Telomere Shortening and Growth Inhibition of Human Cancer Cells by Novel Synthetic Telomerase Inhibitors MST-312, MST-295, and MST-199[1]

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Abstract

Epidemiological studies suggest potent anticancer effects of tea catechins. Previously, we have reported (I. Naasani *et al.,* Biochem. Biophys. Res. Commun., *249:* 391–396, 1998) that epigallocatechin gallate (EGCG), a major tea catechin, strongly and directly inhibits telomerase, a ribonucleoprotein that maintains telomeres and has been implicated in tumorigenesis. Here, we describe newly synthesized compounds MST-312, MST-295, and MST-199, as more effective telomerase inhibitors than EGCG. Continuous treatment of human monoblastoid leukemia U937 cells with a nontoxic dose of each drug caused progressive telomere shortening and eventual reduction of growth rate accompanied by induction of the senescence-associated β-galactosidase activity. Particularly, in the case of MST-312, the effective dose required for the telomere shortening was 1–2 μM, which was 15- to 20-fold lower than that of EGCG. These compounds may provide a novel chemotherapeutic strategy for the treatment of cancers.

Introduction

Human telomeres, the nucleoprotein complexes at chromosome ends, consist of tandem arrays of TTAGGG repeats that are bound to specific telomeric-repeat binding proteins, such as telomeric repeat binding factors 1 and 2 (1, 2). In most somatic tissues, telomeric DNA shortens after each cell cycle because of the end replication problem of chromosomal DNA. Because impairment of telomere function by loss of telomeric sequences limits the proliferative capacity of cultured cells, it has been proposed that critically shortened telomeres act as a signal for replicative senescence (3). Hemann *et al.* (4) have demonstrated that it is not the average but rather the shortest telomeres that constitute telomere dysfunction and limit cellular survival. Another important role of telomeres is to protect the linear chromosome ends from being recognized as double-strand breaks, which would elicit a checkpoint or an apoptotic response in the cell. Recently, Karlseder *et al.* (5) have reported that replicative senescence is induced by disrupted protection of shortened telomeres rather than by a complete loss of telomeric DNA. In immortal cells, including germ-line cells and most cancer cells, telomeres are maintained by telomerase, a specialized reverse transcriptase that adds the telomeric repeats to the ends of chromosomes (6). Telomerase holoenzyme consists of two essential factors, the TR RNA component and the TERT catalytic subunit. Telomerase activity is regulated by the transcription of TERT (7–10), and our recent study also suggests a posttranslational regulation of telomerase (11).

According to the fact that most (80–90%) of all cancer cells possess telomerase activity (12, 13), the maintenance of telomeres by telomerase has been implicated in tumorigenesis. In this model, cancer cells bypass telomere crisis by activating telomerase and exhibit an infinite replicative capacity. In fact, short dysfunctional telomeres, caused by the absence of telomerase activity, impair tumor development in the INK4aΔ2/3 cancer-prone mouse (14). Also, inhibition of telomerase by its dominant-negative mutant or by oligonucleotides complementary to the hTR[4] RNA template region induces apoptosis of telomerase-positive tumor cells (15–17). One probable advantage of telomerase-targeted therapy would be its specific effect on telomerase-positive tumor cells, because most of human somatic tissues are telomerase-negative (12). Although telomerase inhibition might have effects on regenerative tissues that express telomerase, these effects seem to be minor because stem cells in such tissues have much longer telomeres than do cancer cells. Actually, the time necessary for telomerase inhibition to cause growth arrest or cell death depends on initial telomere length of target cells (15, 17). On the basis of these situations, various types of small compounds that inhibit telomerase activity have been developed. Such inhibitors include nucleoside analogs, *e.g.,* azidothymidine, 6-thio-2′-deoxyguanosine 5′-triphosphate (18, 19); catalytic

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[4] The abbreviations used are: hTR, human TR; TRF, terminal restriction fragment; EGCG, epigallocatechin gallate; PD, population doubling; SA-β-Gal, senescence-associated β-galactosidase; TRAP, telomeric repeats amplification protocol; hTERT, human TERT; GI_{50}, (concentration) inhibiting the cell growth by 50%; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; RPE, retinal pigment epithelial (cell).

inhibitors, *e.g.,* 2-[3-(trifluoromethyl)phenyl]isothiazolin-3-one, BIBR1532 (20, 21); hTR antisense oligonucleotides, *e.g.,* 2′-*O*-methyl-RNA, peptide nucleic acids, and N3′→P5′ phosphoramidates (22–24); ribozymes (25); and G-quadruplex ligands, *e.g.,* TMPyP4, Telomestatin (26, 27).

Several lines of evidence on the anticancer and chemopreventive activities of tea catechins have emerged from animal models and human epidemiological studies (28, 29). As the mechanisms underlying these activities, various factors have been postulated, including its antioxidant activity, interaction with certain enzymes or proteins implicated in tumorigenesis (*e.g.,* urokinase, ornithine decarboxylase, NADPH-cytochrome P450 reductase, protein kinase C, steroid 5α reductase, tumor necrosis factor expression and nitric oxide synthase). However, the concentrations of catechins used for the *in vitro* experiments were largely not relevant to the *in vivo* and epidemiological observations (28, 30). Recently, we have reported that EGCG, a major tea catechin, inhibits telomerase activity at a low concentration in harmony, somewhat, with the plasma levels obtained after drinking a few cups of green tea (31). Continuous treatment of human cancer cells with such a low and nontoxic concentration of EGCG results in growth arrest accompanied by telomere shortening and expression of a senescence-associated phenotype (31).

EGCG is unstable especially in the basic conditions. In addition, it is difficult to obtain pure EGCG in bulk because this compound is a natural product. In this study, to circumvent such disadvantages of EGCG, we have synthesized a series of stable compounds that have EGCG-related moieties and have tested their ability to inhibit telomerase activity. Among them, we have established novel synthetic compounds that possess striking advantages as compared with EGCG in terms of chemical stability, the effective dose for the induction of telomere shortening, and the circumvention of acquired resistance. These compounds may provide a novel chemotherapeutic strategy directed to telomerase of cancer cells.

Materials and Methods

Chemical Synthesis. EGCG and TMPyP4 were purchased from Sigma-Aldrich (St. Louis, MO) and Tokyo Chemical Industry (Tokyo, Japan), respectively. Compounds 1–8 were prepared essentially as described previously (32). Compounds 9–17 were prepared by conventional methods: amidation to phenylenediamine with benzyloxy-substituted benzoyl chloride, followed by deprotection of the benzyl groups.

Cell Culture. Human monoblastoid leukemia U937 cells were grown in RPMI 1640 (Nissui Co. Ltd., Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum and 100 μg/ml kanamycin in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Seeding density for the cells was empirically optimized for passaging twice weekly.

Drug Treatment. For preliminary cell growth inhibition assays, cells were plated in 24-well plates at 4×10^4 cells/well. After incubation for 6 h, cells were grown in the continuous presence of test compounds for 3 days. Cell numbers were counted by a semi-automated hematology analyzer (F-520P; Sysmex, Kobe, Japan). Concentrations at GI_{50} were determined by interpolation from the concentration-response curves. In cases of long-term treatment with the compounds, cells were split exactly 1:16 (*i.e.,* +4 PD) in the presence of the compounds at $\sim 0.5 \times GI_{50}$ concentrations; under these conditions, most subcultures before crisis exhibited growth rates that were comparable with the control and that needed to be split every 3 days. Cells with reduced growth rates at later passages were given fresh medium and compound every 2~3 days. Quantitation of cell numbers was done by hemocytometer counting.

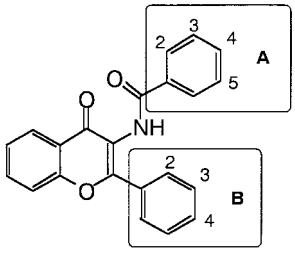
Measurement of Telomerase Activity. The U937 cellular extract was prepared, and TRAP assay was performed essentially as described (12). In this assay, we used a modified set of primers (TS, 5′-AAT CCG TCG AGC AGA GTT-3′; ACX, 5′-GCG CGG CTT ACC CTT ACC CTT ACC CTA ACC-3′; NT, 5′-ATC GCT TCT CGG CCT TTT-3′) and an internal standard, TSNT (5′-AAT CCG TCG AGC AGA GTT AAA AGG CCG AGA AGC GAT-3′), for enhanced accuracy of the quantitation (33). The telomeric products were separated by PAGE and visualized by staining with SYBR Green (Takara, Kyoto, Japan; Ref. 34). The quantitative range of the telomerase reaction was initially determined by serial dilution of cellular lysates. Densitometric analysis was carried out on the photograms using an NIH Image 1.60.

Southern Blot Analysis. Genomic DNA was prepared by using a Blood and Cell culture DNA Midi kit (Qiagen, Valencia, CA) according to the instruction manual. Portions of DNA (4 μg/assay) were digested with *Hin*fI and *Rsa*I and fractionated by agarose gel electrophoresis. The gel was soaked in 0.25 M HCl for 15 min twice and soaked in 0.4 M NaOH for 15 min twice. The DNA in the gel was transferred to the Hybond N+ membrane (Amersham, Buckinghamshire, England) for 4 h. The resulting membrane was rinsed with 1× SSC, dried at 50°C for 1 h, and soaked in the hybridization buffer, consisting of 5× SSC, 5× Denhardt solution, 1% SDS, and 100 μg/ml salmon sperm DNA at 55°C for 30 min. This membrane was hybridized with biotin-conjugated $(TTAGGG)_3$ probe at 45°C overnight, and washed with 2× SSC/0.1% SDS for 5 min twice and 0.2× SSC/0.1% SDS for 15 min twice. The membrane was soaked in the blocking buffer (5% SDS, 17 mM Na_2HPO_4, and 8 mM NaH_2PO_4) for 1 min, and then incubated with avidin-conjugated horseradish peroxidase for 4 min. This membrane was extensively washed with 0.1% Tween 20/PBS, and the specific signals were detected on X-ray films (Kodak, Rochester, NY) by a SuperSignal detection kit (Pierce, Rockford, IL).

SA-β-Gal Staining. Cells were washed with PBS, fixed with 0.5% glutaldehyde/PBS (pH 7.2), and washed with 1 mM $MgCl_2$/PBS. Then, the cells were incubated in the acidic X-gal solution, consisting of 1 mg/ml X-Gal, 0.12 mM each $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$, and 1 mM $MgCl_2$ in PBS (pH 6.0) at 37°C (35). The blue color development in the senescent cells was monitored by photomicroscopy.

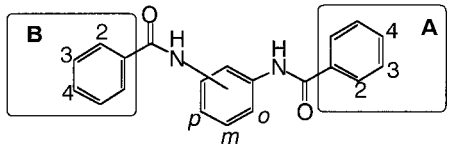
Results

Inhibition of Telomerase by Novel Synthetic Compounds with EGCG-related Moieties. A series of synthetic compounds that overcame the chemical disadvantages of natural EGCG were examined for their ability to inhibit telomerase



Compound	MST-	-OH position of A moiety	-OH position of B moiety	IC_{50} (μM)
1		3,4,5	3,4	0.43
2	199	3,4	3,4	0.38
3		4	3,4	12
4		3,5	3,4	6.6
5		2,4	3,4	5.0
6		None	3 4	27
7		3,4	2,3	1.7
8		3,4	None	>30

Fig. 1. Relationship between structures of synthetic chromone derivatives and their telomerase inhibitory activities. The TRAP lysate was prepared from U937 cells, as a source of telomerase. Telomerase activity was determined by an *in vitro* TRAP assay in the presence of each compound, as described in "Materials and Methods." Concentrations inhibiting the telomerase activity by 50% (IC_{50}) were determined by interpolation from the concentration-response curves.



Compound	MST-	substituted position	-OH position of A moiety	-OH position of B moiety	IC_{50} (μM)
9	295	*o*	3,4	3,4	0.72
10		*m*	3,4	3,4	0.43
11		*p*	3,4	3,4	0.42
12		*o*	3,4,5	3	8.7
13		*m*	3,4,5	3	8.0
14		*o*	3,4,5	4	6.2
15		*m*	3,4,5	4	5.5
16		*o*	3,4,5	None	>10
17		*o*	2,3	2,3	>10
18	312	*m*	2,3	2,3	0.67
19		*p*	2,3	2,3	>10

Fig. 2. Relationship between structures of synthetic phenyl derivatives and their telomerase inhibitory activities. The TRAP assay was performed in the presence of each compound and the IC_{50} was determined as in Fig. 1.

activity *in vitro*. First, we designed the compound 1 as a simpler and more stable EGCG derivative (Fig. 1). In this compound, the chromane ring and the ester linkage were changed to a chromone ring and an amide linkage, respectively, for its chemical stabilization. To simplify the structure, hydroxy groups on the original chromane ring were deleted. We found that this compound potently inhibited telomerase activity (IC_{50}, 0.43 μM). According to our previous report (31), the IC_{50} value of EGCG *in vitro* is ~1 μM. Thus, we presumed that two moieties, pyrogallol (3,4,5-trihydroxy groups on the A moiety) and pyrocatechol (3,4-dihydroxy groups on the B moiety) were important pharmacophores and that other hydroxy groups on the chromone ring were dispensable.

On the basis of this presumption, we examined simpler derivatives, in which hydroxy groups of A and B moieties were changed (Fig. 1). Compound 2 (MST-199), which had 3,4-dihydroxy groups on the A moiety, still exhibited comparable inhibition to compound 1 (IC_{50}, 0.38 μM), whereas compound 3, which had only one hydroxy group on the A moiety, decreased the activity (IC_{50} = 12 μM). Compounds 4 and 5, which had 3,5- and 2,4-dihydroxy groups on the A moiety, respectively, showed decreased activity as compared with compound 1. Compound 6, which had no hydroxy group on the A moiety, further decreased its activity (IC_{50}, 27 μM). Similar to the case of the A moiety, removal of hydroxy groups on the B moiety diminished the inhibitory activity; compound 7 exhibited much stronger activity (IC_{50}, 1.7 μM) than did compound 8 (IC_{50}, >30 μM), in which dihydroxy groups on the B moiety were removed. Together, these observations indicate that the 3,4-dihydroxy groups on each phenyl rings (A and B moieties) are important for the potent inhibition of telomerase activity.

We, therefore, examined the simpler compounds 9 (MST-295), 10, and 11, in which a pair of 3,4-dihydroxybenzamide groups (A and B moieties in Fig. 2) were attached to the *o*-, *m*-, and *p*-position of a phenyl ring, instead of a chromone ring. As the result, we found that all of these compounds showed high activity of telomerase inhibition (IC_{50}, 0.42–0.72 μM). In contrast, compounds 12–15, which had only one hydroxy group on the B moiety, exhibited lower activity (IC_{50}, 5.5–8.7 μM). Compound 16, which had no hydroxy group on the B moiety, further decreased the activity (IC_{50} = >10 μM). These observations of the phenyl derivatives were similar to those of the chromone derivatives (Fig. 1) and support the idea that the existence of more than two hydroxy groups on A and B moieties are required for the effective inhibition of telomerase activity.

Finally, we examined the compounds 17, 18 (MST-312), and 19, which had the same 2,3-dihydroxy groups on both A and B moieties at different positions of the phenyl ring. Interestingly, only the compound 18 (MST-312), in which two moieties were attached at *m*-position, strongly inhibited the telomerase activity (IC_{50}, 0.67 μM). These data suggest that a pair of dihydroxyphenyl moieties (pyrocatechol) is an important pharmacophore and that the arrangement of a pair of pyrocatechol moieties to appropriate a steric conformation is required for the maximal inhibition of telomerase activity.

Fig. 3 shows the representative TRAP assay on selected compounds, 18, 9, and 2; MST-312 [*N*,*N*′-bis(2,3-dihydroxybenzoyl)-1,2-phenylenediamine], MST-295 [*N*,*N*′-bis(3,4-

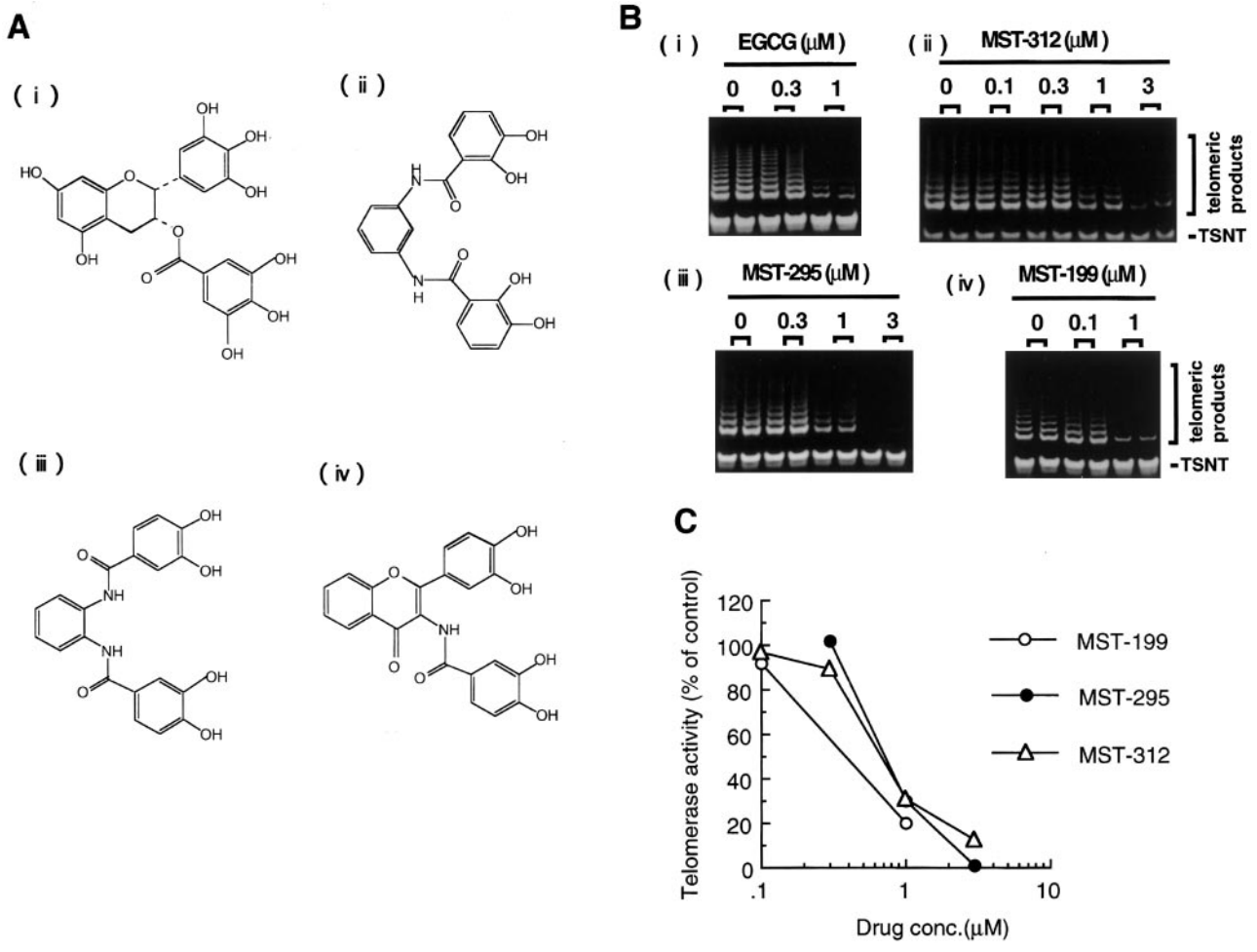


Fig. 3. Inhibition of *in vitro* human telomerase activity by MST-312, MST-295, and MST-199. *A*, chemical structures of EGCG *(i)*, MST-312 *(ii)*, MST-295 *(iii)*, and MST-199 *(iv)*. *B*, inhibition of telomerase activity by the compounds. The TRAP lysate was prepared from U937 cells, as a source of telomerase. Telomerase activity was determined by an *in vitro* TRAP assay. *C*, quantitative data from *B*. Control activity determined in the absence of any compounds was given a value of 100%.

dihydroxybenzoyl)-1,3-phenylenediamine], and MST-199 {*N*-[2-(3,4-dihydroxyphenyl)-4-oxo-4*H*-chromen-3-yl]-3,4 dihydroxybenzamide}, which exhibited similar or stronger activities as compared with the original EGCG. Each compound inhibited telomerase activity in a dose-dependent manner. These compounds did not inhibit *Taq* DNA polymerase used for the TRAP assay, even at much higher concentrations (see relevant amplification of the TSNT fragment, an internal standard for PCR; Fig. 3*B*), which indicated that their inhibitory effects were specifically directed to telomerase.

Inhibition of U937 Cell Growth by Continuous Treatment with the MST Compounds. To determine the *in vivo* capacity of cell growth inhibition by the representative compounds in Fig. 3, we investigated the prolonged passage of U937 cells in the presence of each compound. By our preliminary tests, sublethal GI_{50} concentrations of MST-312, MST-295, and MST-199 were determined as 1.7 μM, 25 μM, and 37 μM, respectively. Subsequent tests revealed that continuous treatment with these compounds at GI_{50} conferred a slight resistance to the cell growth and morphology at ~3 weeks (MST-312), 1 month (MST-295), and 2 months (MST-199), respectively (data not shown). On the basis of these observations, initial concentration of each drug for the long-term cultivation was determined to be the highest that actually did not have any significant effects on cell growth or morphology, at least for more than ten days (*i.e.,* ~0.5 × GI_{50}; 1.0 μM MST-312, 10 μM MST-295, and 20 μM MST-199). To circumvent possible emergence of acquired resistance to each compound, we doubled the concentration at day 20 (MST-312), day 30 (MST-295), or day 50 (MST-199), respectively, in the lineage of cultivation. Essentially, cells in the presence of each compound grew at a rate comparable with that of a control culture. However, at ~PD80 (day 65–70), cultures with these compounds showed a marked reduction in growth rate (Fig. 4). This change could not be observed at the early stage of the passages, which suggested that the growth inhibition was attributable to the eventual loss of the telomeric tracts in the cells.

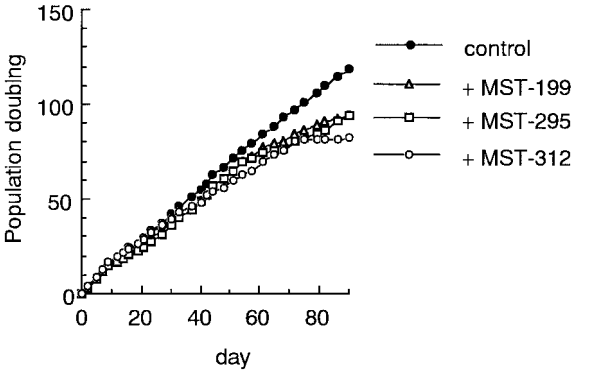


Fig. 4. Long-term treatment with MST-312, MST-295, or MST-199 at nontoxic doses inhibits U937 cell growth. Cells were cultured in the presence of 1.0 μM MST-312, 10 μM MST-295, or 20 μM MST-199. Each dose was doubled at day 20 (2.0 μM; MST-312), day 30 (20 μM; MST-295) or day 50 (40 μM; MST-199), respectively, in the lineage of cultivation.

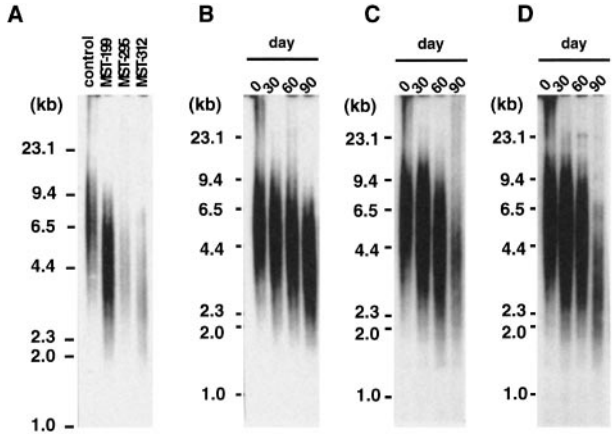


Fig. 5. Long-term treatment with MST-312, MST-295, or MST-199 at nontoxic doses shortens telomere length of U937 cells. Cells were treated as in Fig. 4 legend and the genomic DNA was prepared from the same subculture. Terminal restriction fragment length (TRF) was determined by Southern blot analysis and a subsequent densitometric analysis. Equivalent amounts of genomic DNA were loaded in each lane. *A*, TRF length of the cells treated with MST-312, MST-295, or MST-199 for 90 days. *B–D*, time course monitoring of the TRF length of the MST-312-treated cells (*B*), MST-295-treated cells (*C*), or MST-199-treated cells (*D*).

Telomere Shortening on Addition of the MST Compounds. To support the hypothesis that the decrease in cell proliferation was caused by the MST compounds through a telomerase-inhibitory mechanism rather than being caused by long-term cytotoxicity that was independent of telomerase inhibition, we monitored the effects of the MST compounds on telomere length. Genomic DNA was prepared from the same subcultures as in Fig. 4 and examined by Southern blot analysis. As shown in Fig. 5*A*, cells treated with each compound for 90 days exhibited significantly shortened telomeres. Control cells maintained a mean TRF length of 6.30 kb, whereas those treated with MST-312, MST-295, or MST-199 had TRF lengths of 3.95, 4.63, and 4.70 kb, respectively. Fig. 5*B* shows progressive telomere shortening during the course of cell proliferation. The loss of telomere sequence per each cell division was estimated as 29 bp (MST-312), 18 bp (MST-295), and 17 bp (MST-199), respectively. Furthermore, continuous treatment of human RPE cells that stably expressed hTERT (hTERT-RPE1; Clontech, Palo Alto, CA) with either MST-312, MST-295, or MST-199, also shortened their telomeres by 52 bp (MST-312), 17 bp (MST-295), or 11 bp (MST-199), respectively, per cell division (data not shown; see "Discussion" below). This estimation was somewhat smaller than, but still similar to, previous measurements of telomere loss in telomerase-negative cells (~60 bp; Refs. 15 and 36). These observations suggest that the telomere loss by the MST compounds was caused by the end-replication problem that emerged from the inhibition of telomerase. In fact, lysates prepared from the drug-treated U937 cells exhibited lower levels of telomerase activity; 67% (MST-312), 63% (MST-295), and 35% (MST-199) of nontreated cells (data not shown).

Senescence-like Phenotype Associated with Growth Inhibition by the MST Compounds. To examine whether the growth inhibition and telomere shortening by the MST compounds were associated with senescence-like phenotype, we monitored the expression of the SA-β-Gal activity, a potential biomarker for cellular aging (37). As shown in Fig. 6, continuous treatment of U937 cells with either MST-312, MST-295, or MST-199 for 90 days induced SA-β-Gal activity in 58.3, 24.8, or 24.9% of the total cell population, respectively. As control, only 4.2% of nontreated cells exhibited the SA-β-Gal activity. The frequency of the SA-β-Gal-positive cells correlated with the capabilities of each compound to reduce cell growth and to shorten telomere length; among three compounds, MST-312 had the greatest activities of (*a*) retardation of cell growth, (*b*) telomere shortening, and (*c*) SA-β-Gal induction at the lowest concentration. MST-295 and MST-199 possessed these three activities at comparable levels with each other although the latter required a 2-fold higher dose. Finally, cytometric analysis of DNA derived from the same subculture (*i.e.,* day 90) revealed that these compounds also induced a moderate tendency of either G_0-G_1 arrest of the cell cycle (MST-312) or apoptotic cell death (MST-295 and MST-199); treatment with MST-312 increased the fraction of the cells in G_0-G_1 phase (45→60% of total cells) whereas treatment with either MST-295 or MST-199 increased the fraction of the cells in sub-G_1 (apoptotic) areas (0.3%→4–5% of total cells; data not shown). Taken together, these results suggest that the reduced growth rate of U937 cells by continuous treatment with our compounds is derived from the telomerase-inhibitory action of the compounds and not from unidentified cytotoxic effects.

Discussion

Here, we have developed novel synthetic compounds, MST-312, MST-295, and MST-199, that inhibit telomerase activity, induce telomere shortening, and cause eventual reduction of U937 cell growth. According to previous reports (15, 36), in telomerase-negative backgrounds, telomeres are shortened by only ~60 bp per cell division. This fact indicates that, when treated with a telomerase inhibitor, cells with longer telomeres would require more doublings to exhaust telomeric sequences compared with cells with shorter te-

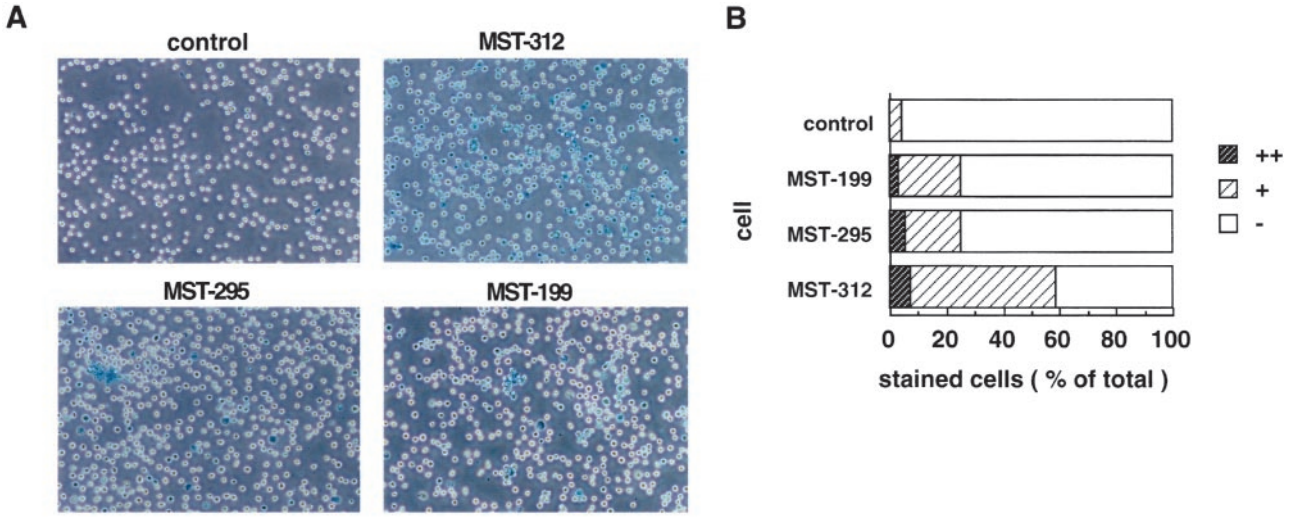


Fig. 6. Senescence-like phenotype induced by MST-312, MST-295, or MST-199. *A*, SA-β-Gal staining of the cells treated with MST-312, MST-295, or MST-199 for 90 days. Cells were treated with each compound as in Fig. 4, fixed with 0.5% glutaldehyde/PBS (pH 7.2), and incubated in the acidic X-gal solution (see "Materials and Methods") at 37°C for 3 h. *B*, percentage of SA-β-Gal positive cells. In each subculture, ~1000–2000 cells were classified into three categories: ++ (in *B*), *dark blue* (in *A*); + (in *B*), *blue* (in *A*); − [negative (in *B*)], *white* (in *A*). Each *bar*, the average of at least four experiments.

lomeres. Continuous treatment of hTERT-RPE1 cells with either MST-312, MST-295, or MST-199, also induced growth arrest, telomere shortening, and SA-β-Gal activity (data not shown). Intriguingly, growth arrest of hTERT-RPE1 cells by the MST compounds emerged much earlier (~PD20) than that of U937 cells (~PD80). This result was easily expectable because nontreated hTERT-RPE1 cells maintained much shorter TRF length (3.9 kb) as compared with U937 cells (6.0 kb; Fig. 5 and data not shown). These observations support the idea that the growth inhibition of U937 or hTERT-RPE1 cells by our compounds results from the eventual loss of telomeric tracts in the cells.

Among other targets for EGCG, urokinase is thought to enhance tumor growth *in vivo* by increasing the invasive and metastatic potential of cancer cells (38). On the other hand, the antiproliferative effect of our compounds was observed even *in vitro*, excluding the possibility that this effect was derived from a reduction of invasive or metastatic potential of the cells through the inhibition of urokinase. Second, the antiproliferative effect achieved by inhibiting other EGCG targets often emerges earlier than in case of our compounds. For instance, the inhibition of ornithine decarboxylase can induce cell death associated with a rapid decline of intracellular polyamine contents (39). Such an acute effect was not observed in the present study. Finally, all of other potential targets (NADPH-cytochrome P450 reductase, and so forth) are thought to be involved in the initial steps of carcinogenesis rather than in the growth inhibition of once-transformed cancer cells (40). Thus, it is unlikely that the eventual growth retardation of U937 leukemia cells by our compounds was caused by the inhibition of the tumor initiation or promotion steps.

Terminal differentiation of cancer cells has been associated with the repression of telomerase activity (41–43). U937 is a monoblastoid leukemia cell line, and its differentiation into macrophage can be induced by treatment with phorbol ester for only 1~3 days (44). Differentiation of this cell line can be easily monitored by its morphological change (adhesion to a culture dish) or growth arrest (G_0-G_1 arrest). If EGCG or its analogues described here induce differentiation of U937 cells, such phenotypic changes would have been observed much earlier than was observed in the case of Fig. 4: antiproliferative effect of our compounds was observed only after continuous treatment for ~65–70 days. In addition, on treatment with these compounds, the cells never adhered to the culture dish even after the SA-β-Gal was induced (data not shown). These observations clearly exclude the possibility that the effect of our compounds on telomere shortening or senescence is simply a result of the antiproliferative or differentiating effect of the compounds.

When U937 cells were treated with MST-312, MST-295, or MST-199, the levels of telomere shortening, reduced growth rate, and emergence of SA-β-Gal activity were well correlated and even predictable from each other phenotype. Considering that the effective dose of MST-312 (1–2 μM) was the lowest among the three compounds, we could conclude that MST-312 was the most potent telomerase inhibitor against intact cells. Meanwhile, when the lysate from the cells treated with each compound was analyzed by an *in vitro* TRAP assay, the MST-312-treated cell lysate did not exhibit the lowest telomerase activity. This observation suggests that the telomerase-inhibitory effect of MST-312 was reversible and that the compound incorporated into the cells was stripped out from telomerase when the lysate was diluted by adding the reaction mixture for the TRAP assay. This idea is consistent with our previous findings that telomerase inhibition by EGCG is direct and reversible and that the lysate from EGCG-treated cells exhibits telomerase activity that is comparable with that from nontreated cells (31).

Telomerase inhibition *in vitro* (Figs. 1–3) did not simply reflect the telomere-shortening activity in intact cells (Fig. 5); MST-199 had the lowest IC_{50} value in an *in vitro* TRAP assay, whereas MST-312 most efficiently induced a telomere crisis of U937 cells even at ~10- to 20-fold lower concentration than those of MST-295 or MST-199. A more striking example would be that the compound 1 was initially found to be a telomerase inhibitor that was comparable to MST-199 *in vitro* (Fig. 1) but did not shorten telomeres in U937 cells at all.[5] These discrepancies might be derived from the differential properties of each compound, including stability in the culture medium, permeability across the cell membrane, and susceptibility to intracellular metabolism. In the case of compound 1, the 5-hydroxy group on the A moiety might critically reduce permeability across the cell membrane and/or enhance susceptibility to intracellular metabolism as compared with MST-199.

Thus far, various compounds have been reported to inhibit telomerase activity *in vitro*. In most cases, however, their ability to shorten telomeres *in vivo* remains to be determined. As control for our experiments, we also examined the effect of a previously reported telomerase inhibitor, 5,10,15,20-tetra-(*N*-methyl-4-pyridyl)porphine (TMPyP4; Refs. 26, 45, and 46) on telomerase activity and telomere length in U937 cells. We confirmed that this compound inhibited telomerase activity more potently than any of our MST compounds *in vitro* (IC_{50} of TMPyP4, 0.16 μM). However, continuous treatment with 5 or 10 μM TMPyP4 for 90 days failed to shorten telomeres in U937 cells probably because of higher cytotoxicity against this cell line.[5] These observations establish the advantage of the compounds described here in term of their ability to induce telomere shortening *in vivo*.

If the mode of action for a telomerase inhibitor is reversible, continuous treatment with such a drug would be necessary to induce telomere crisis in target cells. Actually, if inhibitor addition is terminated, telomeres regain their initial lengths (17). One probable concern that emerges from continuous exposure to a compound is acquired resistance of cells (or the whole body) to the compound. For example, continuous treatment of U937 cells with FJ5002, which we have previously reported as a potent telomerase inhibitor, confers a 4- to 20-fold resistance to the cells (47). In this respect, a telomerase inhibitor that easily allows cancer cells to be resistant would not be desirable for successful induction of telomere crisis. During the long-term cultivation (~90 days) of U937 cells in the presence of MST-312, MST-295, or MST-199, we increased the dose only 2-fold for the induction of telomere crisis. This observation further establishes a striking advantage of these compounds in comparison with the original EGCG, because it was often necessary to increase the dose of EGCG even more than 10-fold for induction of telomere crisis.[5]

Thus far, the precise mechanism by which EGCG and our MST compounds inhibit telomerase activity remains to be determined. It has been postulated that the guanine-rich single-strand telomeric DNA (3′-overhang) can form a characteristic structure called G-quadruplex, at least, *in vitro* and in *Stylonychia lemnae* (48, 49). Some compounds, such as cationic porphyrins (*e.g.,* TMPyP4), stabilize the quadruplex structure and inhibit the extension reaction by telomerase (26, 45). In the TRAP assay, because a G-quadruplex cannot be formed in the TS primer until after several rounds of extension, typical G-quadruplex ligands exhibit little effect on the amounts of the shorter extension products. As shown in Fig. 3*B*, EGCG and our MST compounds reduced the formation of the shorter extension products as well as that of the longer products. Thus, it is unlikely that these compounds are G-quadruplex ligands.

The telomeres are also postulated to participate in processes of chromosomal repairs, as evidenced by the capture or *de novo* synthesis of telomeric repeats at double-strand breaks (50, 51). Recently, this idea has been supported by the finding that telomere dysfunction in late-generation *mTR* knockout mice impairs DNA repair and enhances sensitivity to ionizing radiation (52). The pathogenic link between telomere dysfunction and impaired DNA repair suggests a combination of telomerase inhibitors and DNA-damaging anticancer drugs (including radiotherapy) as a rational strategy for the treatment of cancers. In fact, telomerase inhibition by an anti-hTR antisense strategy has been shown to increase the susceptibility of malignant glioblastoma cells to cisplatin-induced apoptosis (53). Furthermore, the exposure of solid tumor cells to hypoxia, the microenvironmental stress commonly observed in solid tumor masses, results in an increase in telomerase activity that is correlated with an increased resistance to apoptosis (34). On the basis of these observations, it might be worth trying the combination of our compounds and DNA-damaging anticancer drugs to see whether any synergistic effect could be obtained.

[5] Unpublished observations.

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