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G-Quadruplex ligands: Potent inhibitors of telomerase activity and cell proliferation in *Plasmodium falciparum*

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ABSTRACT

Telomeres are DNA and protein structures located at the ends of eukaryotic chromosomes. These structures maintain chromosomal stability by impeding chromosomal ends from being recognized and processed as fragmented DNA. They also support the complete replication of the genome by providing mechanisms that solve the end-replication problem. Telomeric DNA is formed of short, repeating, guanine-rich sequences. The G-rich sequence extends towards the 3′ end, forming a protruding single-stranded end that can acquire a conformation known as G-quadruplex. The ligands stabilizing this structure are potent inhibitors of telomerase activity, a catalytic activity necessary to compensate for the loss of telomeric DNA that occurs in each round of replication. In the absence of telomerase, telomeres shorten after a given number of cell divisions, after which the cell enters a senescence state and finally dies. In the presence of telomerase activity, telomeres are preserved, and cells reach a state of indefinite replication or immortalization.

This study analyzed the effect of two ligands of the G-quadruplex (TMPyP4 and Telomestatin) on the telomerase activity and cell proliferation of *Plasmodium falciparum*, given that this parasite has a high proliferation rate and an almost indefinite replication capacity. The effect of the ligands on telomerase activity was evaluated using the TRAP (Telomere Repeat Amplification Protocol) activity assay, which was performed in the presence of increasing concentrations of each ligand. In this study, TMPyP4 showed the highest inhibitory effect, reaching 50% inhibition at a 5 μM concentration. Regarding proliferation, both ligands drastically affected parasite growth, but Telomestatin had a stronger effect. After three days of treatment, parasite growth decreased by 90%. Thus, it is possible that this compound interferes with other vital pathways for the parasite beyond the elongation of telomeric DNA by telomerase.

1. Introduction

The telomere is a complex structure made of DNA and protein and is located at the ends of eukaryotic linear chromosomes. In general, telomeres are formed by short, repeating sequences (5–26 bp) rich in guanine (TTAGGG in vertebrates, some filamentous fungi and some parasites, such as *Leishmania* and *Trypanosoma*). In most organisms, the G-rich strand extends towards the 3′ end, generating a protruding strand of single-chain DNA (overhang) [1,2].

The double-stranded terminal region of the telomeric DNA is not packed in nucleosomes, but rather has a special structure. The presence of specific binding sites allows some proteins to anchor, which then allow other proteins to bind, creating a multi-protein structure that covers and protects the DNA [3,4]. Thus, the DNA-protein complex at the end of the chromosome forms a *cap* whose main function is to preserve the stability of the chromosome. When the protecting cap is removed, the telomeres are recognized and processed as fragmented DNA [5,6]. The length of the telomeric DNA varies among organisms, cell type, and chromosomes since few bases in ciliates (24–50) and up to several kilobases in mammals (15 kb–150 kb) [7]. Maintaining the length of the telomere within a defined range in a given organism is crucial for cell stability [8]. Nonetheless, a loss of 50–150 bp of telomeric DNA is associated with each cell division because conventional DNA polymerases cannot synthesize the ends of linear DNA molecules. This phenomenon is known as the end replication problem [9]. To compensate for this loss, three mechanisms have been reported, of which the de novo synthesis of telomeres by telomerase activity predominates [10,11]. Telomerase activity is undetectable in most animal somatic cells but is generally present in highly reproducing tissues and in most human tumor cells [12]. In somatic cells, the telomeres shorten with every cell

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division, reaching a critical length at which they are not functional. At this point, the cell stops its growth and enters a state of replicative senescence [13,14]. In the presence of telomerase, telomeres extend or are preserved, replicative senescence is avoided, and the cell reaches an indefinite multiplication state or "immortalization" [15].

Telomerase activity resides in a catalytic core formed by an RNA subunit known as TER (Telomerase RNA) and a protein subunit TERT (Telomerase Reverse Transcriptase). The elongation of the telomere by the addition of telomeric sequences is a reverse transcription reaction in which the RNA subunit serves as the template, the protruding 3′ end of the telomere as the primer and the protein subunit provides the catalytic activity [10,11,16].

Telomerase activity is detected in 90% of human tumor cells but is undetectable in most normal somatic cells [17]. This correlation has suggested that the reactivation of the telomerase is necessary for the sustained cell proliferation typical of tumor cells, and this activity has become a potential target for the development of chemotherapeutic agents [18]. This enzymatic activity offers different action targets: TERT is the target for reverse transcriptase inhibitors; TER is the target for anti-sense oligos that block the region that serves as the template; and telomeric DNA is the target for G-quadruplex ligands [18,19]. Telomerase inhibition can be performed by "sequestering" the protruding 3′ end which can adopt a conformation known as G-Quadruplex. This conformation is made up of 4 strands and consists of a tetrad of guanines stabilized by hydrogen bonds and by the presence of monovalent cations, particularly K^+ [20,21]. This conformation is recognized and maintained by certain ligands, impeding the access of the telomerase to its substrate and, therefore, the elongation of the telomere. Several stabilizers of the G-quadruplex have been reported, including porphyrin derivatives [22,23]. One of the most powerful is Telomestatin, a molecule of natural origin obtained from *Streptomyces anulatus* 3555-SV4, which has high structural similarity to the G-Quadruplex structure [24]. *Plasmodium falciparum* is the causal agent of the most severe form of human malaria, a disease that constitutes one of the most serious health issues in the tropical world. The treatment and control of this disease is becoming more and more difficult because the parasite has developed resistance to commonly used medicines [25]. Given that *P. falciparum* is a parasite with a high proliferation rate and can undergo an indefinite number of divisions, it can be assumed that the molecular machinery conferring this almost unlimited proliferation capacity might be an excellent target against the parasite.

P. falciparum telomeres consist of a tandem array of the degenerate G-rich heptamer GGGTT(T/C)A with an approximate length of 1.3 kb [26]. Since the telomerase complex is active in different development stages of the parasite [26–29], telomerase activity could be the mechanism used by the parasite to compensate for the loss of telomeric sequences associated with each replication process and to avoid replicative senescence. In humans, several telomeric specific proteins have been shown to regulate telomerase activity [3;4;6], however in *P.falciparum*, the homologues of these well-known telomeric binding proteins have not been identified yet. This study focused on establishing whether the telomeric DNA of *P. falciparum* acquires a G-quadruplex structure and if it is susceptible to ligands that induce or stabilize these structures and therefore block telomerase activity. The study also sought to evidentiate whether treating *in vitro*-cultured parasites with these ligands affects cell proliferation.

2. Materials and methods

Two G-quadruplex ligands were used: the cationic porphyrin (TMPyP4: 5,10,15,20-tetra-(*N*-methyl-4-pyridyl) porphyrin, Calbiochem San Diego, CA,USA) and the macrocyclic polyoxazole Telomestatin, donated by Dr. Kazuo Shin-ya of the Biosciences Institute at Tokyo University.

2.1. Induction of G-quadruplex conformation using oligos to simulate the P. falciparum telomere

An EMSA (Electrophoretic Mobility Shift Assay) was performed with biotinylated oligos simulating the guanine-rich strand of the telomeric DNA of *P. falciparum* or the C-rich complementary strand in the presence or absence of each ligand. For this purpose, 200 fmol of the oligo **Tel Rep G** $(GGGTTCA)_4$ or **Tel Rep C** $(TGAACCC)_4$ were incubated in 20 μL of binding buffer (50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM EDTA, 5% Glycerol) at room temperature for 15 min. Then, increasing concentrations of each ligand were added and incubated for an additional 45 min. The reactions were then placed in non-denaturing 10% acrylamide gels and run in 0.5X TBE for 45 min at 100 V. The gels were transferred to membranes, and the detection was performed using a LightShift™ Chemiluminescent EMSA Kit (Thermo) following the manufacturer's instructions.

2.2. Effect of the G-quadruplex ligands on the 3′ protruding end

To detect the 3′ protruding end, hybridization in solution was performed [23]. A total of 2 μg of genomic DNA was incubated in the hybridization buffer (20 mM Tris pH 8.0, 0.5 mM EDTA, 50 mM NaCl, 10 mM $MgCl_2$) with 10 pmol of the biotinylated Tel RepC oligo at 55 °C for 16 h. The samples were separated in 0.8% agarose gels in 0.5X TBE and visualized under UV light after staining with ethidium bromide. The gel was transferred to a nylon membrane, and detection was performed using a LightShift™ Chemiluminescent EMSA Kit (Thermo). To determine the effects of TMPyP4 and Telomestatin, increasing concentrations of the inhibitors were added to the reaction mix [23]. Tel Rep C*: 5′-TKAACCCTKAACCCTKAACCC TKAACCC-3′ K: A/G.

2.3. Effect of the G-quadruplex ligands on telomerase activity

2.3.1. Obtaining protein extracts as a source of telomerase

A total of 2–8 × 10^9 parasites at trophozoite stage were resuspended in 1 mL of TMG Buffer (10 mM Tris pH 7.5, 1.5 mM MgCl2, 10% Glycerol, and 10 mM beta mercaptoethanol), protease inhibitors (Sigma) and RNAse inhibitors (RNAse OUT, Invitrogen) and were lysed by incubation with 0.2% NP40 at 4 °C for 30 min. Cellular debris were removed by centrifuging at 14,000 rpm for 30 min at 4 °C, and the supernatant (i.e., the cytosolic fraction) was distributed into vials and stored at −70 °C [27,28].

2.3.2. TRAP

Telomere Repeat Amplification Protocol assay. A method for absolute quantification with real-time PCR was implemented based on the use of an antisense oligo named Amplifluor® in the PCR reaction [30]. The conditions of the non-radioactive TRAP assay [28] were modified to achieve extension via telomerase and then amplification using the fluorogenic oligo as follows: 500 ng of protein extract was incubated at 37 °C for 30 min with 20 ng of TS oligo AATCCGTCGAGCAGAGTTCA in 20 μL of 1X TRAP Buffer (20 mM Tris pH 8.3, 50 mM KCl, 0.1 mg/mL BSA, 0.005% Tween, 50 μM dNTPs, 1.0 mM $MgCl_2$). After extension via telomerase activity, samples were denatured at 94 °C for 3 min, 20 ng of the CX oligo $\mathbf{A}^{FAM}$CGCAATGTATGCG$\mathbf{T}^{DAB}$GGCTT$(TRAACCC)_3$ was added, and amplification was performed under the following temperature profile: 94 °C for 15 s, 65 °C for 30 s for 40 cycles.

To determine the effect of the G-quadruplex ligands on the telomerase activity of *P. falciparum*, the activity assay was performed