Tankyrase 1 as a target for telomere-directed molecular cancer therapeutics

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Summary

Telomere elongation by telomerase is repressed in *cis* by the telomeric protein TRF1. Tankyrase 1 poly(ADP-ribosyl)ates TRF1 and releases it from telomeres, allowing access of telomerase to telomeres. Here we demonstrate that tankyrase 1 inhibition in human cancer cells enhances telomere shortening by a telomerase inhibitor and hastens cell death. Conversely, either tankyrase 1 upregulation or telomere shortening, each of which decreased TRF1 loading on a chromosome end, attenuated the impact of telomerase inhibition. These results are consistent with the idea that telomeres having fewer TRF1s increase the efficiency of their elongation by telomerase. This study implies that both enzyme activity and accessibility to telomeres can be targets for telomerase inhibition.

Introduction

Telomeres are specialized nucleoprotein complexes that protect the ends of eukaryotic chromosomes (reviewed in Blackburn, 2001). Since DNA polymerases cannot replicate the ends of linear DNA (end replication problem), telomeric DNA would gradually shorten after each cell cycle unless this problem was solved by a telomere-synthesizing enzyme, telomerase. In most human somatic tissues, telomerase activity is not detectable or is so weak (Kim et al., 1994; Shay and Bacchetti, 1997; Masutomi et al., 2003) that it cannot overcome telomere attrition. Critically shortened telomeres directly elicit a DNA damage checkpoint response (d'Adda di Fagagna et al., 2003), which results in replicative senescence. Functionally intact telomeres can form a potentially protective structure, called a t loop, in which the single-stranded telomeric terminus (3′-overhang) folds back and invades the double-stranded region (Griffith et al., 1999). Loss of the 3′-overhang leads to prompt decapping of telomeres even if they are not critically short (van Steensel et al., 1998). Thus, both length of the telomeric tract and status of its distal end would contribute to telomere capping.

In contrast to human somatic cells, telomerase is highly activated in germ and most cancer cells (Kim et al., 1994; Shay and Bacchetti, 1997). Telomerase is composed of a ubiquitously expressed RNA template, TR (or TERC), and a catalytic subunit, TERT, whose expression correlates with telomerase activity (reviewed in Nugent and Lundblad, 1998). Introduction of the *TERT* gene into human somatic cells produces telomerase activity and extends their replicative capacity (Bodnar et al., 1998). In mice, genetic disruption of *TR* abolishes telomerase activity and shortens telomeres (Blasco et al., 1997), resulting in reduced oncogenesis in later generations (Greenberg et al., 1999). In the early stages of human carcinogenesis, increased cell division accelerates telomere shortening. Consequently, human cancer cells often maintain shorter telomeres than do cells in surrounding normal tissues (reviewed in Maser and DePinho, 2002). Thus, cancer cells bypass telomere crisis by activating telomerase.

Telomerase could be not only a powerful marker for cancer diagnosis, but also a rational target for anticancer therapeutics (reviewed in Shay and Wright, 2002). In fact, overexpression of dominant negative mutants of TERT represses telomerase activity, progressively shortens telomeres, and eventually kills cancer cells (Hahn et al., 1999; Zhang et al., 1999). Continuous exposure to chemical telomerase inhibitors also shortens telomeres, followed by senescence or apoptosis (Naasani et al., 1999; Damm et al., 2001; Seimiya et al., 2002; Asai et al., 2003). One concern in this strategy is that there will be a lag phase between the time telomerase is inhibited and when telomeres shorten sufficiently to disrupt their capping function. Furthermore, the potentially long treatment time could allow for drug resistance to occur, which is a severe challenge to chemotherapy. Although there is no evidence that telomerase inhibition is toxic prior to the point at which short telomeres start triggering

SIGNIFICANCE

Telomere maintenance by telomerase allows human cancer cells to divide indefinitely. Telomerase inhibitors are expected to selectively kill cancer cells by resuming the "end replication problem" of linear chromosomal DNA. However, therapeutic outcome as a result of this strategy must await the emergence of a critically shortened telomere, which would require a long treatment duration. Since cancer has a robust ability to evolve, continuous exposure to the same anticancer stress carries the risk of acquired drug resistance, a serious problem in cancer chemotherapy. We demonstrate that pharmacological targeting of tankyrase 1 enhances telomere shortening by means of a telomerase inhibitor and results in earlier cellular crisis. This study provides insight into strategies for telomere-based molecular cancer therapeutics.

DNA damage responses, intracellular uptake of a telomerase inhibitor is sometimes reduced during prolonged treatment of cancer cells (Naasani et al., 1999). Also, in general, increased hepatic metabolism often explains reduced action of drugs. Accordingly, the potential ability to accelerate the rate of telomere shortening has profound implications for earlier induction of crisis and thereby reduced risk of drug resistance. So far, factors that modulate the effects of telomerase inhibitors have not been described.

In telomerase-positive cells, telomere length is maintained at a constant average value. This equilibrium is mediated by a negative feedback regulation of telomerase where a "protein-counting" mechanism discriminates the number of telomeric proteins bound to a telomere (reviewed in Smogorzewska and de Lange, 2004). Thus, longer telomeres recruit a greater number of telomeric proteins that inhibit telomere elongation in *cis*. In humans, this mechanism involves TRF1, a duplex telomeric repeat binding protein. Overexpression of TRF1 in telomerase-positive cells results in telomere shortening, whereas its dominant negative mutant induces elongation (van Steensel and de Lange, 1997). Poly(ADP-ribosyl)ation of TRF1 by a telomeric poly(ADP-ribose) polymerase (PARP), tankyrase 1, leads to loss of its DNA binding activity (Smith et al., 1998; Loayza and de Lange, 2003) and subsequent degradation of TRF1 (Chang et al., 2003). Although tankyrase 1 has no recognizable nuclear localization signal (NLS), it is present in the nucleus as well as the cytoplasm (Smith and de Lange, 1999; Cook et al., 2002; Seimiya and Smith, 2002). Nuclear localization of tankyrase 1 requires interaction with TRF1, which contains an NLS (Smith and de Lange, 1999). Overexpression of NLS-tagged tankyrase 1 in telomerase-positive cells releases TRF1 from telomeres and induces their elongation (Smith and de Lange, 2000). This suggests that tankyrase 1 may alleviate the effect of telomerase inhibitors by accelerating access of residual telomerase activity to telomeres. Since shortened telomeres increase the efficiency of telomere elongation (Marcand et al., 1999; Ouellette et al., 2000; Hemann et al., 2001), telomere shortening per se may also result in decreased sensitivity to telomerase inhibitors.

This study demonstrates that tankyrase 1 diminishes the effect of telomerase inhibitors in telomerase-positive cells. Furthermore, during progressive shortening, telomeres became more resistant to a telomerase inhibitor. PARP inhibitors that could inhibit tankyrase 1 eliminated these problems and enhanced telomere shortening. This report demonstrates a specific enzyme that can modulate the impact of telomerase inhibition on cancer cells.

Results

Telomere elongation by tankyrase 1 is blocked by telomerase inhibition

Telomere elongation by tankyrase 1 correlates with telomerase activity (Cook et al., 2002; Chang et al., 2003). Thus, we first examined whether telomere elongation by tankyrase 1 is blocked by telomerase inhibition. Since the amount of intracellular TRF1, which is required for nuclear localization of tankyrase 1, is usually too small to interact with excess amounts of exogenous tankyrase 1, we introduced an NLS at the N terminus of tankyrase 1 (Smith and de Lange, 2000). While tankyrases are thought to have an innate role in cytoplasmic events (Chi and Lodish, 2000; Lyons et al., 2001), this modification allows us to see the effect of exogenous tankyrase 1 (FN-tankyrase1; tagged with a FLAG epitope and an NLS at the N terminus) exclusively in the nucleus. HTC75 fibrosarcoma cells were infected with FN-tankyrase 1 retrovirus, and the transduced populations were further infected with a dominant negative mutant of human TERT (DN-hTERT) (Hahn et al., 1999). Consistent with previous reports (Hahn et al., 1999; Zhang et al., 1999), overexpression of DN-hTERT repressed telomerase activity (Figure 1A) and decreased the telomere length (Figure 1B). On the other hand, overexpression of FN-tankyrase 1 downregulated TRF1 and progressively increased telomere length (Smith and de Lange, 2000) without affecting telomerase activity (Figures 1A and 1B). As expected, telomere elongation by FN-tankyrase 1 was completely abolished by DN-hTERT in spite of TRF1 loss. After 60 population doublings (PD) of the FN-tankyrase 1/DN-hTERT-double infectants, DN-hTERT expression was lost and telomerase was reactivated (data not shown); these phenomena are often observed in DN-hTERT-transfected cancer cells (Zhang et al., 1999). The resulting populations maintained a high level of FN-tankyrase 1 expression (data not shown) and exhibited telomere elongation (Figure 1B). These observations verify that telomere elongation by tankyrase 1 depends on telomerase activity and is therefore blocked by telomerase inhibition.

Overexpression of a dominant negative TRF1 (DN-TRF1), which inhibits the telomere binding of endogenous TRF1, also induces telomere elongation without affecting telomerase activity in HT1080 cells (Figures 1C and 1D, van Steensel and de Lange, 1997). DN-hTERT also inhibited telomere elongation by DN-TRF1 (Figures 1C and 1D). In agreement with an earlier report that TRF1 inhibits telomeric association of hTERT (Sharma et al., 2003), these observations indicate that FN-tankyrase 1 and DN-TRF1 elongate telomeres by allowing access of telomerase to telomeres.

Tankyrase 1 confers resistance to telomerase inhibitors

While dominant negative mutants (Hahn et al., 1999; Zhang et al., 1999) or small interfering RNAs (Masutomi et al., 2003) for hTERT are probably the most specific and powerful agents for telomerase inhibition, there would be no effect on a small fraction of the cells that lost or did not incorporate those exogenous genes. This situation makes it difficult to explore the intrinsic mechanisms that modulate the impact of continuous telomerase inhibition. Thus, we next used a synthetic telomerase inhibitor, MST-312 (Seimiya et al., 2002; Supplemental Table S1), that had been developed by taking a hint from telomerase-inhibitory activity of (−)-epigallocathechin gallate (EGCG) (Naasani et al., 1998). Continuous treatment of HTC75 cells with nontoxic doses of MST-312 induced gradual shortening of telomeres (−22.5 bp/PD, Figure 2A). Since shortened telomeres tended to be more resistant to further shortening (see below), we increased the concentration from 1 μM to 2 μM during the course of cultivation. Essentially, the cells in the presence of MST-312 grew at a comparable or slightly slower rate than that of untreated cells. However, at ~PD85, MST-312 reduced the growth rate (Figure 2B). At the time, the cells exhibited a flattened morphology and expressed the senescence-associated β-galactosidase (SA-β-Gal), both of which are potential biomarkers for cellular aging (Figure 2C). Finally, the cells externalized phosphatidylserine and exhibited a sub-G1 fraction, both of which are characteristic of apoptosis (Figure 2D). Furthermore, the

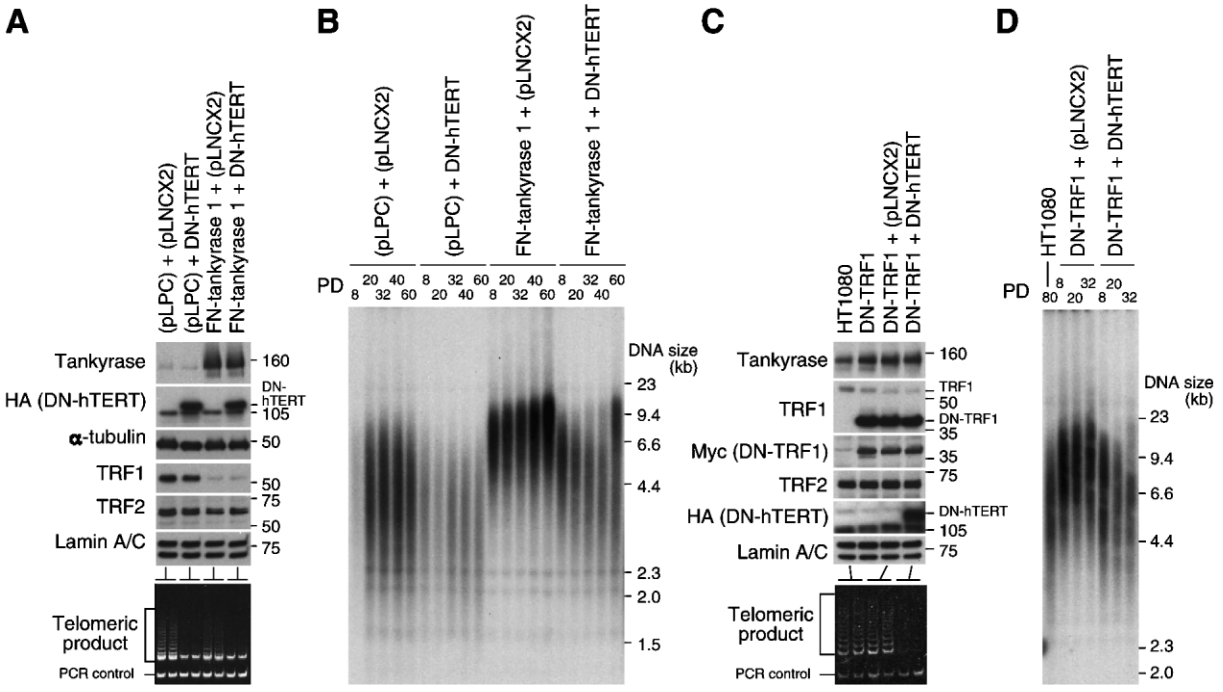

Figure 1. DN-hTERT inhibits telomere elongation by FN-tankyrase 1 and DN-TRF1

A: HTC75 cells were infected with pLPC (empty) or FN-tankyrase 1 retroviruses and then with pLNCX2 (empty) or HA-tagged DN-hTERT retroviruses. Upper panels, Western blot analysis (20 μg of proteins per lane) of whole cell (tankyrase, DN-hTERT, α-tubulin) or nuclear extracts (TRF1, TRF2, lamin A/C) at PD8. Molecular mass markers (kDa) are indicated at the right. Bottom, telomerase activity determined by in vitro TRAP assay. Each lysate was examined in duplicate.

B: Change in telomere length of the cells in **A**. Genomic DNA was prepared at indicated population doublings (PD) and digested with HinfI and RsaI. Telomeric restriction fragments (TRF) were detected by Southern blot analysis.

C: HT1080 cells were infected with a Myc-tagged DN-TRF1 retrovirus and then with pLNCX2 or HA-tagged DN-hTERT retroviruses. Upper panels, Western blot analysis of whole cell or nuclear extracts at PD8. Bottom, telomerase activity in each cell lysate.

D: Change in telomere length of the cells in **C**.

p21 cyclin-dependent kinase inhibitor was induced, and the proapoptotic caspase-3 was activated (data not shown). These changes were not observed at the earlier stage of passage. MST-312 had no effect on telomere length (determined by Southern blot [Figure 2E] and quantitative FISH [data not shown]) or proliferative capacity (Figure 2F) of GM847 cells, which maintain their telomeres by telomerase-independent recombination (Dunham et al., 2000).

MST-312 inhibited telomere elongation by FN-tankyrase 1 (Figure 2G). During the drug treatment, there was no change in expression, self-ADP-ribosylation, or intracellular localization of FN-tankyrase 1 (data not shown). While the conventional PARP inhibitor, 3-aminobenzamide (3AB), inhibited poly(ADP-ribosyl)ation of TRF1 by FN-tankyrase 1 in vitro, MST-312 did not (Figure 2H). Hence, it is unlikely that MST-312 directly inhibits tankyrase 1 (see also Figure 3). These observations support the idea that telomere elongation by tankyrase 1 depends on telomerase activity.

However, FN-tankyrase 1 disturbed telomere attrition (Figure 2G) and cellular crisis (Figures 4E and 4H, left) by MST-312. Similarly, FN-tankyrase 1 conferred resistance to telomere shortening through another telomerase inhibitor, EGCG, and DN-TRF1 also diminished the effect of MST-312 (data not shown). Overexpression of FN-tankyrase 1 had no significant effect on acute cytotoxicity of MST-312 (data not shown). These observations suggest that decrease in the number of telomere-bound TRF1 allows access of residual telomerase activity to telomeres and therefore alleviates the effects of telomerase inhibitors.

Because overexpressed tankyrase 1 that lacks the NLS accumulates in the cytoplasm, it does not release TRF1 from telomeres (Smith and de Lange, 1999; T.O., H.S., and T.T., unpublished data). We established a stable HTC75 cell line that overexpressed FLAG-tagged tankyrase 1 without NLS (designated as FLAG-tankyrase 1). As shown in Supplemental Figure S1, FLAG-tankyrase 1 did not affect TRF1 protein level or telomere length. Furthermore, MST-312 effectively shortened telomeres of FLAG-tankyrase 1-overexpressing cells. These findings clearly exclude the possibility that MST-312 resistance by FN-tankyrase 1 is attributable to enhanced poly(ADP-ribosyl)ating events that are unrelated to telomere length regulation by TRF1.

Inhibition of tankyrase 1 by PARP inhibitors in intact cells

Since telomere elongation by FN-tankyrase 1 depends on poly(ADP-ribosyl)ation of TRF1 (Cook et al., 2002; Seimiya et al., 2004), PARP inhibitory agents may block tankyrase 1 in intact cells. To explore the possibility, we monitored the "TRF1-releasing" activity of FN-tankyrase 1 in the presence of PARP inhibi-

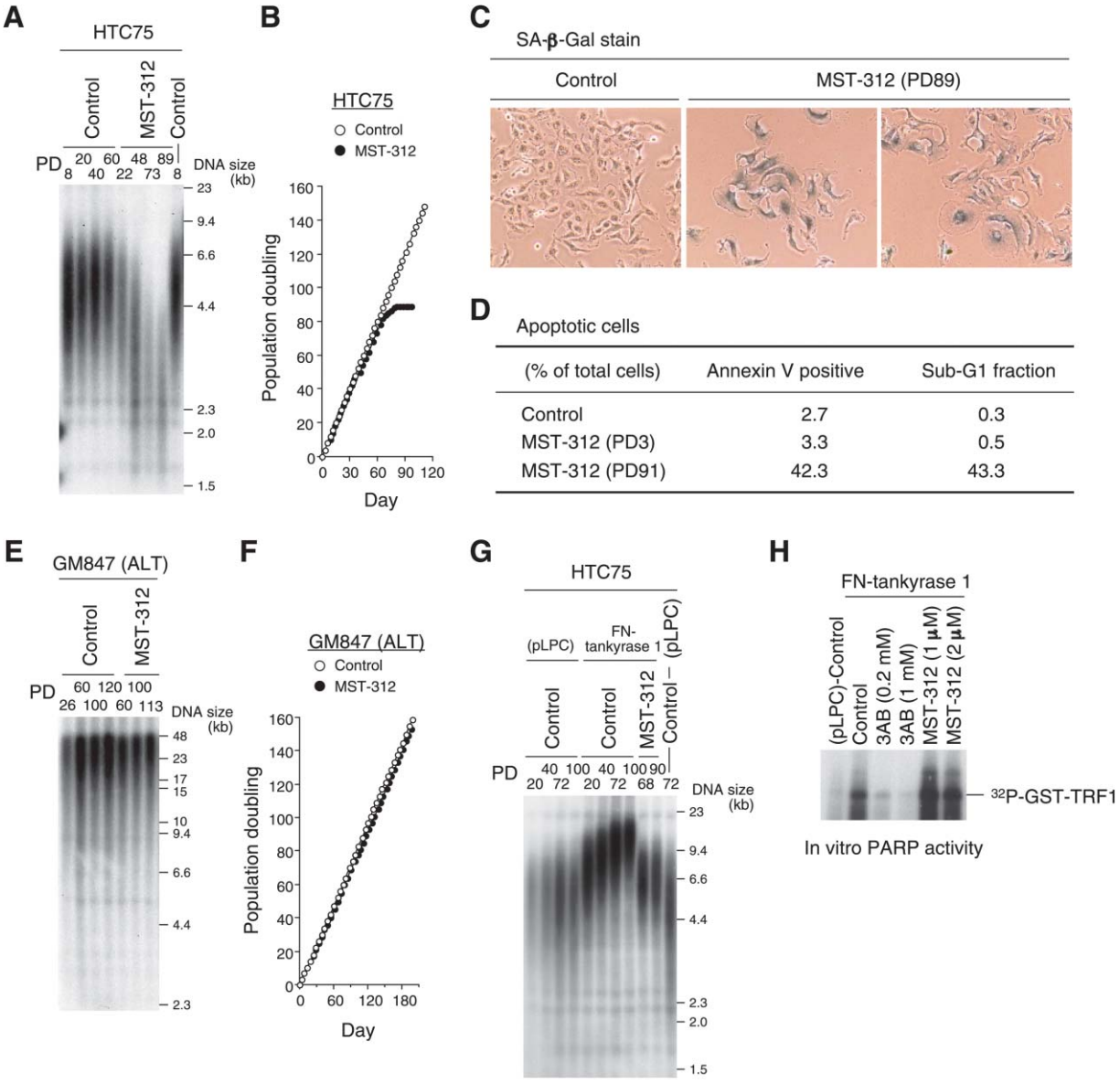

Figure 2. Telomere shortening by MST-312 is disturbed by FN-tankyrase 1

A: HTC75 cells were propagated in the presence of MST-312 at 1 μM (day 0–33), 1.25 μM (day 33–36), 1.5 μM (day 36–40), and 2 μM (from day 40). TRFs at indicated PD were detected by Southern blot analysis.
B: Growth curve of MST-312-treated HTC75 cells.
C: SA-β-Gal activity in senescent HTC75 cells at PD89. Glutaldehyde-fixed cells were incubated in the acidic X-gal solution at 37°C for 3 hr.
D: Apoptosis of MST-312-treated HTC75 cells at PD91. Each value indicates percentage of apoptotic cells quantitated by flow cytometry.
E: Effect of MST-312 on telomere length of GM847 cells. Cells were treated as in **A**. ALT, alternative lengthening of telomeres.
F: Growth curve of MST-312-treated GM847 cells.
G: Effect of MST-312 on telomere length of FN-tankyrase 1-overexpressing HTC75 cells. Cells were treated as in **A** from PD20.
H: In vitro PARP assay of FN-tankyrase 1. Whole extracts of mock (pLPC) or FN-tankyrase 1-overexpressing HTC75 cells were immunoprecipitated with anti-FLAG M2 agarose. The washed beads were incubated with 4 μg of GST-TRF1 and 1.3 μM [^{32}P]-NAD$^+$. 3AB and MST-312 were added prior to addition of GST-TRF1. Poly(ADP-ribosyl)ated GST-TRF1 was fractionated by SDS-PAGE and detected by autoradiography.

tors. Transient overexpression of FN-tankyrase 1 in HeLa I.2.11 cells results in release of TRF1 from telomeres, which is illustrated by disappearance of the immunofluorescent spots of TRF1 (Figures 3Aa, 3Af, and 3Ak, and Smith and de Lange, 2000). 3AB at 3 mM inhibited the disappearance of these spots (Figure 3Ab and 3Ag). Since 3AB did not inhibit the interaction of FN-tankyrase 1 with TRF1, it enhanced colocalization of the two proteins at the spots (Figure 3Al). Such colocalization is observed when the PARP activity of FN-tankyrase 1 is abolished by point mutations (Cook et al., 2002). 3AB did not affect the expression level or stability of FN-tankyrase 1, which were determined by Western blot analysis (data not shown). On the other hand, neither MST-312 nor 3-aminobenzoic acid (3ABA), a 3AB analog that does not inhibit PARP activity, affected TRF1 release

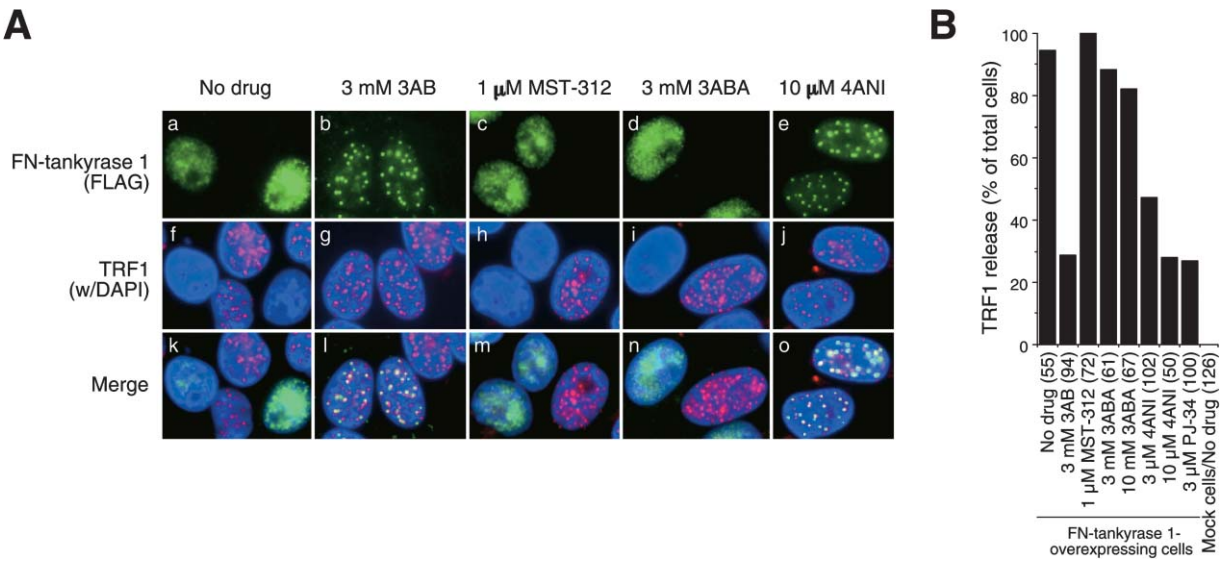

Figure 3. PARP inhibitors block the TRF1 release from telomeres

A: HeLa I.2.11 cells were transiently transfected with FN-tankyrase 1. After 20 hr incubation in the presence of the drugs indicated, cells were fixed with paraformaldehyde. FN-tankyrase 1 and TRF1 were detected by indirect immunofluorescence stain with anti-FLAG M2 (green; a–e) and anti-TRF1 5747 (red; f–j) antibodies, respectively. 4,6-diamino-2-phenylindole (DAPI) staining of DNA is shown in blue.

B: Quantitation of the TRF1-releasing activity of FN-tankyrase 1. Column indicates a percentage of the cells in which the telomeric TRF1 spots completely disappeared. Only the cells that expressed FN-tankyrase 1 were counted. Value in the parentheses indicates the number of counted cells.

(Figures 3Ac, 3Ad, 3Ah, 3Ai, 3Am, and 3An). Consistent with these observations, 3AB blocked telomere elongation by FN-tankyrase 1 in HTC75 cells (Figures 4A and 4D and Table 1). We could not determine the effect of 3ABA on telomere length, since the cells suffered acute cytotoxicity when treated for more than a week (data not shown). Meanwhile, 3AB did not inhibit telomere elongation by DN-TRF1 at all (Figure 4B). Together, these observations indicate that the repressive effect of 3AB on FN-tankyrase 1 is derived from inhibition of its PARP activity.

Other PARP inhibitors, such as 4-amino-1,8-naphthalimide (4ANI, 10 μM) (Banasik et al., 1992) and PJ-34 (3 μM) (Abdelkarim et al., 2001), also inhibited the TRF1 release at levels comparable with 3 mM 3AB (Figures 3Ae, 3Aj, 3Ao, and 3B). These PARP inhibitors diminished auto-poly(ADP-ribosyl)ation of FN-tankyrase 1 (data not shown). Furthermore, continuous treatment of FN-tankyrase 1-overexpressing cells with PJ-34 significantly reduced telomere elongation (Figures 4C and 4F and Table 1). These agents at concentrations used in this study did not affect cell growth or telomerase activity (Figures 4E and 4G, Supplemental Figure S2, and data not shown). These observations verify that certain PARP inhibitors block FN-tankyrase 1 in intact cells. On the other hand, some other PARP inhibitors affected telomerase activity (Y.M., H.S. and T.T., unpublished observation), and we did not use such inhibitors in this study.

PARP inhibitors reverse the MST-312 resistance in tankyrase 1-overexpressing cells

To determine if the PARP inhibitors mentioned above restore the impact of telomerase inhibition, we examined the combined effects of such inhibitors and MST-312. While either MST-312 or 3AB alone inhibited telomere elongation by FN-tankyrase 1, neither of them induced efficient telomere shortening (Figures 4A and 4D, and Table 1) or cell crisis (Figures 4E and 4H, left). However, a combination of two drugs progressively shortened telomeres (Figures 4A and 4D, and Table 1) and eventually induced crisis at ~PD60 (Figures 4E and 4H, left). 3AB at 3–5 mM did not enhance any acute cytotoxicity of MST-312 (data not shown). 3AB may also inhibit PARP-1, which is involved in genomic stability (reviewed in Shall and de Murcia, 2000). However, while genetic disruption of *PARP-1* causes hypoploidy and hyperploidy (Kanai et al., 2003), 3 mM 3AB did not affect the profile of DNA content in HTC75 cells, at least that could be detected by flow cytometry (data not shown). PJ-34 also cooperated with MST-312 to shorten telomeres and induce crisis of FN-tankyrase 1-transduced cells (Figures 4C, 4F, 4G, and 4H, left, and Table 1). Thus, these PARP inhibitors can reverse MST-312 resistance that results from FN-tankyrase 1 overexpression.

While the parental HTC75 cells treated with MST-312 underwent senescence and apoptosis at ~PD90, when the mean telomeric restriction fragment (TRF) length was less than 3 kb (Figure 2), FN-tankyrase 1-overexpressing cells treated with MST-312 and 3AB reached crisis with longer TRFs (around 4 kb). For a more precise view of each chromosome end, we performed telomere peptide nucleic acid (PNA) FISH. As shown in Figures 4I and 4J, combination of MST-312 and PARP inhibitors increased frequency of the telomere signal-free chromosome/chromatid ends. Furthermore, combination of two drugs induced chromosomal abnormalities (i.e., concomitant treatment with MST-312 and 3AB gave one dicentric chromosome, 3 fusions, and 5 breaks per 10 metaphases at crisis, whereas none of these abnormalities were detected in 10 metaphases of nontreated cells; data not shown). These observations explain why FN-tankyrase 1-overexpressing cells treated with two drugs

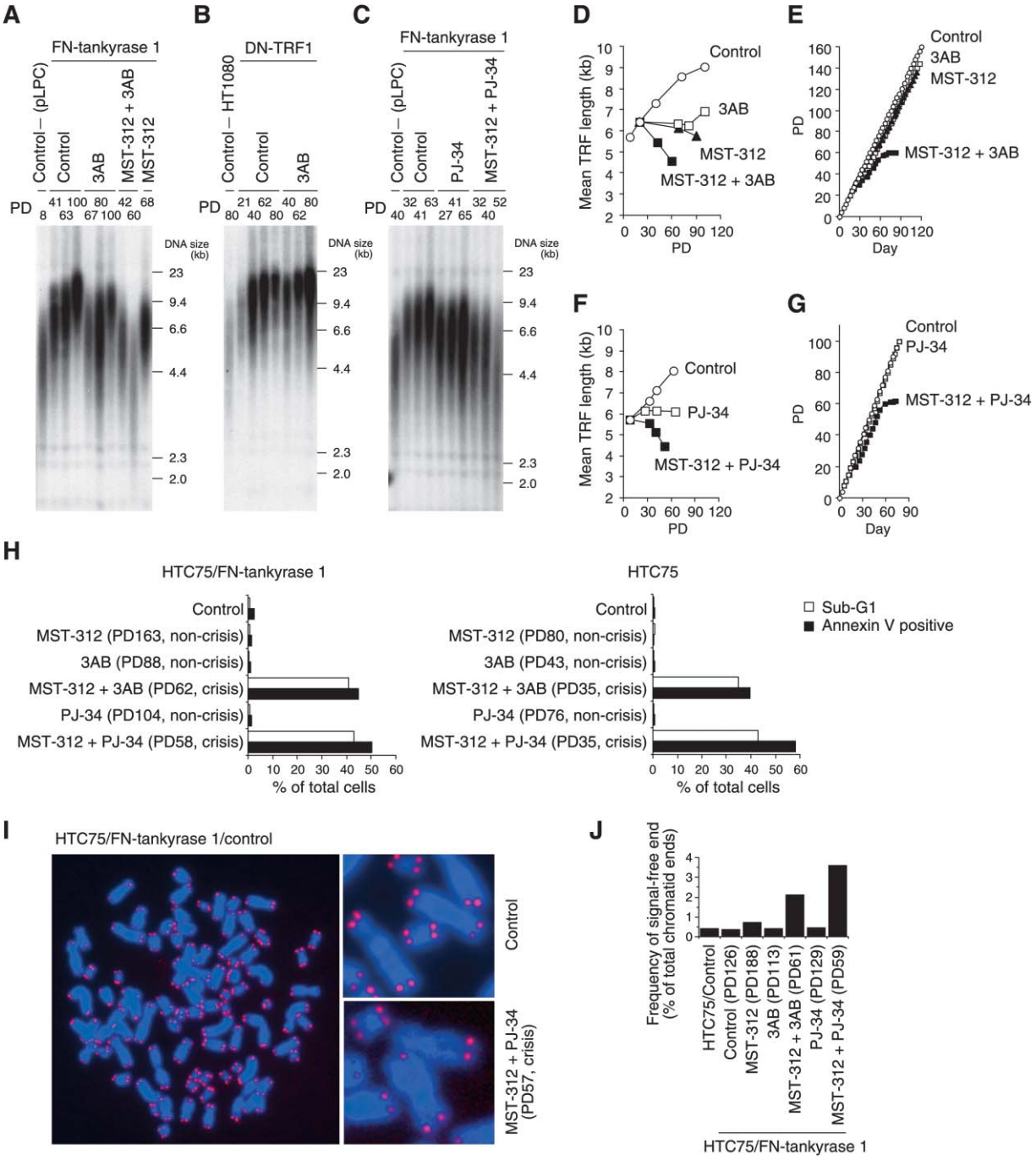

Figure 4. PARP inhibitors block FN-tankyrase 1 and reverse the MST-312 resistance

A: Effects of 3AB (3 mM) and MST-312 (1–2 μM) on telomere elongation by FN-tankyrase 1. FN-tankyrase 1-overexpressing HTC75 cells were treated with each drug from PD20.

B: Effect of 3AB (3 mM) on telomere elongation by DN-TRF1. Cells were treated with 3AB from PD8.

C: Effects of PJ-34 (3 μM) and MST-312 (1–2 μM) on telomere elongation by FN-tankyrase 1. Cells were treated with each drug from PD8.

D and **F:** Graphic representations of telomere length change in FN-tankyrase 1-overexpressing HTC75 cells in the presence of MST-312 and 3AB (**D**) or PJ-34 (**F**).

E and **G:** Growth curves of the cells in **D** and **F**, respectively.

H: FN-tankyrase 1-overexpressing (left) and the parental (right) HTC75 cells were treated with MST-312 as in Figure 2A in the presence or absence of 3 mM 3AB or 3 μM PJ-34. Percentages of apoptotic cells were monitored at indicated PD (parentheses).

I: Telomere PNA FISH of FN-tankyrase 1-overexpressing cells. Telomere DNA (red) was visualized on the metaphase chromosomes (blue). Right, magnified views of typical chromosomes in nontreated cells (upper) and abnormal chromosomes without detectable telomere signals in MST-312 and PJ-34 cotreated cells (lower).

J: Frequency of the telomere signal-free ends. Percentages of total chromatid ends were shown: one chromosome has four chromatid ends.

Table 1. Change in telomere length by treatment with MST-312 and PARP inhibitors

	Cell	
Compound	HTC75/FN-tankyrase 1	HTC75
Non	35.9 ± 8.2 (6)[a]	1.2 ± 4.9 (7)
MST-312	−6.6 ± 3.8 (4)[b]	−21.1 ± 2.6 (4)
3AB	6.8 ± 2.6 (3)[b]	−8.1 ± 0.1 (2)
PJ-34	6.4 (1)[c]	−3.7 ± 0.1 (2)
MST-312 + 3AB	−46.0 ± 6.2 (3)[b]	−40.4 ± 0.9 (2)
MST-312 + PJ-34	−26.7 (1)[c]	−40.0 (1)

Cells were continuously treated with each compound for 50–90 PD (treatment with a single compound, which induced later or no crisis) or 30–40 PD (concomitant treatment with two compounds, which induced earlier crisis). Genomic DNA was prepared at several points of population doublings (PD), and the mean TRF lengths were determined by Southern blot analysis. Values indicate the rates of change in the telomere length (bp) per PD, which represent slopes of the graphs drawn by PD (x axis) and the mean TRF length (y axis). Data are presented as means ± SD. Repetitions of identical experiments are indicated in parentheses.

[a] Monitored within a range between PD8–PD72 because elongation was saturated with an increasing number of cell divisions at >PD80.

[b] Treatment started at PD20.

[c] Treatment started at PD8.

reached crisis with longer average telomeres. These findings are consistent with the idea that telomere crisis is triggered by the shortest telomere but not by the average telomere length (Hemann et al., 2001).

PARP inhibitors promote the effect of MST-312 in the parental HTC75 cells

We next examined the effects of PARP inhibitors on the parental HTC75 cells that did not overexpress exogenous tankyrase 1. HTC75 cells expressed tankyrase 1 at a level that was easily detectable but was much lower than that seen in FN-tankyrase 1-overexpressing cells (Figure 1A). Continuous treatment of the cells with 3 mM 3AB or 3 μM PJ-34 shortened telomeres to a small extent (Table 1; see also Figure 7C). These effects were marginal but were observed in multiple experiments. Furthermore, these PARP inhibitors enhanced telomere shortening by MST-312 (Table 1) and induced earlier cell crisis onset, which was observed at PD35–PD38 (MST-312 + 3AB) and PD34–PD35 (MST-312 + PJ-34) (Figure 4H, right). Neither 3AB nor PJ-34 increased the percentage of apoptotic cells (Figures 2D and 4H, right), indicating that they had little direct effect on cellular survival or apoptosis. The average rates of telomere shortening per PD (Table 1) became similar to those observed in DN-hTERT-transduced HTC75 cells (−39.9 bp/PD). On the other hand, 3AB did not enhance telomere shortening by DN-hTERT (data not shown), suggesting that DN-hTERT by itself completely inhibited telomerase activity.

Telomere shortening attenuates the effect of telomerase inhibition

Since a chromosome end with a shorter telomere has fewer TRF1 molecules (Loayza and de Lange, 2003), telomere shortening would be functionally equivalent to FN-tankyrase 1 overexpression with respect to TRF1 loading on the chromosome end. Thus, telomere shortening itself may decrease the effect of telomerase inhibitors by accelerating access to residual telomerase activity. Actually, the rate of telomere shortening by a telomerase inhibitor in the latter period of treatment is sometimes much lower than that in the earlier period, although this issue has not been fully addressed in the original report (Asai et al., 2003). To reproduce this phenomenon, we used a lower concentration (0.75 μM) of MST-312 to moderately inhibit telomerase activity in HTC75 cells. In vitro, 2, 1, and 0.75 μM MST-312 inhibited telomerase activity 78%, 69%, and 62%, respectively (data not shown). We could not determine the diminished levels of telomerase activity in intact cells: because MST-312 is a reversible inhibitor, telomerase activity in the drug-treated cells revives during preparation of the lysate (Seimiya et al., 2002). In the presence of 0.75 μM MST-312, telomeres primarily shortened at a rate of −30.0 bp/PD (PD0–PD46, Figures 5A and 5B). When the PD exceeded ~50, however, MST-312 could not induce further shortening or cellular crisis, and the average telomere length was stabilized at ~3.8 kb. These results would not be due to acquired drug resistance, such as reduced uptake or enhanced metabolism of MST-312, since acute cytotoxicity of MST-312 did not differ before or after drug treatment at PD88 (data not shown). A chromatin immunoprecipitation assay of telomere DNA (see below) estimated that average TRF1 loading on each chromosome end in MST-312-treated cells at PD94 was about 50% of that in untreated cells (data not shown). These observations suggest that telomere shortening per se is a resistance factor for telomerase inhibition.

3AB did not accelerate the initial rate of telomere shortening by MST-312 (−32 bp/PD, PD0–PD46, Figure 5B). Strikingly, however, it maintained the rate of telomere shortening even beyond the 3.8 kb equilibrium length that was caused by MST-312 alone (−28 bp/PD, from PD46). Furthermore, even after the effect of MST-312 alone was saturated, combination of 3AB with MST-312 (from PD108) restored additional shortening of telomeres (−10 bp/PD). By using breast cancer HBC4 cells, we repeated the same experiments and verified that 3AB enhanced telomere shortening by MST-312 (Figure 5C). In contrast, 3AB had no effect on telomere length in a telomerase-independent GM847 cells (Figure 5D). Similarly, 3AB did not accelerate telomere shortening in normal foreskin fibroblasts (Figure 5D). Thus, 3AB specifically enhanced telomere shortening by MST-312 in telomerase-positive cells.

3AB enhances telomeric association of TRF1

Tankyrase 1 releases TRF1 from telomere DNA in its PARP activity-dependent manner (Cook et al., 2002; Loayza and de Lange, 2003). To determine if 3AB restores the telomere binding of TRF1, we performed a chromatin immunoprecipitation (ChIP) assay. As shown in Figure 6A, anti-TRF1 antibody precipitated the telomeric TTAGGG but not Alu repetitive elements in HeLaI.2.11 and HTC75 cells. Neither anti-TAB182 (tankyrase 1 binding protein of 182 kDa) (Seimiya and Smith, 2002) N terminus antibody nor preimmune serum precipitated telomere DNA; because TAB182 does not colocalize with telomeres, we used this antibody as a negative control. Consistent with a previous report (Loayza and de Lange, 2003), FN-tankyrase 1-overexpressing HTC75 cells reduced the telomere binding of TRF1 (Figure 6B). 3AB partially, but statistically significantly, recovered the telomere binding of TRF1, whereas 3ABA had no effect. Although the efficiency of ChIP was much lower than that with the anti-TRF1 antibody, anti-tankyrase antibody also precipitated telomere DNA in FN-tankyrase 1-overexpressing cells (Figure 6C). The low values for telomere DNA in ChIP by anti-

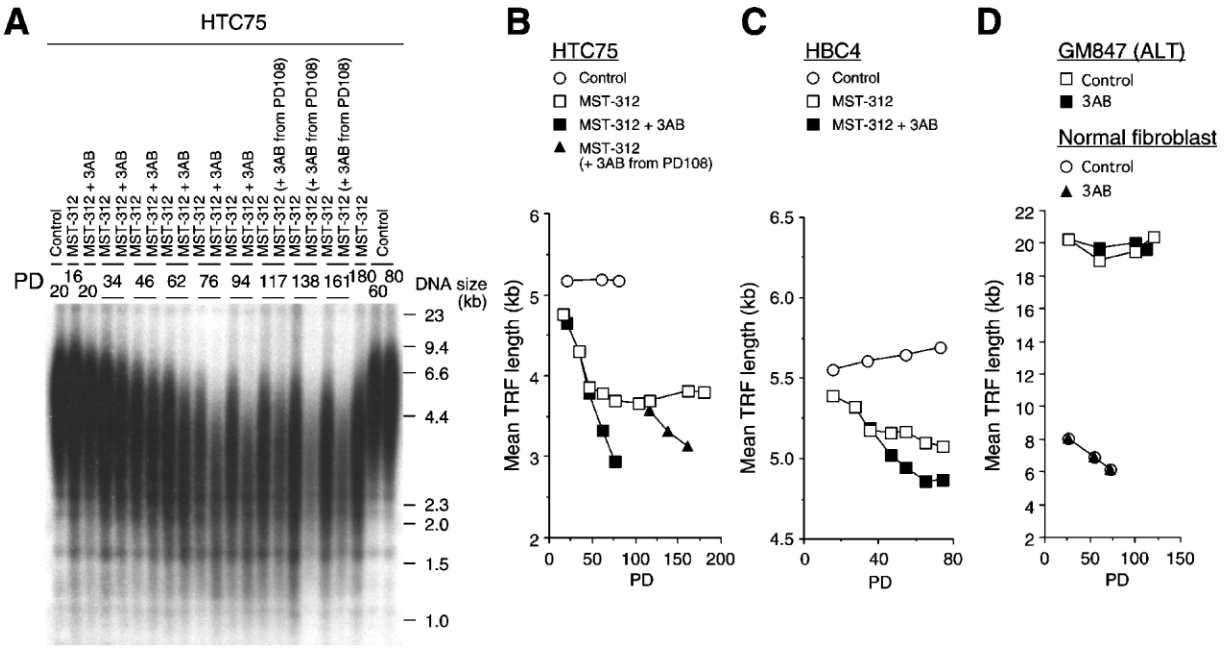

Figure 5. Telomere shortening attenuates the effect of MST-312

A: HTC75 cells were treated with MST-312 (0.75 μM) in the presence or absence of 3AB (3 mM). TRFs at indicated PD were detected by Southern blot analysis.

B: Quantitative representation of **A**.

C: Breast cancer HBC4 cells were treated as in **A**. TRFs at indicated PD were detected by Southern blot analysis and the quantitative data are shown.

D: Effect of 3AB (3 mM) on telomere length in GM847 cells and normal foreskin fibroblasts. The quantitative data of Southern blot are shown.

tankyrase antibody are consistent with the previous observation that tankyrase 1 is much less abundant on telomeres than TRF1 (Smith et al., 1998; Loayza and de Lange, 2003; Ye and de Lange, 2004). 3AB increased the tankyrase-bound telomere DNA in the FN-tankyrase 1-overexpressing cells. A similar trend was observed in the parental cells, although it was not statistically significant, and the efficiency of ChIP was very low. This is consistent with the observation that catalytically inactivated FN-tankyrase 1 colocalizes with the telomeric TRF1 spots (Figure 3A, Cook et al., 2002). Consistent with the observations that 3AB inhibited telomere elongation by FN-tankyrase 1 but not by DN-TRF1 (Figures 4A and 4B), these results indicate that 3AB settles TRF1 on telomere DNA by inhibiting the PARP activity of FN-tankyrase 1.

In the parental HTC75 cells or in the MST-312-treated HTC75 cells (at PD80–PD90 in Figure 5B), ChIP has failed to detect a statistically significant effect of 3AB on telomere binding of TRF1, even under synchronized culture conditions (data not shown). This may be explained by the hypothesis that under physiological conditions, TRF1 dissociates from telomeres only briefly (e.g., ~5 min), if at all, during replication of telomere DNA (Ohki and Ishikawa, 2004).

Telomere shortening by a mutant allele of tankyrase 1

We next examined if moderate telomere shortening by the PARP inhibitors (Table 1) is mimicked by a mutant allele of tankyrase 1. The ankyrin domain of tankyrase 1 has five independent TRF1 binding sites, designated as ANK repeat clusters (ARC) I to V (Seimiya and Smith, 2002; Seimiya et al., 2004). Among the five ARCs, the C-terminal ARC V is essential, whereas ARCs I to IV play an auxiliary role for telomere elongation (Seimiya et al., 2004). We generated the FN-tankyrase 1 deletion mutant, FN-tank-ARC V, which retained ARC V but lacked other four ARCs (Figure 7A). As shown in Figure 7B, overexpression of FN-tank-ARC V in HTC75 cells shortened telomeres. The rate of shortening was moderate (−9.3 bp/PD) and very similar to that in HTC75 cells overexpressing TRF1 (van Steensel and de Lange, 1997) or treated with 3AB or PJ-34 (Table 1 and Figure 7C). In contrast, neither FN-tank-ARC I, which retained a nonessential ARC I but lacked ARC II to V, or FN-tank-ΔANK, which lost all the five ARCs (Figure 7A), affected telomere length (Figure 7D). Telomere shortening by FN-tank-ARC V was not accelerated by 3AB (Figure 7B), indicating that FN-tank-ARC V and 3AB shortened telomeres through the same mechanism.

Discussion

This study has demonstrated that either tankyrase 1 upregulation or telomere shortening lessens the impact of a telomerase inhibitor. Concomitant inhibition of tankyrase 1 PARP activity restored telomere shortening with a telomerase inhibitor. These findings suggest that tankyrase 1, as well as telomerase, may be a rational target for telomere-directed cancer therapeutics. One striking difference between telomerase and tankyrase 1 is the expression profile of each protein. While telomerase activity correlates well with human cancer, tankyrase 1 transcripts were almost equally detected in various normal and tumor tissues (H.S. and T.T., unpublished data). Since tankyrases exist in various intracellular loci (Smith and de Lange, 1999; Chi and Lodish, 2000; Lyons et al., 2001), monitoring the nuclear (or

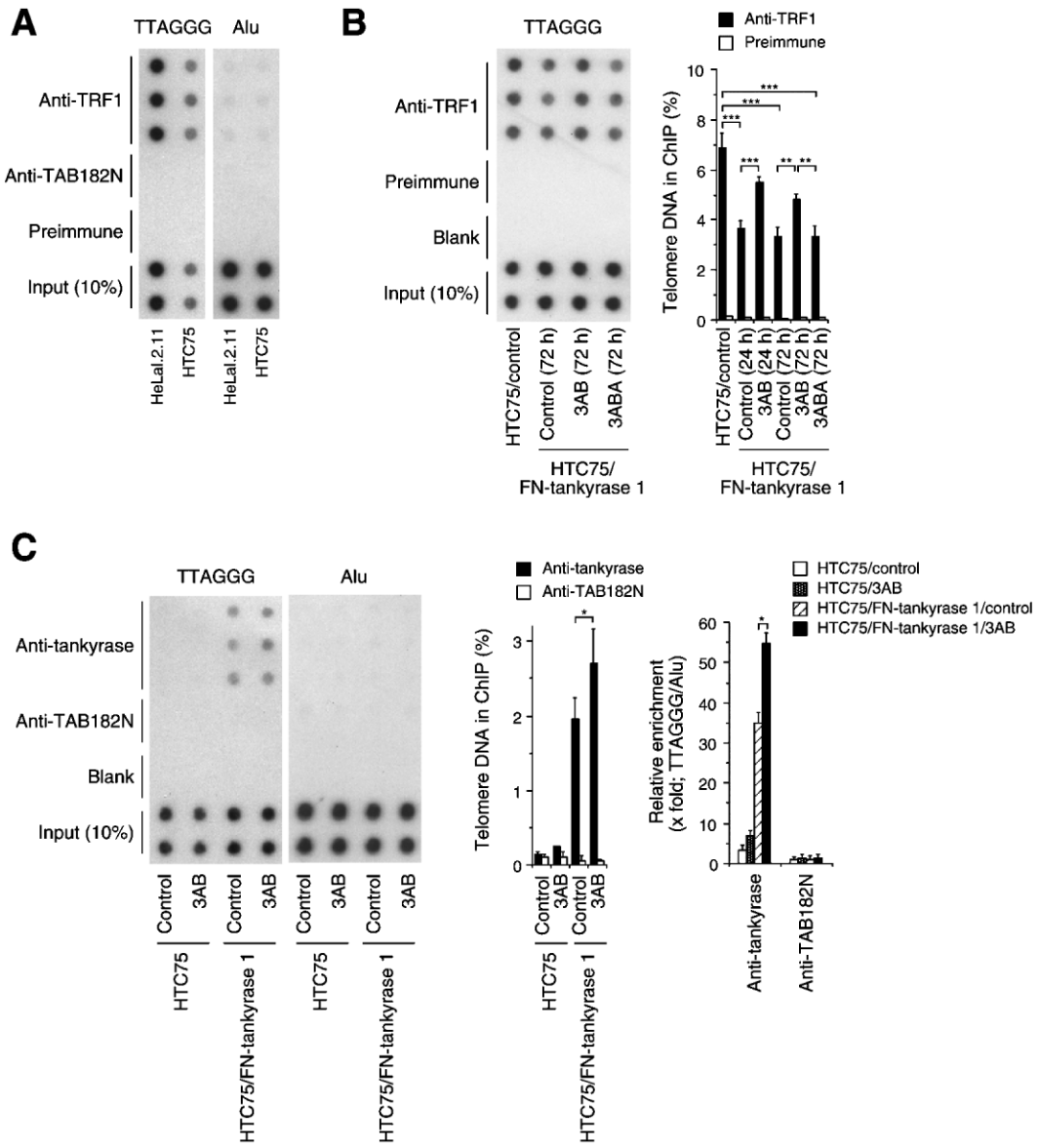

Figure 6. 3AB restores telomere binding of TRF1 in FN-tankyrase 1-overexpressing cells

A: ChIP analysis was performed by using HeLa1.2.11 and HTC75 cell lysates. Cells were fixed with formaldehyde and were sonicated. The resulting lysates were immunoprecipitated with antibodies or preimmune serum as indicated in triplicate or duplicate. The bead-bound DNA was blotted onto nylon filters. The filters were hybridized with the probes for TTAGGG (left) or Alu (right) repetitive sequences. For input, 10% of the total DNA samples were applied.

B: FN-tankyrase 1-overexpressing HTC75 cells were treated with 3 mM 3AB or 3ABA for 24 hr or 72 hr. ChIP was performed with anti-TRF1 antibody or preimmune serum. Quantitative values of the bead-bound telomere DNA (% precipitation relative to the total DNA) are shown in the right. ***$p < 0.005$, **$p < 0.01$ (unpaired *t* test).

C: Parental and FN-tankyrase 1-overexpressing HTC75 cells were treated with 3 mM 3AB for 24 hr. ChIP was performed with indicated antibodies. Quantitative values of the bead-bound telomere DNA are shown in the center panel. To verify the specificity of ChIP, enrichment of telomere DNA relative to Alu elements (% telomere DNA in ChIP/% Alu elements in ChIP) is also shown (right). *$p < 0.05$ (unpaired *t* test).

more directly, telomeric) accumulation of tankyrase 1 might reveal the different distribution between normal and cancer cells. Whether or not this is the case, 3AB enhanced telomere shortening by MST-312 in telomerase-positive cells but not in normal or telomerase-independent cells. Thus, although tankyrase 1 expression might not be tumor-specific, we still could expect that its inhibitory effect on telomere length is specific to telomerase-positive target cells.

This work has been originated from the assumption that telomerase inhibition would not be complete at doses achievable in vivo. We have demonstrated that incomplete inhibition of telomerase primarily shortens telomeres to some extent, but ultimately equilibrates, at which point stabilized telomeres are shorter than those in nontreated cells. If the telomeres at the equilibrium point were longer than those at crisis ("limit of shortening"), the cells would maintain the protective function of telomeres and would not undergo crisis. This situation has been exemplified by treatment of HTC75 cells with an intentionally

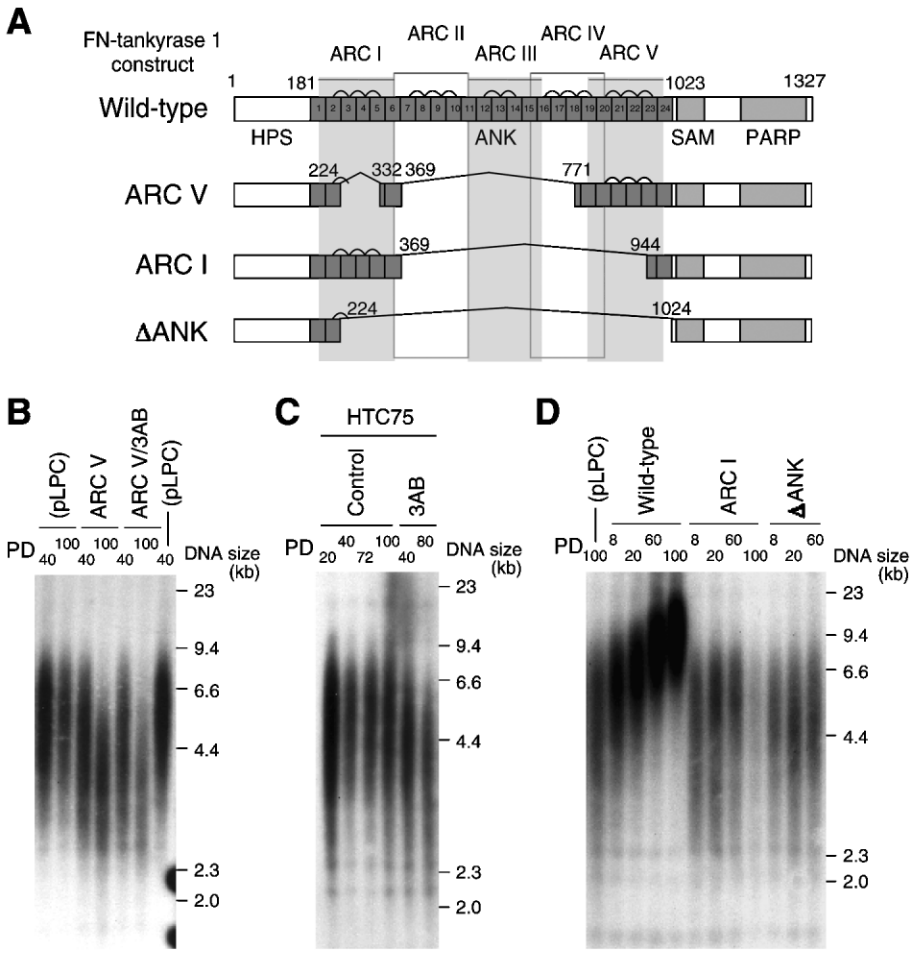

Figure 7. Tankyrase 1 deletion mutant that shortens telomeres

A: Schematic views of FN-tankyrase 1 deletion mutant constructs. These constructs contain a FLAG epitope tag and an NLS at the N termini (not shown). The numbers indicate the positions of amino acid residues. HPS, region containing homopolymeric runs of His, Pro, and Ser; ANK, ankyrin domain; SAM, multimerization domain homologous to the sterile α motif; PARP, PARP catalytic domain; ARC, ANK repeat cluster working as an independent TRF1 binding site; bridge above two adjacent ANK repeats indicates presence of a conserved histidine, presumably contributing to inter-repeat stabilization.

B: Effect of overexpression of FN-tank-ARC V on telomere length in HTC75 cells in the presence or absence of 3 mM 3AB. The drug treatment started at PD20. TRFs at indicated PD were detected by Southern blot analysis.

C: Effect of 3 mM 3AB on telomere length in HTC75 cells.

D: Effects of overexpression of the wild-type FN-tankyrase 1, FN-tank-ARC I, and FN-tank-ΔANK on telomere length in HTC75 cells.

lower dose (0.75 μM) of MST-312 (Figures 5A and 5B). On the other hand, if the telomeres at the equilibrium point were shorter than the limit of shortening, telomere dysfunction would emerge before the effect of the telomerase inhibitor was saturated. This has been exemplified by treatment of HTC75 cells with standard doses (1–2 μM) of MST-312 (Figures 2A–2D). Since the rates of telomere shortening in both experiments are slower than those observed in DN-hTERT-infected cells, MST-312 does not completely inhibit telomerase activity in the cells. Thus, even if complete inhibition of telomerase cannot be accomplished, one can still expect telomere crisis, as exemplified in Figures 2A–2D. Actually, several groups have reported that telomerase inhibitors successfully inhibit the growth of human cancer cells that are implanted in nude mice (Damm et al., 2001; Asai et al., 2003).

For induction of telomere crisis in HTC75 cells, MST-312 alone required 90 PD, whereas concomitant treatment with PARP inhibitors required 34–38 PD. Thus, the combined inhibition of tankyrase 1 and telomerase induced telomere dysfunction 52–56 PD earlier. This means that MST-312 alone allows a tumor to grow 4.5×10^{15}- to 7.2×10^{16}-fold larger than the combined inhibition. Since cancer cells often exhibit rather short average telomere length, one can expect that the combined inhibition produces meaningful clinical results when it is properly applied to cancers with short telomeres. Actually, MST-312 can induce telomere crisis much earlier (i.e., at PD20) in other cells that maintain shorter telomere length (Seimiya et al., 2002).

The rate of telomere shortening in telomerase-negative cells is proportional to the length of the telomeric 3′-overhang (Huffman et al., 2000). Our study indicates that 3AB did not accelerate telomere shortening in normal fibroblasts at all. Hence, enhancement of telomere shortening by 3AB in MST-312-treated cells would not be due to extension of the 3′-overhang, although other alterations in telomeric structure cannot be excluded.

PARP inhibitors could affect the functions of multiple PARP family members. Genetic disruption of PARP-1, the most abundant isozyme, causes hypersensitivity to DNA damaging agents (reviewed in Shall and de Murcia, 2000). So far, we have no evidence that MST-312 triggers a DNA damaging response and, in fact, 3AB did not enhance acute cytotoxicity of MST-312. Furthermore, PARP-1 does not poly(ADP-ribosyl)ate TRF1 in vitro (Cook et al., 2002). Therefore, combined effects of PARP inhibitors and MST-312 could not be explained by these mechanisms, although PARP-1 and PARP-2 still could be involved in regulation of telomeric functions (d'Adda di Fagagna et al., 1999; Dantzer et al., 2004). Since the catalytic efficiency of tankyrase 1 is 150-fold lower as compared to the basal activity of PARP-1 (Rippmann et al., 2002), PARP inhibitors would differentially affect the functions of these two enzymes. Furthermore, we have used a lower concentration of 3AB (3 mM) as compared to previous papers, such as Kanai et al. (2003), in which higher concentration (7 mM) was used to observe the PARP-1-blocked phenotype. We have shown that FN-tank-ARC V, a mutant allele

of tankyrase 1, caused moderate telomere shortening (Figure 7), presumably by sequestering the functionally crucial ARC V of endogenous tankyrase 1 from TRF1 binding. Since this phenotype was not enhanced by 3AB, telomere shortening by PARP inhibitors would mainly be derived from tankyrase 1 inhibition. Recently, TIN2 (another TRF1-interacting factor) has been reported as a negative regulator for tankyrase 1-mediated poly (ADP-ribosyl)ation of TRF1 (Ye and de Lange, 2004). According to the report, knockdown of TIN2 by small interfering RNA leads to derepression of endogenous tankyrase 1 at telomeres, resulting in release of TRF1 from telomeres. Importantly, 3AB restores the telomeric localization of TRF1 in TIN2 knockdown cells. This indicates that 3AB truly inhibits the activity of endogenous tankyrase 1 at telomeres and enhances the telomeric function of TRF1. Because shorter telomeres are going to have fewer TRF1s, telomere shortening may derepress tankyrase 1 action by reducing the TIN2 loading on a chromosome end. This may explain why 3AB had a greater impact on shortened telomeres than on longer telomeres, although additional involvement of other PARPs cannot be excluded.

It remains to be determined if tankyrase inhibition causes considerable side effects in clinical therapy. Tankyrases are not only found on telomeres, but also in other intracellular loci with additional binding partners, such as insulin-responsive aminopeptidase, Grb14, and TAB182 (Smith and de Lange, 1999; Chi and Lodish, 2000; Lyons et al., 2001; Seimiya and Smith, 2002). These facts suggest yet unidentified physiological functions of tankyrases. Interestingly, knockdown of tankyrase 1 by RNA interference prevents resolution of sister telomere association during mitosis (Dynek and Smith, 2004). As compared with its function on telomere length control, the role of tankyrase 1 for progression of mitosis would be less sensitive to PARP inhibitors, because no alteration was observed in mitosis of the PARP inhibitor-treated cells in this study.

TRF1 stalls a replication fork at telomere DNA (Ohki and Ishikawa, 2004), suggesting that TRF1 may need to dissociate from telomeres during its replication. Even if this were the case, it might be technically difficult, from a physiological standpoint, to detect the release of TRF1 during a very narrow window in the cell cycle. It would be interesting to determine if any release of TRF1 from telomeres is coupled with TIN2-mediated modulation of tankyrase 1 PARP activity (Ye and de Lange, 2004) in a cell cycle-dependent manner.

From a pharmacokinetic aspect, it might be difficult to keep an enzyme completely static for a long time. We expect that inhibition of tankyrase 1 will compensate for incomplete inhibition of telomerase. Consequently, this strategy would shorten the time period of drug treatment that is required for the onset of telomere crisis and reduce the potential risk of acquired drug resistance. This study also provides a novel application of PARP inhibitors, and would have an impact on the field of drug development for stroke, myocardial ischemia, diabetes, central nervous system injury, and various other forms of PARP-related pathogenesis.

Experimental procedures

Plasmids

pLPC/FN-tankyrase 1 constructs were made as described previously (Seimiya et al., 2004). The mutant hTERT(D712A/V713I)-HA (DN-hTERT-HA) (Hahn et al., 1999) was created using the HA-tagged hTERT fragment and a QuikChange XL site-directed mutagenesis kit (Stratagene) and cloned into pLNCX2 (BD Biosciences). For Myc-DN-TRF1, the partial fragment of TRF1(66–385) was amplified by PCR and cloned into pLHCX (BD Biosciences) with an N-terminal Myc epitope tag.

Cell culture and drug treatment

HT1080, HTC75 (van Steensel and de Lange, 1997), and HeLaI.2.11 (van Steensel et al., 1998) cells were grown in DMEM medium supplemented with 10% heat-inactivated calf serum. Retroviral infection was performed essentially as described (Cook et al., 2002). Infected cells were selected with 2 μg/ml of puromycin (FN-tankyrase 1), 500 μg/ml of G418 (DN-hTERT-HA), or 200 μg/ml of hygromycin (Myc-DN-TRF1). Cells that expressed two exogenous constructs were cultured with 25% original concentrations of each drug. Foreskin fibroblasts, GM847, and HBC4 cells were grown in DMEM medium with 10% heat-inactivated fetal bovine serum. In cases of long-term treatment with test compounds, concentrations of puromycin, G418, and hygromycin were further reduced to 25% of the nontreatment conditions. Cells were split exactly 1:16 (i.e., +4 PD) with test compounds at concentrations of less than 0.5 × IC_{50}, under which conditions cells exhibited almost comparable growth rates with the control; cells needed to be split every 3–4 days (5–6 days in case of GM847 and HBC4 cells). Cells with reduced growth rates at later passages were split 1:2 to 1:8 (i.e., +1 to +3 PD) or given fresh medium with the compounds every 3–4 days.

Western blot analysis

Cell lysates were prepared as described (Seimiya et al., 2004). Western blot analysis was performed as previously described (Seimiya and Smith, 2002) with the following primary antibodies: rabbit anti-tankyrase (H-350, 2 μg/ml, Santa Cruz Biotechnology), rabbit anti-HA (Y-11, 2 μg/ml, Santa Cruz Biotechnology), mouse anti-α-tubulin (B-5-1-2, 1:1,000, Sigma), rabbit anti-TRF1 (5747, 0.5 μg/ml) (Seimiya et al., 2004), mouse anti-TRF2 (4A794, 2.5 μg/ml, Imgenex), or mouse anti-lamin A/C (636, 2 μg/ml, Santa Cruz Biotechnology).

Detection of telomere DNA

Telomere restriction fragments (TRF) were detected by Southern blot analysis with a [^{32}P]-TTAGGG probe, as previously described (see references in Seimiya et al., 2004). The mean length of TRFs was determined on an Atto densitoscan analyzer, using scanned images of autoradiograms. For telomere PNA FISH, cells were treated with 0.25 μg/ml colcemid for 3 hr, trypsinized, and swollen in 0.6% sodium citrate for 30 min at 37°C. Metaphase spreads were prepared on slide glass and dehydrated as described (Dynek and Smith, 2004). Telomere PNA FISH was performed essentially as described (Meeker et al., 2002).

Enzyme assays

TRAP assay for estimation of telomerase activity was performed as described (Seimiya et al., 2002). We used a modified set of primers, TS and ACX, and an internal standard, TSNT, for enhanced accuracy of the quantitation (Kim and Wu, 1997). As pilot studies, we determined the quantitative range of the reaction by serial dilution of cellular lysates. In vitro PARP assay for estimation of tankyrase 1 activity was performed as described (Seimiya et al., 2004).

Detection of cellular senescence and apoptosis

SA-β-gal staining was performed as described (Seimiya et al., 2002). For quantitation of apoptotic cells, propidium iodide staining of cellular DNA and annexin V-fluorescein staining of externalized phosphatidylserine were performed as previously described (Seimiya and Tsuruo, 1998).

Immunofluorescence microscopy

HeLaI.2.11 cells were transfected with pLPC/FN-tankyrase 1 using a standard electroporation method. After incubation for 20 hr at 37°C, cells were fixed with 2% paraformaldehyde/PBS for 10 min and permeabilized with 0.5% NP-40/PBS. Indirect immunofluorescence staining was performed as described (Seimiya et al., 2004) with the primary antibodies mouse anti-FLAG (M2, 2 μg/ml) and affinity purified rabbit anti-TRF1 (5747, 1 μg/ml) (Seimiya et al., 2004).

Chromatin immunoprecipitation

ChIP was carried out essentially as described by Loayza and de Lange (2003), except that sonication of lysates was performed at a density of 5 ×

10^6 cells/ml. For immunoprecipitation, 50 μl of preimmune serum, anti-GST-TRF1 5747, or anti-GST-TAB182 N terminus (a rabbit polyclonal antibody 418 raised against a recombinant protein for the N-terminal 731 amino acids of TAB182; H.S. and S. Smith, unpublished data) antiserum, or 2 μg of anti-tankyrase antibody (H-350) was used per sample. Signals were quantitated by phosphorimaging with a Fuji BAS Imaging Analyzer. We confirmed that an excess amount of antibody was added to each ChIP and that the values for telomere DNA in ChIP (%) were maximally saturated.

Supplemental data

Supplemental data for this article can be found at http://www.cancercell.org/cgi/content/full/7/1/25/DC1/.

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