The topoisomerase II catalytic inhibitor ICRF-193 preferentially targets telomeres that are capped by TRF2

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Chen L, Zhu X, Zou Y, Xing J, Gilson E, Lu Y, Ye J. The topoisomerase II catalytic inhibitor ICRF-193 preferentially targets telomeres that are capped by TRF2. Am J Physiol Cell Physiol 308: C372–C377, 2015. First published December 17, 2014; doi:10.1152/ajpcell.00321.2014.—The increased level of chromosome instability in cancer cells is not only a driving force for oncogenesis but also can be the Achilles’s heel of the disease since many chemotherapies kill cells by inducing a nontolerable rate of DNA damage. A wealth of published evidence showed that telomere stability can be more affected than the bulk of the genome by several conventional antineoplastic drugs. In the present study, HT1080 cell lines compromised for either telomere repeats binding factor 2 (TRF2) or POT1 were treated with ICRF-193 (3 μM, 24 h) or bleomycin (1 μM, 24 h). DNA damage was assayed by combining telomeric DNA staining of a (CCCTAA)n PNA probe with immunofluorescence of 53BP1 to score the rate of telomere colocalization with 53BP1 foci. We found that ICRF-193, but not bleomycin, leads to DNA damage preferentially at telomeres, which can be rescued by TRF2 inhibition. POT1 inhibition exacerbates telomere dysfunction induced by ICRF-193. Thus, ICRF-193 induces damage at telomeres properly capped by TRF2 but not by POT1. These findings are expected to broaden our view on the mechanism by which conventional therapeutic molecules act to eliminate cancer cells and how to use TRF2 and POT1 levels as surrogate markers for anti-topoisomerase II sensitivity.

Topoisomerase II; ICRF-193; telomere; TRF2; POT1

Telomeres are key features of chromosome termini that preserve genome integrity (5). In many organisms, their protective functions depend on at least two pathways. The first relies on telomerase, which can compensate for replicative erosion (8); the second relies on the binding to telomeric DNA of the shelterin protein complex composed of six polypeptides [telomeric repeat binding factor 1 (TRF1), TRF2, Rap1, Tin2, TPP1, and Pot1] (11). Three of the shelterin components recognize directly telomeric DNA; TRF1 and TRF2 bind telomeric DNA duplexes, while Pot1 binds single-stranded 3’-overhands. Among the shelterin subunits, TRF2 (3) is at the heart of the molecular events that maintain telomere integrity in mammals by preventing at telomeres different events normally occurring during DNA damage response: activation of ATM pathway (15), nonhomologous end-joining (NHEJ) (2), and homologous recombination (9). Moreover, TRF2 helps to fold the telomeric DNA into a t-loop, which may contribute to telomere protection by masking the 3’-overhang (1, 12).

A correct function of telomere seemingly displays antagonistic functions as far as tumorigenesis is concerned (7). On one hand, reactivation of telomerase in cancer cells is crucial for tumor progression. On the other hand, telomere shortening cooperates with p53 deficiency to favor carcinogenesis in aged mice (16). TRF2 appears also to play an important role in oncogenesis as it is upregulated in several human tumors (4, 14) and has been found to exert oncogenic properties when overexpressed in mouse keratinocytes (6). Recently, we showed that the high level of TRF2 found in cancer cells positively regulates the transcription of HS3ST4, a gene encoding a heparan sulfate (glucosamine) 3-O-sulfotransferase that is involved in the recruitment of NK-cells, revealing that TRF2 upregulation can have oncogenic properties through cell nonautonomous mechanisms (4).

Inhibition of telomerase provides an interesting “universal” strategy for cancer therapy: immunological and pharmacological inhibition of telomerase is currently used in a series of clinical protocols. However, with many but not all telomerase therapeutic approaches, growth arrest has been observed only when telomeres reach a critically short length. An alternative approach, although still poorly developed, is to target key components of telomere structure such as the use of G4 ligand (17). Interestingly, although DNA damages induced by drugs used in conventional chemotherapy are usually believed to occur stochastically in the genome, a wealth of evidence indicates that telomere stability is more affected than the bulk of the genome by several genotoxic molecules (e.g., cisplatin, spindle poisons as well as anti-topoisomerase I and II and replication inhibitors) (13). For instance, hydroxyurea (HU) is a chemotherapeutic agent commonly used for various malignancies and hematological disorders, including chronic myelogenous leukemia and sickle cell anemia. Chronic, low-level treatment with HU preferentially decreased the rate of telomere DNA synthesis and dissociated TRF2 from telomere DNA. All these indicate that a broad spectrum of genotoxic drugs can have specific DNA damage at telomeres either by binding directly to the DNA structure, or by occupancy of the interactive domain of telomeres to specific telomeric nucleoproteins. Collectively, these findings linking conventional chemotherapies to telomeres open new avenues for innovative combinations of chemotherapy drugs, for the use of telomere parameters as surrogate markers for therapy response and toxicity as well as for the introduction of future drugs against telomerase and telomere structural components. Therefore, there is a need...
for a precise understanding of the impact of genotoxic drugs on telomere functions. In this context, this work was designed to investigate the effects of the topoisomerase II catalytic inhibitor ICRF-193 [meso-2, 3-bis (2, 6-dioxopiperazin-4-yl) butane] on telomere functions and conversely to study the impact of specific telomere dysfunctions on ICRF-193 effects. We found that dysfunctional telomeres as a consequence of TRF2 inhibition, but not of POT1 inhibition, are less sensitive to ICRF-193 than control ones. This might be due to the fact that TRF2 has seemingly opposed roles regarding telomere topology: it can create topological barriers, rendering telomeres more sensitive to topoisomerase depletion, and it can cooperate with topoisomerase II to prevent replicative damage at telomeres (19). These findings are expected to broaden our view on the mechanism by which conventional therapeutic molecules act to eliminate cancer cells and how to use TRF2 and POT1 levels as surrogate markers for anti-topoisomerase II sensitivity.

MATERIALS AND METHODS

Cell culture, transfections, and lentiviral or retrovirus production and infections. Human embryonic kidney 293 Phoenix cells and HT1080 fibrosarcoma cells were grown in DMEM with 10% fetal calf serum and penicillin-streptomycin at 37°C. Transfections of plasmid DNA were performed with lipofectamine 2000 reagent (Invitrogen) as described by the protocol. Empty vector or expressing TRF2B/H9004M or shPOT1 vector were produced by transient transfection of human embryonic kidney 293 Phoenix cell lines with two other packaging plasmids, p8.91 and pVSVg. At 48 and 72 h after transfection, the infectious supernatant was collected and applied to the target cells. The efficiency of infection was determined by flow cytometry analysis of GFP expression. The stable HT1080 cell line with POT1 knockdown induced by shPOT1 was selected by 100 nM puromycin for 3 wk. HT1080 cells were treated with ICRF-193 (3 μM, 24 h) or bleomycin (1 μM, 24 h) before further experiments were performed.

Fig. 1. A flow cytometry analysis of the DNA content after the treatment of genotoxic drugs in HT1080 fibrosarcoma cell lines. A: representative FACS profiles of HT1080 fibrosarcoma cell lines treated either with ICRF-193 (3 μM, 24 h) or with bleomycin (1 μM, 24 h). B: quantitative analysis of DNA contents in cell cycles. Data are from three repeat experiments.

Fig. 2. DNA damage response induced by genotoxic drugs. A: representative images of confocal sections used in the detection of 53BP1 in the indicated situations of HT1080 cells. B: quantification of the mean number of 53BP1 foci per nucleus. The number below the x-axis indicates the scored nuclei for each condition. *P < 0.05.

Fig. 3. Telomere dysfunction induced by ICRF-193. A: representative images of confocal sections used in the detection of PNA-telomere signal (green) and 53BP1 colocalization at telomere damage-induced foci (TIF, marked by white arrows) in the indicated situations in HT1080 cells. Scale bar, 5 μm. B: quantification of the mean number of TIF per nucleus of A. The number below the x-axis indicates the scored nuclei for each condition. *P < 0.05.
Fig. 4. Telomeric repeat binding factor 2 (TRF2) inhibition, not POT1 inhibition, rescues telomere damage induced by ICRF-193. 

A: relative mRNA level of POT1 dividing to β-actin in HT1080 cells infected with a lentivirus expressing a scramble shRNA and a shRNA directed against mRNA POT1. B–E: representative images of confocal sections and quantification of the mean number of TIF and 53BP1 foci per nucleus in the indicated situations in HT1080 cells. shCtrl indicates empty vector; shPOT1 indicates knockdown of POT1; pWPIR indicates empty vector; pWPIR-TRF2ΔBΔM indicates expression of the dominant negative form of TRF2, also known as pWPIRTRF2DN. Scale bar, 5 μm. The number below the x-axis indicates the scored nuclei for each condition.
**Immunofluorescence detection.** Slides were fixed either by MeOH at −20°C or by 4% formaldehyde at room temperature (RT) for 10 to 15 min, then incubated 1 h with blocking buffer (0.8× PBS, 50 mM NaCl; 0.5% Triton X-100, 3% milk), followed by 1 h of incubation at 37°C with first antibody rabbit polyclonal to 53BP1 (Novus Biologicals; NB 100-305) in blocking buffer. Cells were then washed with 0.8× PBS, 50 mM NaCl, 0.1% Triton X-100, followed by donkey polyclonal anti-mouse Alexa488 (A21202; Molecular Probes) and donkey polyclonal anti-rabbit Alexa555 (A-31572; Molecular Probes). After washing with 0.8× PBS, 50 mM NaCl, 0.1% Triton X-100, the nucleus was labeled with DAPI and/or TOTO3.

Combined immunofluorescence detection and PNA or DNA fluorescence in situ hybridization. Cells were permeabilized in Triton X-100 buffer (20 mM Tris-HCl pH 8.0, 50 mM NaCl, 3 mM MgCl2, 0.5% Triton X-100 and 300 mM sucrose) at RT for 5 min, followed by 15 min fixation in 3% (para) formaldehyde and 2% sucrose, then again permeabilized in Triton X-100 buffer at RT for 10 min. Cells were blocked in 2% BSA in PBS overnight at 4°C. We then followed immunofluorescence steps as shown above. After final washes, slides were fixed for 2 min at RT in 4% (para) formaldehyde, followed by dehydration through an ethanol series (50%, 75%, and 100%, 5 min each). Fluorochrome-coupled PNA probe (AATCCC) was then applied to the slides [0.3 ng PNA probe/μl dissolved in 70% formamide] or with immunofluorescence in situ hybridization. First denaturing at 80°C for 3 min, then hybridization for 2 h at RT. Alternatively, slides were hybridized either with the digoxigenin-labeled ITS probe (8 ng/μl in 50% formamide/2xSSC/10% dextran sulfate) or with a probe labeling the subtelomeric region of the long arm of chromosome 4 (TelVysion DNA Probes, Vysis). Washes were the following: 2× 15 min at RT in 70% formamide, 10 mM Tris pH 7.2, 1% Blocking Reagent (Roche Biochemicals) first denaturing at 80°C for 3 min, then hybridization for 2 h at RT. Finally, slides were counterstained with DAPI and mounted in Vectashield (Vector Laboratories).

**Microscopy.** Images of metaphase preparations and cytokinesis-block micronucleus cytome assay were recorded on an AxioPlan microscope (Zeiss) equipped with a Plan-Apochromat ×63, NA 1.4, oil immersion lens and a cooled CCD camera (CoolSNAP HQ, Photometrics). Image acquisition, processing, and analysis software were from MetaMorph (Molecular Devices).

Confocal images were made with the Zeiss LSM-510 confocal scanning laser microscope. Optical sections were recorded at an interval of <200 nm, with a Plan-Apochromat ×63, NA 1.4, oil immersion lens and a cooled CCD camera (CoolSNAP HQ, Photometrics). Image acquisition, processing, and analysis software were from MetaMorph (Molecular Devices).

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**Statistical analysis.** According to the results of univariate test, continuous normal variables are expressed as means ± SD. Categor-
negative allele of TRF2 (also known as TRF2<sup>ABAM</sup> or TRF2<sup>DN</sup>) induced telomere dysfunction, including TIF and end-to-end chromosome fusions detectable in metaphase and anaphase cells (18). The inhibition of POT1 expression by siRNA targeting POT1 gene (Fig. 4A) has been shown to induce TIF and single telomere loss. As expected, both TRF2 and POT1 inhibition lead to an increased rate of TIFs (Fig. 4, D and E). In the presence of ICRF-193, POT1-compromised cells exhibit a rate of TIF formation (P < 0.05, Fig. 4B) that corresponds roughly to the addition of the POT1 depletion effect and the one of ICRF-193 treatment (P > 0.05, Fig. 4B), indicating that POT1 depletion and ICRF-193 uncap telomeres through separate pathways (Fig. 4, B and D). Remarkably, the rate of ICRF-193-dependent telomere damage decreases upon TRF2 inhibition to a level that is similar, even slightly lower (P < 0.05, Fig. 4C), than the one scored in TRF2-compromised cells without ICRF-193 treatment (Fig. 4, C and E). This indicates that TRF2 inhibition protect cells from ICRF-193 treatment. This result is concordant with a cross-talk between TRF2 and topoisomerase II actions for telomere protection (19).

**DISCUSSION**

Many drugs targeting genome stability are widely used in chemotherapies, but it is often unclear how they actually kill cancer cells. As a eukaryotic topoisomerase II catalytic inhibitor, ICRF-193 is a bis-dioxopiperazine that interferes with ATPase and DNA strand-passage reactions leading to stabilization of the closed clamp form of the enzyme. Not like other topoisomerase II inhibitors, ICRF-193 has been shown not to cause DNA damage (19). In agreement with this previous study, our results demonstrate that ICRF-193 fails to induce global DNA damages as bleomycin, a well-known genotoxic drug. As expected, we found that ICRF-193 acts as a genotoxic drug that preferentially damages telomeres. This effect is not a secondary effect of DNA damage response and cell cycle alteration since adding a sublethal dose of bleomycin to the cells triggers a marked enhancement of DNA damage and a G2/M delay but does not significantly increase the number of dysfunctional telomeres. This result opens the new avenue of understanding the mechanism of topoisomerase II inhibitor in the elimination of cancer cells.

Another important finding of this study is that ICRF-193 induces damage at telomeres properly capped by TRF2 but not by POT1. This rescue of ICRF-193 damage by TRF2 inhibition could be explained by a direct effect of ICRF-193 on the telomere-protective abilities of TRF2. However, we have previously shown that TRF2-telomere association is in fact increased upon ICRF treatment, probably as a result of an increased amount of superhelical stress triggered by ICRF-193 (19), rendering unlikely a direct effect of ICRF-193 on TRF2. Since TRF2 cooperates with topoisomerase II to protect from telomere damage (19) and is involved in t-loop formation (1), we propose that t-loop forms a topological barrier against telomere replication and/or transcription progression, leading to an increased rate of positive supercoiling requiring more topoisomerase II to be relaxed (Fig. 5).

The findings of this work validate the interest to explore telomere functions in relevant conventional chemotherapies. First, it increases our knowledge on the mode of action of the topoisomerase II inhibitor ICRF-193 at telomeres. Second, it suggests that TRF2 level could be used as a surrogate marker to predict and follow treatment efficiency. Third, these results suggest a potential personalized chemotherapy with or without topoisomerase inhibitors based on the TRF2 level in cancer tissues. Fourth, the results of this study pave the way for future prospective clinical studies addressing the contribution of telomere dysfunction induced by chemotherapy to the development of second cancers and to posttreatment ageing-like syndromes. Fifth, drugs of the same class as ICRF-193 have only a modest anti-tumor activity, which might be explained by the fact that TRF2 is often upregulated in cancer cells. Targeting POT1 rather than TRF2 might help to increase the sensitivity of cancer cells to catalytic inhibitors of topoisomerase II.

Possible future work will be to prospectively assess dysfunctional telomeres and correlation with long-term effects of chemotherapy among cancer survivors, which would contribute to the understanding of the interface between cancer and aging.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

L.C. and X.Z. performed experiments; L.C., X.Z., Y.Z., and J.X. analyzed data; L.C., E.G., and J.Y. prepared figures; L.C., X.Z., Y.Z., J.X., E.G., Y.L., and J.Y. approved final version of manuscript; E.G., Y.L., and J.Y. conception and design of research; E.G. and J.Y. interpreted results of experiments; E.G. and J.Y. drafted manuscript; E.G. and J.Y. edited and revised manuscript.

**REFERENCES**


