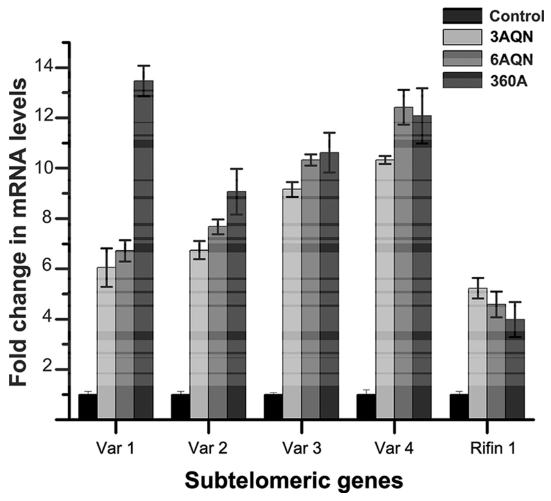


**Figure 4.** Telomere dysregulation-induced transcriptional activation of subtelomeric genes. Real-time PCR analysis of the fold change in the mRNA expression of subtelomeric genes upon ligand treatment (5 $\mu$M, 24 h). Error bars represent the standard deviation ($n$ = 4).

## DISCUSSION

In this study, we have investigated the molecular events involved in telomeric quadruplex−ligand interaction mediated parasite inhibition. A major concern for exploring the telomeric quadruplex of *P. falciparum* as an antimalarial target is that this motif is also formed by the human telomeric sequence, which has attracted an immense amount of attention as a drug target for cancer.[14,15] Therefore, to examine the *P. falciparum* telomeric quadruplex as an antimalarial drug target, it is important to understand the differences in the architecture and dynamics of telomeric ends in both the parasite and the human host. First, the average parasite telomere length is 1−2 kb, while the average length of the human telomere is 10−15 kb in a normal healthy cell.[30,49] Second, the parasite telomerase is 2.5 times larger and has a telomerase mRNA (Pf−TERC) that is longer than its human counterpart.[50] Third, the parasite subtelomeric region encodes multigene families involved in cytoadherence and antigenic variation,[44] whereas the human subtelomeric region encodes pseudogenes, putative RNA helicases, and immunoglobulin heavy chains.[51] Last, the telomerase and other telomere/subtelomere binding proteins in the parasite are highly diverged from the human orthologs.[32,42,50] Taking together these notable differences in the structural and functional complexity of telomeric ends in *P. falciparum* and in humans, we can explore quadruplex−ligand interaction to selectively achieve parasite inhibition.

Our biophysical experiments showed that bisquinolinium derivatives of 1,8-naphthyridine and pyridine remarkably stabilize and induce structural changes in the molecular recognition properties of telomeric and subtelomeric quadruplexes (Figure 1 and Figure S1). However, sometimes promising biophysical properties do not translate into efficacious biological responses because of the inability of ligands to cross the cell membranes. This problem becomes even more challenging while targeting the malaria parasite, which resides in human RBCs. A promising antimalarial molecule targeting the parasite telomere should be able to cross the plasma membrane of RBCs, parasitophorous vacuolar membrane, parasite plasma membrane, and nuclear membrane to reach its target site.[52] We checked whether 3AQN, 6AQN, and 360A selectively inhibit parasite proliferation. Our parasite inhibition and cytotoxicity assays indicate that these ligands induced parasite death without any adverse effects on normal human cells (Figure 2 and Table 1). Cellular effects of quadruplex interacting ligand 360A have been studied in different human cancerous cell lines, which generally have a shorter telomere (1−4 kb)[53] and are highly dependent on telomerase to maintain telomeric length for cellular proliferation. 360A exhibits potent antiproliferation activity by stabilizing the human telomeric quadruplex and prevents the binding of telomerase for telomere maintenance.[54−57] Like human cancerous cells, the parasite has short telomeres (1−2 kb) and is critically dependent on telomerase for its survival.[30,50] Our experiments suggest that quadruplex interacting ligands (3AQN, 6AQN, and 360A) induce telomere


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erosion that consequently leads to parasite death (Figures 2 and 3). Another striking observation was that parasites do not recover even upon removal of drug pressure, suggesting that loss of telomere homeostasis is associated with persistent DNA damage and irreversible loss of cell division potential (Figures S4 and S6). Telomere shortening and degeneration also influence the transcriptional activation of subtelomeric genes (Figure 4 and Figure S5) that are epigenetically regulated in the silenced state by telomere binding proteins through the telomere positioning effect (TPE) in a healthy cell.[32,42,58,59] Furthermore, many subtelomeric genes (especially the *Var* and *Rifin* multigene family) harbor quadruplex-forming sequences, which may facilitate recombination events to generate antigenic diversity.[25] Similarly, the occurrence of potential quadruplex-forming sequences in antigenic variation genes in pathogenic microorganisms has been suggested to facilitate hyper-recombination for expansion of the surface antigen repertoire to escape from host immune surveillance.[17] Interestingly, expression of the antigenic variation genes in subtelomeric loci can be perturbed by disturbing their recombination status or chromatin dynamics in the subtelomeric/telomeric regions. The observed effects of G-quadruplex ligand-mediated parasite inhibition could be due to cumulative effects of ligand-induced telomeric and subtelomeric dysregulation resulting in an irreversible collapse of cellular homeostasis.

In this study, we provide a proof of concept for targeting parasite telomeres using G-quadruplex interacting ligands (3AQN, 6AQN, and 360A). Though these ligands have $IC_{50}$ values that are higher than those of chloroquine (CQ), they exhibit promising antimalarial activity in both CQ-sensitive and -resistant strains with minimal toxicity in a mammalian host (Table 1). Chloroquine, a 4-aminoquinoline derivative, has been discontinued because of an increased number of cases of drug resistance in various malaria-infected regions. Additional structure−activity relationship (SAR) experiments are required to further improve the antimalarial activity of the investigated ligands so that they can be taken forward for evaluation of their efficacy in animals (rodent and monkey models) infected with multidrug-resistant strains. To date, malaria drug discovery programs have mainly focused on the identification of lead molecules that specifically target essential parasite proteins. However, the parasite has progressively acquired random mutations in these drug target proteins that either cause efflux of the drug (e.g., resistance to Chloroquine)[60] or prevent the binding of the drug to their respective protein targets (e.g., resistance to Cycloguanil, Pyrimethamine, and Atovaquone).[33] Recent cases of drug resistance for the current frontline drug Artemisinin[33] have raised an alarm for the urgent need to identify new scaffolds and biological targets. Hence, pharmacological targeting of noncanonical structures in telomeres may offer an alternative strategy for combatting emerging pleiotropic drug resistance problems, as acquiring random mutations to disrupt the secondary structures in its telomeric region will dramatically reduce the survival fitness of the parasite.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.7b00964.

Details of sequences used for biophysical and molecular biology experiments, CD spectra and thermal stability data for telomeric/subtelomeric quadruplexes and duplex DNA upon interaction with G-quadruplex interacting ligands, microscopy data for stage-specific ligand treatment and removal of drug pressure, and real-time PCR data for subtelomeric, DNA repair, and histone deacetylase genes (PDF)

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### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS

CD, circular dichroism; $T_m$, thermal melting temperature; RBC, red blood cell.

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