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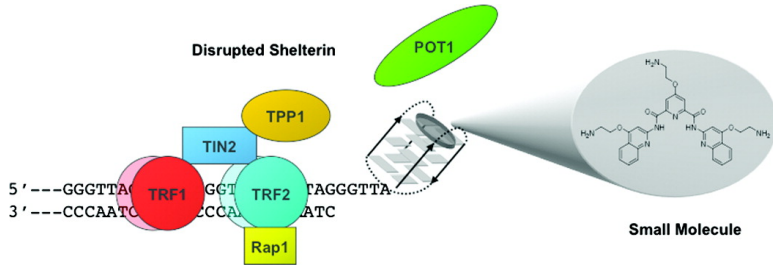

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A Novel Small Molecule That Alters Shelterin Integrity and Triggers a DNA-Damage Response at Telomeres

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The protection and maintenance of the genome in human cells is critical. The cell uses signaling pathways that can down regulate the cell cycle when DNA-damage occurs, giving an opportunity for efficient repair before division.[1] However, this raises the question of how cells manage to distinguish the telomeres from DNA double-strand breaks. A six-protein complex named "telosome" or "shelterin" (Figure 1) binds to the double-stranded telomeric DNA and single-stranded telomeric G-overhang at the chromosome ends.[2,3] The complex shelters telomeres from the DNA-damage response machinery and protects chromosomes from shortening, nonhomologous end-joining, and homology-directed repair. Moreover, the shelterin component POT1 (protection of telomeres 1)[4] has been shown to modulate the activity of telomerase,[5] the enzyme capable of conferring infinite proliferative capacity on cells by extension of the G-overhang.[6] It has been shown that the telomeric G-overhang, when folded into G-quadruplexes, is resistant to extension by telomerase,[7] and that synthetic small molecules that stabilize these structures can decrease the enzyme efficiency.[8−10] Gomez et al. showed that the potent G-quadruplex binding natural product telomestatin induces apoptosis of cancer cells via a mechanism proposed to involve the uncapping of POT1 from telomeres.[11] Herein, we describe a novel synthetic small molecule (compound **1**, Figure 1), which exhibits unprecedented G-quadruplex stabilization leading to an alteration of shelterin at the telomeres of human cancer cells.

Compound **1** was designed following intensive research on the biology of G-quadruplex nucleic acids.[12] The design rationale comprises certain structural features shared by known quadruplex binding small molecules, with particular emphasis on an electron rich aromatic surface, the potential for a flat conformation, and an ability to participate in hydrogen bonding.[13] The small molecule is readily accessible in six synthetic steps that are easily scalable and amenable to molecular diversity (see Supporting Information).

We first evaluated the potential for **1** to stabilize the telomeric G-quadruplex by FRET-melting experiments.[14] Compound **1** stabilized the human telomeric G-quadruplex with a maximum ΔT_m of 35 K in 60 mM K^+ and 44 K in 100 mM Na^+ at 0.18 and 0.34 μM compound, respectively. In contrast, the ligand-induced double-stranded DNA stabilization was negligible with a ΔT_m of 0.5 K in 60 mM K^+ at 1 μM compound. It is noteworthy that the G-quadruplex melting profile was almost unaffected by the presence of 25 mol equiv of unlabeled double-stranded DNA competitor (see Supporting Information). By comparison, the maximum ΔT_m induced by telomestatin was 30 K in 60 mM K^+ at 1.2 μM compound.[15] The data recorded for **1** represent the highest induced shifts in melting temperature for the telomeric G-quadruplex that we are aware of, accompanied by a high level of selectivity over duplex DNA.[16]

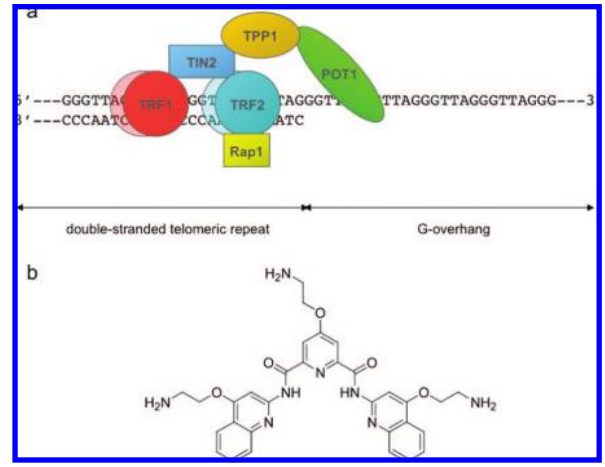

Figure 1. (a) The six-protein complex shelterin bound to telomeric DNA; (b) structure of **1**.

We next explored the ability of **1** to uncap POT1 from telomeric single-stranded DNA. The dissociation of a complex formed by POT1 and the telomeric DNA, in the presence of increasing amounts of small molecule, was evaluated by electrophoretic mobility shift assay on a native agarose gel.[17] We found that **1** uncaps POT1 from the DNA in a dose-dependent manner with an $IC_{50(POT1)}$ of 200 nM (Figure 2), the lowest reported value for a small molecule. In contrast, telomestatin exhibits an $IC_{50(POT1)}$ of 500 nM (see Supporting Information). We then assessed telomerase inhibition by direct assay using $d(T_2AG_3)_3$ as primer (see Supporting Information). Compound **1** exhibits an $IC_{50(Telo)}$ of 21 μM, which represents a relatively weak inhibition considering the high ΔT_m recorded. These results suggest that stabilization of the telomeric G-quadruplex by the small molecule has a stronger potential to perturb the binding of a shelterin component to telomeric DNA than to inhibit extension of the DNA by telomerase.

To investigate whether **1** could uncap POT1 in cells, we used a model human cancer cell line HT1080 modified to express a GFP-POT1 fusion protein that colocalizes with TRF2 at telomeres.[11] The incubation of HT1080GFP-POT1 cells with 1 μM of compound **1** for 72 h, conditions under which most cells were still viable,[18] resulted in a strong disappearance of GFP signal associated with telomeres compared to the untreated control as observed by fluorescence microscopy (Figure 3). This result is consistent with the uncapping of POT1 in vitro, and a model whereby the folding of the telomeric G-overhang into quadruplexes induced by **1** leads to POT1 uncapping from the telomeres in cells.

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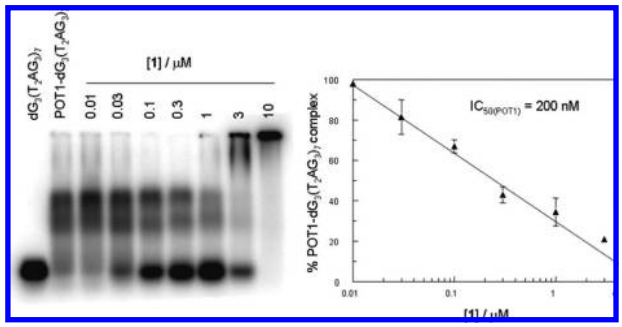

Figure 2. POT1 uncapping: inhibition of POT1 binding to the telomeric sequence $dG_3(T_2AG_3)_7$ induced by **1** in vitro.

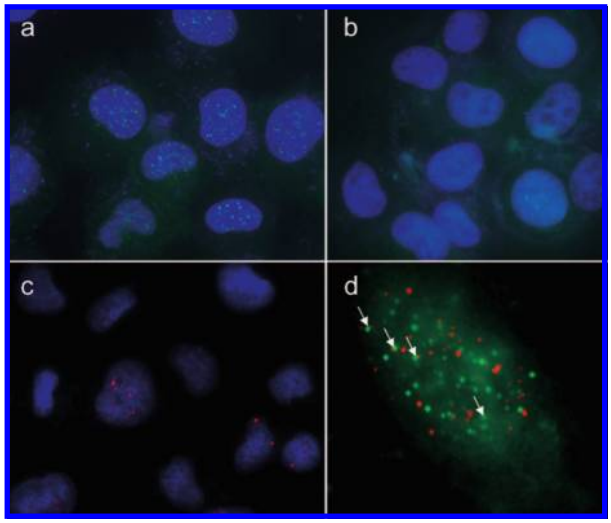

Figure 3. Effect of **1** on HT1080GFP-POT1 cells: (a) Untreated control, fluorescent GFP-POT1 (green); (b) after treatment with **1** (1 μM) for 72 h; (c) γH2AX foci in cells treated with **1** (3 μM) for 24 h (red); (d) colocalization of γH2AX foci (red) and GFP-POT1 (green) at telomeres (marked with arrowheads). DAPI DNA staining (blue) throughout.

Dysfunctional telomeres that are no longer protected by shelterin have been shown to activate the DNA-damage response machinery, which can trigger cell cycle arrest, senescence and apoptosis.[19] Such dysfunction has been associated with the appearance of nuclear foci of phosphorylated histone H2AX (γH2AX), an early DNA-damage response marker. To evaluate the consequence of POT1 uncapping from telomeres induced by **1**, we performed γH2AX immunofluorescence microscopy[11] on cells incubated with 3 μM compound for 24 h, conditions where POT1 is only partially removed from telomeres. A strong increase in γH2AX foci compared to the untreated control was observed in the nucleus, and partially co-localized with GFP-POT1 at telomeres (Figure 3). This observation suggests that **1** induces a DNA-damage response through the removal of POT1 from telomeres.

In conclusion, we have described a novel synthetic small molecule that stabilizes the folded human telomeric quadruplex with an unprecedented induced shift in the melting temperature, and very good selectivity relative to double-stranded DNA. We have shown that the small molecule interacts with telomeres and alters the integrity of shelterin in cells through POT1 uncapping resulting in a DNA-damage response. Compound **1** is therefore a small molecule with considerable potential to dissect the biological processes occurring at telomeres. Such studies are ongoing and will be reported in due course.

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Supporting Information Available: Experimental details for the synthesis of **1**, FRET-melting, direct telomerase extension assay, in vitro POT1 uncapping assay, POT1 and γH2AX in cellulo experiments, growth inhibition assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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