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Interaction of Quindoline derivative with telomeric repeat–containing RNA induces telomeric DNA-damage response in cancer cells through inhibition of telomeric repeat factor 2

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ABSTRACT

Background: Telomeric repeat–containing RNA (TERRA) is a large non-coding RNA in mammalian cells, which forms an integral component of telomeric heterochromatin. TERRA can bind to an allosteric site of telomeric repeat factor 2 (TRF2), a key component of Shelterin that protect chromosome termini. Both TERRA and TRF2 have been recognized as promising new therapeutic targets for cancer treatment.

Methods: Our methods include FRET assay, SPR, CD, microscale thermophoresis (MST), enzyme-linked immunosorbent assay (ELISA), chromatin immunoprecipitation (ChIP), colony formation assays, Western blot, immunofluorescence, cell cycle arrest and apoptosis detection, and xCELLigence real-time cell analysis (RTCA).

Results: In our routine screening of small molecule libraries, we found that a Quindoline derivative, CK1-14 could bind to and stabilize TERRA G-quadruplex structure, which could bind more tightly with an allosteric site of a telomeric binding protein TRF2, resulting in dissociation of TRF2 from telomeric DNA. Further in cellular studies indicated that the above effect of CK1-14 on TERRA G-quadruplex could activate DNA-damage response and cause cell cycle arrest, resulting in inhibition of U2OS cell proliferation and causing cell apoptosis.

Conclusions: Our mechanistic studies indicated that interaction of CK1-14 with TERRA induces telomeric DNA-damage response in U2OS cancer cells through inhibition of TRF2. CK1-14 could be further developed as a promising lead compound targeting telomere for cancer treatment.

General significance: Our present study provides the first evidence that allosteric modulation of TRF2 by TERRA G-quadruplex with a binding ligand could become a promising new strategy for cancer treatment especially for ALT tumor cells.

1. Introduction

Guanine-rich single-stranded DNA can form four-stranded DNA secondary structures called G-quadruplexes (G4) [1]. The formation of G-quadruplex has been initially proposed to occur at telomeric region where G-rich repeats are present within an extended 3′ single-strand protruding portion involving the interaction of four guanine bases in a square planar arrangement stabilized by central cations [2,3]. G-quadruplexes can sequester the 3′ end of the telomeric DNA and prevent it from being extended by telomerase, making this structure promising anti-cancer drug target [3]. Some studies on telomeric DNA G-quadruplex binding ligands show that these ligands can inhibit telomerase activity and induce telomere shortening and replicative senescence by blocking the binding of telomerase with G-overhang [4–6]. It is clear how the telomeric DNA G-quadruplex binding ligands work in telomerase positive cancer cells. Significantly, tumor cells stabilize their telomeres, either rely on upregulating telomerase or activating an alternative lengthening of telomeres (ALT) mechanism [7]. Although recent data report that G-quadruplex binding ligands induce an important telomere degradation of ALT cell lines, it has been suggested that additional mechanisms may explain the biological activity of the G-quadruplex binding ligands in these tumor cell lines [8–10]. However,

Abbreviations: ALT, alternative lengthening of telomeres; ATM, ataxia telangiectasia mutated; CD, circular dichroism; ChIP, chromatin immunoprecipitation; DAPI, 4′,6-diamidino-2-phenylindole; DDR, DNA damage response; DMSO, dimethyl sulfoxide; DSBs, DNA double-strand breaks; ELISA, enzyme-linked immunosorbent assay; FAM, 6-carboxyfluorescein; MST, microscale thermophoresis experiment; MTT, methyl thiazolyl tetrazolium; OD, optical density; PBS, phosphate buffer saline; PI, propidium iodide; RTCA, real time cellular analysis; SPR, surface plasmon resonance; TAMRA, carboxytetramethylrhodamine; TERRA, telomeric repeat–containing RNA; TRF1, telomeric repeat factor 1; TRF2, telomeric repeat factor 2

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how these G-quadruplex binding ligands induce replicative mortality in telomerase negative cancer cells remains unclear.

To address this question, it is necessary to mention telomeric repeat–containing RNA (TERRA) and telomeric repeat factor 2 (known as TRF2 or TERF2). Telomeric repeat-containing RNA (TERRA) is a large non-coding RNA in mammalian cells, which forms an integral component of telomeric heterochromatin [11,12]. Individual TERRA molecules start with a subtelomeric RNA tract followed by a variable number of telomeric G-rich repeats (5′-UUAGGG-3′ in vertebrates) [13]. Phan and co-workers found that long telomeric RNAs form G-quadruplexes comprising four UUAGGG repeats, adopting a parallel conformation containing three G-tetrad layers [14]. TERRA G-quadruplexes are ideal therapeutic targets, because they are required for telomere heterochromatin formation in all cancer cells, even in those that do not require telomerase (ALT-positive tumors) [15]. Several types of TERRA G-quadruplex binding ligands have been reported, which may inspire new strategies for targeting telomere-related diseases [16–19].

Several TERRA-binding proteins have also been discovered, especially telomeric duplex DNA binding proteins TRF1 and TRF2, pointing to a significant role of TERRA in more general chromosome biology [14,20,21]. TRF2 is a key component of Shelterin that protect chromosome termini [22]. Telomeres can fold into t-loops, which is facilitated by TRF2, and their formation contribute to telomere protection by masking the 3′-end overhang from being recognized as damaged DNA, preventing activation of a DNA damage response (DDR) at telomeres and inappropriate repair [23,24]. TRF2 GAR domain has a relatively high affinity for the G-rich RNA capable of forming G-quadruplex [21,25]. Balasubramanian and co-workers have found that TRF2 binds TERRA via interactions that necessitate the formation of a G-quadruplex structure rather than TERRA liner sequence. They also show that TRF2 simultaneously binds TERRA G-quadruplex and telomeric duplex DNA. Besides, TRF2 binds to telomeric duplex DNA more tightly than its binding to telomeric duplex DNA in the presence of TERRA G-quadruplex [26]. TRF2 helps the folding of telomere to form T-loop structure and the suppression of ATM-dependent DNA damage response activation.

Telomere interacting agents are being exploited and have proven to be a new class of anticancer agents [27,28]. It should be noted that allosteric binding inhibitors of proteins have been previously studied for inhibition of other enzymes [29,30]. In the present study, after screening our G-quadruplex binding ligands, we found a Quindoline derivative, CK1-14, could bind selectively with and stabilize TERRA G-quadruplex. This binding complex could interact strongly with an allosteric binding pocket of TRF2, which consequently inhibited the binding of TRF2 to telomeric duplex DNA both in vitro and *in cells*. The resulting delocalization of TRF2 from telomere induced DNA damage response at telomere region, which could effectively induce acute cell growth arrest in cancer cells. CK1-14 could effectively inhibit the proliferation of osteosarcoma cancer cells (U2OS cells), cause the cell G2/M phase arrest and induce apoptosis of U2OS cells. These findings illustrated that a TERRA G-quadruplex binding ligand could control the binding interaction of TRF2 with telomeric duplex DNA in ALT cell line. CK1-14 induced DNA-damage response in U2OS cells through inhibition of TRF2. TERRA G-quadruplex with bound ligand CK1-14 was found to be a novel allosteric modulator of TRF2, which possibly elicited a telomeric t-loop uncapping effect resulting in DNA damage response.

2. Materials and methods

2.1. Cell culture, TRF2 expression and purification

Detailed information is provided in the Supplementary information.

2.2. FRET spectroscopy

FRET assays were performed following previously described methods. 5′-FAM and 3′-TAMRA dual labeled F-Tel22-T (5′-AGGGTTAGGGTTAGGGTTAGGG-3′), F-TERRA22-T (5′-rArGrGrGrUrUrArGrGrGrUrUrArGrGrGrUrUrArGrGrG-3′), F-TERRA12 (5′-rUrArGrGrGrUrUrArGrGrGrU-3′), and Tel12-T (5′-TAGGGTTAGGGT-3′) at a final concentration of 0.2 μM were incubated with 1 μM compounds in Tris-HCl buffer (10 mM, pH 7.4) containing 100 mM KCl at room temperature for 6 h. The fluorescence melting curves were determined using a Roche LightCycler2® real-time PCR machine. Fluorescence readings with excitation at 470 nm and detection at 530 nm were taken at intervals of 1 °C from 37 to 99 °C, with a constant temperature maintained for 30 s prior to each reading to ensure a stable value. Final analysis of the data was conducted using Origin 8.0 (OriginLab Corp.).

2.3. Surface plasmon resonance

SPR measurements were performed on a ProteOn XPR36 Protein Interaction Array system (Bio-Rad Laboratories, Hercules, CA) using a Neutravidin-coated GLH sensor chip. In a typical experiment, biotinylated duplex DNA and biotinylated TERRA22, Tel22, mutant TERRA (5′-rArGrGrGrUrUrArGrUrGrUrUrArGrUrGrUrUrArGrGrG-3′), and hairpin DNA (5′-dTATAGCTATA-HEG-TATAGCTATA-3′) were folded in filtered and degassed running buffer (50 mM Tris-HCl, 150 mM KCl, pH 7.4, 0.005% Tween20). The DNA samples were then captured (~1000RU) in flow cells 1 and 2, leaving the third flow cell as a blank. Ligand solutions (at 0.0625, 0.125, 0.25, 0.3125, 0.5, 0.625, 1, 1.25, 2, 2.5, 5, and 10 mM) were prepared with running buffer by serial dilutions from stock solutions. Six concentrations were injected simultaneously at a flow rate of 25 mL/min for 240 s of association phase, followed with 240 s of dissociation phase at 25 °C. The GLH sensor chip was regenerated with short injection of 50 mM NaOH solution between consecutive measurements. The final graphs were obtained by subtracting blank sensorgrams from the duplex or quadruplex sensorgrams. Data were analyzed with ProteOn manager software, using the Langmuir model for fitting kinetic data.

2.4. Circular dichroic (CD) spectroscopy and CD-melting experiments

A final concentration of 2 μM oligomers were re-suspended in CD buffer (10 mM Tris-HCl, pH 7.4) with varying amounts of compounds and incubated for 6 h. CD spectra were recorded on a Chirascan® CD spectrophotometer (Applied Photo-physics, UK) at 25 °C. CD spectra were collected from 225 to 340 nm with a 1.0 cm path length cylindrical quartz cuvette. A buffer blank was subtracted for all spectra, and the final analysis was carried out by using Origin 8.0. For CD melting temperature experiments, samples were annealed at first, G-quadruplex formation was induced, and molar ellipticity at 280 nm was measured over a temperature range of 25–95 °C.

2.5. Microscale thermophoresis experiments

Labeling procedure: the NT-647-NHS dye was mixed with the protein in a 2:1 ratio and was incubated for 30 min at room temperature in the dark. Unreacted ‘free’ dye was removed by using a gel filtration column (Sephadex G25, GE Healthcare). The purity was monitored by measuring the ratio of protein to dye after the clean-up procedure. Measuring procedure: the concentration of NT-647 labeled TRF2 was kept constant at 100 nM, while the concentrations of compounds were initially 1000 μM and half-diluted 15 times in DMSO. A similar process was performed for the detection of the compounds interaction with constant concentrations of FAM-TERRA22 at 200 nM. MST analyses were performed using a Monolith NT.115, and the fitting curve was obtained using NT Analysis 1.4.23. The K_D value is the numeric equivalent of the concentration of CK1-14 when the response is half of the plateau response (R_{max}) on the fitting curve.