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RESEARCH ARTICLE

MST-312 induces G2/M cell cycle arrest and apoptosis in APL cells through inhibition of telomerase activity and suppression of NF-κB pathway

Ahmad Fatemi[1] · **Majid Safa**[1] · **Ahmad Kazemi**[1]

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Abstract Telomerase-targeted therapy for cancer has received great attention because telomerase is expressed in almost all cancer cells but is inactive in most normal somatic cells. This study was aimed to investigate the effects of telomerase inhibitor MST-312, a chemically modified derivative of epigallocatechin gallate (EGCG), on acute promyelocytic leukemia (APL) cells. Our results showed that MST-312 exerted a dose-dependent short-term cytotoxic effect on APL cells, with G2/M cell cycle arrest. Moreover, MST-312 induced apoptosis of APL cells in caspase-mediated manner. Telomeric repeat amplification protocol (TRAP) assay revealed significant reduction in telomerase activity of APL cells following short-term exposure to MST-312. Interestingly, MST-312-induced telomerase inhibition was coupled with suppression of NF-κB activity as evidenced by inhibition of IκBα phosphorylation and its degradation and decreased NF-κB DNA binding activity. In addition, gene expression analysis showed downregulation of genes regulated by NF-κB, such as antiapoptotic (survivin, Bcl-2, Mcl-1), proliferative (c-Myc), and telomerase-related (hTERT) genes. Importantly, MST-312 did not show any apoptotic effect in normal human peripheral blood mononuclear cells (PBMCs). In conclusion, our data suggest that dual inhibition of telomerase activity and NF-κB pathway by MST-312 represents a novel treatment strategy for APL.

Keywords APL · MST-312 · Telomerase · NF-κB

✉ Ahmad Kazemi
a.kazemi@iums.ac.ir

[1] Department of Hematology, School of Allied Medical Sciences, Iran University of Medical Sciences, Tehran, Iran

Introduction

Acute promyelocytic leukemia (APL), a unique subtype of acute myelogenous leukemia (AML), is characterized by a specific chromosomal translocation t(15;17), which fuses the promyelocytic leukemia (PML) gene to retinoic acid receptor (RARα) gene. The PML-RARα fusion gene plays a central role in leukemogenesis, including arrest of differentiation and evasion of apoptosis [1, 2]. One of the most important hallmarks of tumor cells is deregulation of signaling pathways that contribute to tumor formation and progression as well as tumor resistance to chemotherapeutic agents. Therefore, identification of the molecular mechanisms that contribute to tumor survival and apoptosis is of great importance for rational development of molecular targeted therapies [3–5].

The telomerase holoenzyme, composed of both RNA subunit (hTR or hTERC) and catalytic subunit (hTERT), maintains telomere length through reverse transcriptase activity [6, 7]. Telomerase activity is suppressed during embryonic differentiation but remains active in highly proliferative cells such as stem cells and germ cells. Reactivation of telomerase has been observed in 90 % of all human cancers, suggesting that its activation is a critical step in human carcinogenicity [8, 9]. Although telomerase overcomes replicative crisis by regulation of telomere length, upregulation of its activity in cancer cells promotes cell proliferation, invasion, and resistance to apoptosis. Increasing evidence has highlighted non canonical (non-telomeric) activities of telomerase that are independent of its conventional function in telomere maintenance [10–12]. These activities included regulation of cell cycle [13], regulation of gene expression [12, 14, 15], inhibition of apoptosis [16–18], and modulation of cellular signaling [19, 20].

The nuclear factor-κB (NF-κB) signaling pathway plays a key role in regulating cellular and developmental events. This pathway is commonly deregulated in human cancers and can

affect main features of cancer such as proliferation, survival, apoptosis resistance, angiogenesis, metastasis, and inflammation through transcriptional activation of the genes involved in these processes [21–23]. Upregulation of anti-apoptotic NF-κB target genes is a key mechanism in evasion of apoptosis in cancers with constitutively activated NF-κB signaling pathway [24, 25]. Intriguingly, the mechanisms underlying the constitutive NF-κB activation are poorly understood and likely to be important in cancer development and progression. Given that both telomerase and NF-κB are hyperactivated in a wide variety of cancers, interaction between them might be a key missing molecular link that less well appreciated.

Telomerase-targeted therapy for cancer has received great attention because telomerase is expressed in almost all cancer cells but is inactive in most normal somatic cells [26]. Although stem cells in regenerative tissues also express telomerase, the effect of telomerase inhibition on these cells seem to be minor because they have much longer initial telomeres compared with cancer cells. Actually, the responsiveness and the time needed for effect of telomerase inhibition on growth arrest or cell death would be different based on initial telomere length of target cells: The longer the telomere, the later the effect [27, 28]. Consistently, cancer cells with varying telomere lengths also indicate different responsiveness to telomerase inhibitors. Therefore, the cells with already short telomeres appear to be ideal target cells for an anti-telomerase therapy [29, 30]. Given that most APL patients have significant shortened terminal restriction fragment (TRF) lengths [31], APL may be a good candidate for a therapeutic intervention with telomerase inhibitors. In order to investigate the efficiency of telomerase inhibition in APL, both APL cell lines NB4 and HL-60 were treated with telomerase inhibitor MST-312.

MST-312 is a chemically modified derivative of epigallocatechin gallate (EGCG), a major catechin of green tea. As compared with EGCG, MST-312 possesses prominent advantages in terms of chemical stability, the effective dose for the induction of telomere shortening, and the circumvention of acquired resistance [32]. Although previous studies have shown that MST-312 inhibits telomerase activity in tumor cells [32–34], its mode of mechanism is yet to be fully understood.

In the present study, we demonstrated that treatment of APL cells with telomerase inhibitor MST-312 is sufficient to induce G2/M cell cycle arrest and apoptosis. Moreover, inhibition of telomerase activity was accompanied with downregulation of NF-κB and NF-κB-regulated gene products.

Materials and methods

Cell culture and MST-312 treatment

APL cell lines, NB4 and HL-60, were cultured in suspension in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10 % fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5 % CO2 at 37 °C. Normal human peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood of healthy donors by density gradient centrifugation on Lymphodex (Inno-Train Diagnostik, Kronberg, Germany) and cultured in the same condition used for APL cells. Stock solution of MST-312 (Sigma-Aldrich, USA) was prepared in dimethyl sulfoxide (DMSO), and suitable working concentration was made from the stock using complete medium. For MST-312 treatment, APL and PBMC cells were treated with relevant amounts of MST-312 working solution to achieve concentrations of 0.5, 1, and 2 μM.

Cell viability measured by trypan blue exclusion assay

To investigate the effect of MST-312 on cell viability, APL cells (NB4 and HL-60) were subjected to trypan blue exclusion assay. Briefly, cells were seeded into a 12-well culture plate at a density of 2×10^5 cells/well and treated with various indicated concentrations of MST-312. After treatment at different incubation times (24, 36, and 48 h), the cell suspension was mixed with 0.4 % trypan blue solution at a 1:1 ratio. After 1–2-min incubation at room temperature, the mixture was loaded onto one chamber of Neubauer hemocytometer and squares of the chamber are observed under a light microscope. The viable/live (clear) and non-viable/dead (blue) cells were counted, and the viability was calculated using the formula (number of live cells counted/total number of cells counted)×100.

Metabolic activity measured by MTT assay

The effect of various concentrations of MST-312 on metabolic activity of APL cells was assayed by the MTT colorimetric method. Briefly, NB4 and HL-60 cells were seeded into a 96-well culture plate (5×10^3 cells/well) and incubated with desired concentrations of MST-312. Thirty-six hours after treatment, the medium was removed and cells were incubated with MTT solution (5 mg/ml in PBS) for 4 h at 37 °C. The resulting formazan crystals were solubilized by addition of 100 μL dimethylsulfoxide (DMSO) at each well, and the absorbance was measured at 570 nm by ELISA reader.

Cell cycle analysis

To explore the effect of MST-312 on cell cycle progression, cells were subjected to DNA content/cell cycle analysis. Briefly, cells were seeded into a 12-well culture plate at a density of 2×10^5 cells/well and incubated in presence of the various indicated concentrations of MST-312 for 36 h. After incubation, cells were harvested, washed twice with PBS and fixed in precooled 70 % ethanol overnight at −20 °C. After washing

with PBS, the cells were treated with 0.5 μg/ml RNase in PBS and incubated at 37 °C for 30 min before staining with 50 μg/ml propiduium iodide (PI) for 30 min. Then the cells were analyzed using a FACScan flow cytometer (Becton Dickinson).

Apoptosis assay

The effect of MST-312 on cell death of APL and PBMC cells was assayed by apoptosis analysis. In brief, cells were seeded into 12-well cell culture plates at a density of 2×10^5 cells/well, and 36 h after MST-312 treatment, the cells were collected. Then the cells were washed with PBS and stained using FITC Annexin V Apoptosis Detection Kit II (BD Biosciences, USA), according to the manufacturer's instructions. The percentage of apoptotic cells was quantified using Becton–Dickinson FACS. Annexin V-positive and PI-negative cells were considered to be in early apoptotic phase, and cells having positive staining both for Annexin-V and PI were deemed to undergo late apoptosis or necrosis.

Telomerase activity assay

The effect of MST-312 on telomerase activity was assayed by the TeloTAGGG Telomerase PCR ELISA kit (Roche, Germany), according to the manufacturer's instructions. The kit method is a photometric enzyme immunoassay for the detection of telomerase activity, utilizing the telomeric repeat amplification protocol (TRAP). Briefly, cells were seeded into 6-well cell culture plates at a density of 5×10^5 cells/well, and 36 h after MST-312 treatment, the cells were harvested. For protein extraction, 2×10^5 cells were lysed in lysis buffer and the protein extracts were subjected to TRAP assay. The kit includes specific telomere primers bound to biotin, which allows measuring the PCR-amplified telomerase products (and the telomerase activity) by ELISA. In addition, the PCR products were also run through 12 % polyacrylamide gel electrphoresis (PAGE) and the ladder was visualized by staining with silver nitrate.

Western blot analysis

Cell suspensions were centrifuged at 36 h after MST-312 treatment, and cell pellets were washed with cold PBS and lysed (5×10^6 cells/aliquots) in 0.2 mL of RIPA buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 % Triton X-100, 0.1 % sodium dodecyl sulfate, and 0.5 % sodium deoxycholate) containing protease and phosphatase inhibitor cocktails (Sigma). The cell lysate was centrifuged at 13, 000 rpm for 20 min at 4 °C, and the supernatant was collected. Total protein of the supernatant was quantified using the Bradford protein assay, and equal amounts of total cellular protein were separated by 10 % SDS–PAGE, according to the method of Laemmli. The resolved proteins were then transferred from the gels to nitrocellulose membranes (Hybond-ECL, Amersham Corp). Afterward, membranes were incubated in blocking buffer (1×Tris buffered saline [TBS], 0.1 % Tween-20 with 5 % nonfat dry milk) for 1 h and probed overnight at 4 °C with specific primary antibodies (Cell Signaling Technology, UK) against cleaved caspase-3 (9664), caspase-9 (9502), cleaved PARP (5625), phospho-IκB (2859), and IκBα (4814). After washing five times in 1×TBS containing 0.1 % Tween-20 (TBST), membranes were incubated with horse radish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. The immunoreactive proteins were then visualized with a chemiluminescence detection system (Amersham ECL Advance Kit, GE Healthcare) according to the manufacturer's protocol.

Nuclear fragmentation

The preparation of nuclear extracts was performed using the Nuclear Extract Kit (Active Motif, Carlsbad, CA), according to the manufacturer's instructions. In brief, APL cells were centrifuged at 36 h after MST-312 treatment and cellular pellets were washed with 1 ml ice-cold PBS/phosphatase inhibitors, lysed in 250 μl hypotonic buffer, and then centrifuged at 14,000 rpm for 30 s at 4 °C. The cell pellets were resuspended in 30 μl complete lysis buffer and centrifuged at 14,000 rpm for 10 min at 4 °C. Finally, the supernatants (nuclear fraction) were saved.

NF-κB DNA-binding assay

The DNA-binding activity of NF-κB was quantified in the APL cells by TransAM™ NF-κB p65 Transcription Factor Assay Kit (Active Motif), according to the manufacturer's instructions. Briefly, equal amounts of the nuclear extracts were incubated in 96-well plates coated with immobilized oligonucleotide (5 -AGTTGAGGGGACTTTCCCAGGC-3) containing a consensus (5 -GGGACTTTCC-3) binding site for the p65 subunit of NF-κB. NF-κB binding to the target oligonucleotide was detected by incubation with primary antibody specific for p65 subunit and HRP-conjugated secondary antibody. For quantification of NF-κB activity, the optical densities were measured at 450 nm by a microplate reader.

RNA isolation and preparation of cDNA

RNA from APL cells (NB4 and HL-60) was isolated at 36 h after treatment with MST-312 by using TriPure Isolation Reagent according to the instructions of the manufacturer (Roche). Reverse transcription (RT) reaction was performed using the RevertAid First Strand complementary DNA (cDNA) Synthesis kit from Fermentas. A 20-μl reaction contained 9-μl nuclease-free water, 1 μl Random Hexamer

Primer, 4 μl 5× Reaction Buffer, 2 μl dNTP Mix (10 mM), 1 μl RiboLock RNase Inhibitor (20 U/μl), and 1 μl RevertAid M-MuLV Reverse Transcriptase (200 U/μl) as a master mix, and 2 μl of total RNA (1 μg per reaction) was added prior to reaction start. Adapted times and temperature profiles for the reverse transcription were used: incubation 5 min at 25 °C followed by 60 min at 42 °C. The reaction was terminated by heating at 70 °C for 5 min.

Quantitative real-time PCR

Real-time PCR was performed using 7.5 μl of RealQ Plus 2× Master Mix Green (Ampliqon), 1.5 μl of the cDNA product, 1 μl of each of forward and reverse primers (10 pmol), and 4 μL of nuclease-free water in a total volume of 15 μl. Thermal cycling conditions included an initial activation step at 95 °C for 15 min followed by 40 cycles including a denaturation step at 95 °C for 15 s and a combined annealing/elongation step at 60 °C for 60 s. The reaction took place in the Rotor Gene 6000 Real-time PCR System (Corbett Research). A melting curve analysis was performed to verify the specificity of the products. The fold induction or repression was measured relative to the control and calculated after adjusting for the GAPDH reference gene using the comparative Ct ($2^{-\Delta\Delta CT}$) method. Primer sequences are available upon request.

Statistical analysis

Two-tailed Student's *t* test was used to determine if there is a significant difference between experimental variables. A *P* value <0.05 was considered statistically significant.

Results

MST-312 decreases cell viability and metabolic activity of APL cells

The effect of various concentrations of MST-312 on cell viability of APL cells was assessed by trypan blue exclusion assay. As shown in Fig. 1a, the viability of NB4 and HL-60 cells after exposure to MST-312 was reduced substantially in a dose- and time-dependent manner. Over 50 % decrease in the

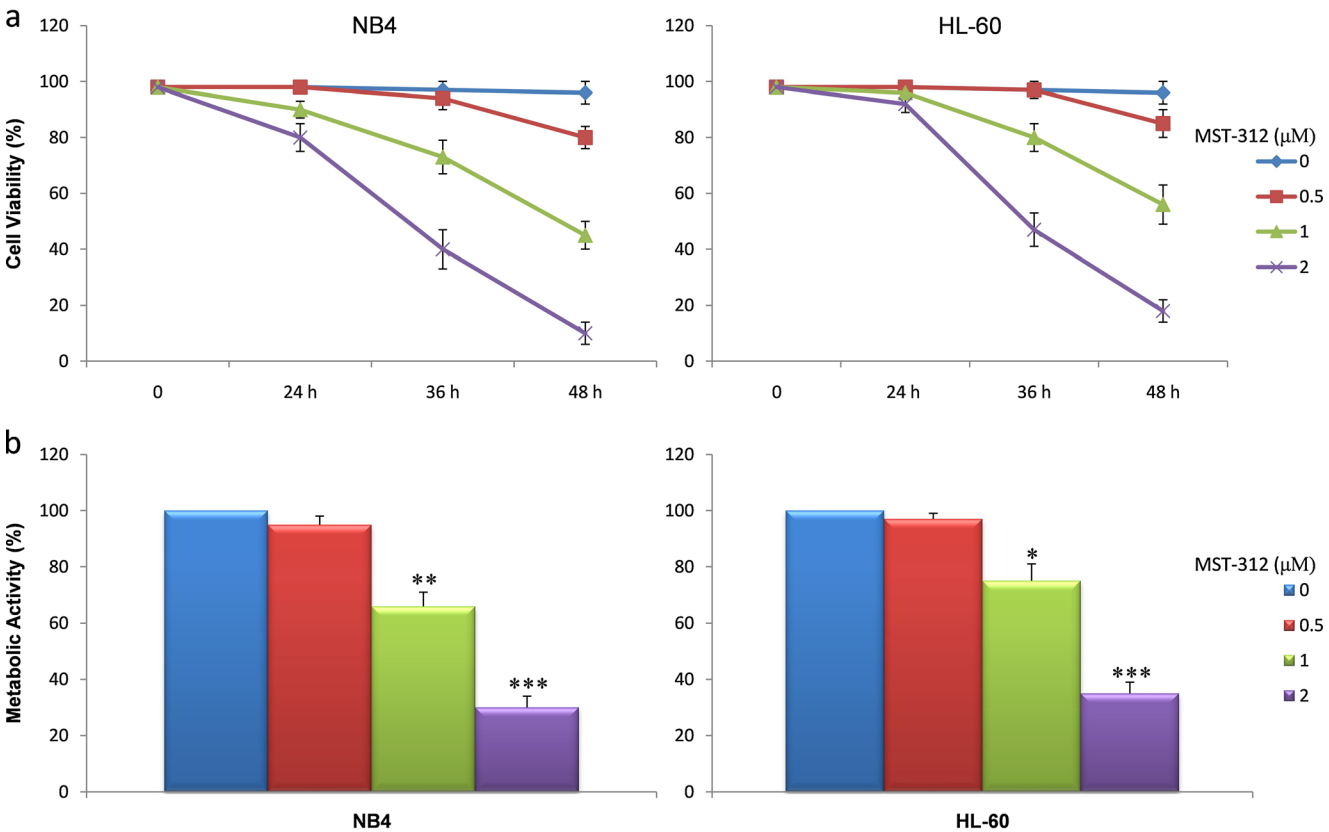

Fig. 1 MST-312 decreases cell viability and metabolic activity of APL cells. **a** APL cells (NB4 and HL-60) were exposed with various concentrations of MST-312, and cell viability was assessed by trypan blue exclusion assay after different times of exposure (mean±SE, *n*=3). The viability of APL cells after short-term exposure to MST-312 was reduced substantially in a dose-dependent manner. **b** APL cells were treated with various concentrations of MST-312 and metabolic activity was assessed using MTT assay 36 h after treatment (mean±S.E., *n*=3). The metabolic activity of APL cells exposed to MST-312 was significantly decreased in a dose-dependent manner (**P*<0.05; ***P*<0.01, ****P*<0.001, relative to cells untreated with MST-312)

viability of treated cells was seen in 2 μM concentration of MST-312 within 36 h, indicating a direct short-term cytotoxic effect of MST-312 on APL cells. With consideration of severe decrease in cell viability following longer exposure of MST-312, the short-term exposure of 36 h was used in the following experiments. To investigate the effect of MST-312 on metabolic activity, APL cells were subjected to MTT colorimetric method. As presented in Fig. 1b, cell metabolic activity which is related to the number of viable cells was significantly decreased in APL cells treated with MST-312. The cytotoxic effect of MST-312 was concentration-dependent, with approximately 5, 34, and 70 % reduction in metabolic activity for NB4 cells and about 3, 25, and 65 % reduction for HL-60 cells after 36 h exposure to 0.5, 1, and 2 μM of MST-312, respectively. Taken together, these findings suggest that MST-312 could exert a dose-dependent short-term cytotoxic effect on APL cells. In addition, NB4 cells appeared to be more sensitive to cytotoxic effect of MST-312, compared with HL-60 cells.

MST-312 leads to G2/M cell cycle arrest and increased apoptotic sub-G1 population in APL cells

To better understand the mechanisms underlying the cytotoxic effect of MST-312 on APL cells, we examined the effect of MST-312 on the cell cycle. As illustrated in Figs. 2 and 3, there was a significant increase in the number of cells at the G2/M phase in both NB4 and HL-60 cells treated with 1 μM concentration of MST-312 compared with control (untreated) cells ($P<0.05$). Furthermore, the proportion of sub-G1 apoptotic cells was also significantly increased after MST-312 treatment ($P<0.05$, 1 μM MST-312-treated cells compared with untreated cells). Taken together, these findings suggest

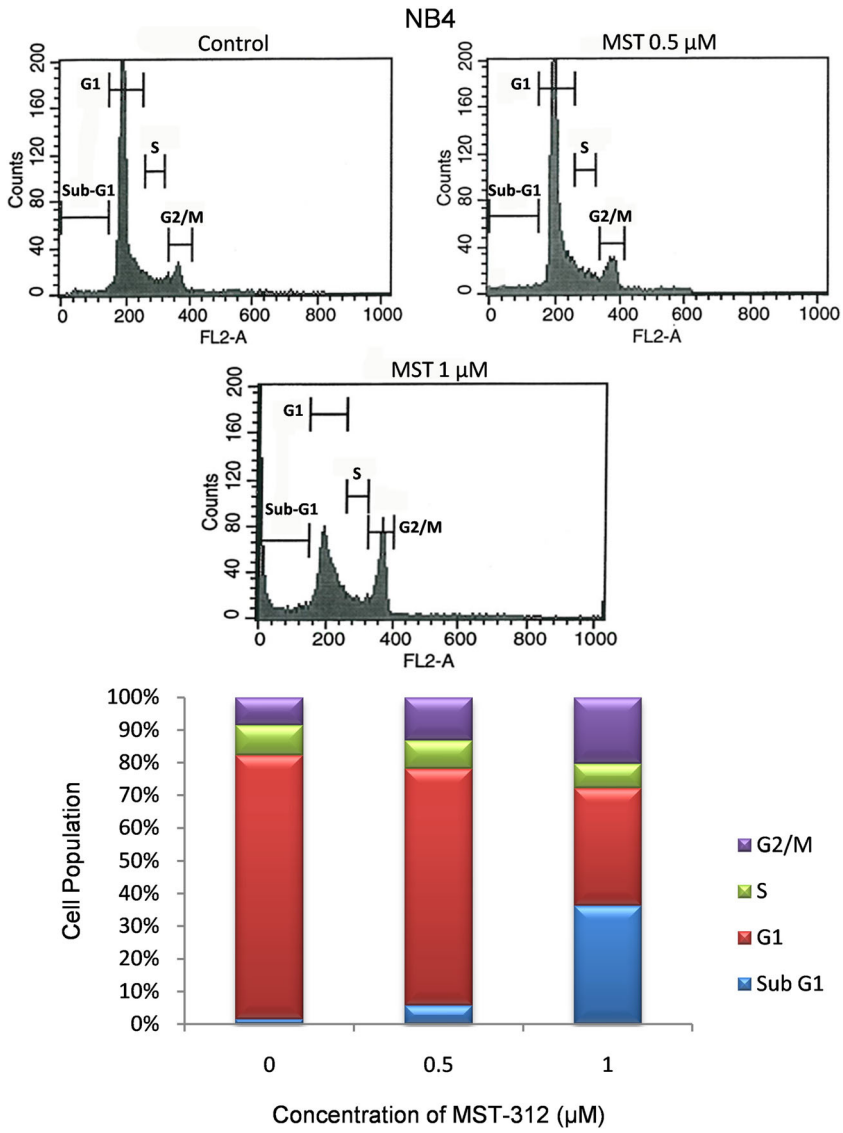

Fig. 2 MST-312 leads to G2/M cell cycle arrest and increased apoptotic sub-G1 population in NB4 cells. NB4 cells were treated with various concentrations of MST-312 for 36 h. The changes in cell cycle phase distribution were assessed by cell cycle analysis. One representative experiment among three independent assays is shown

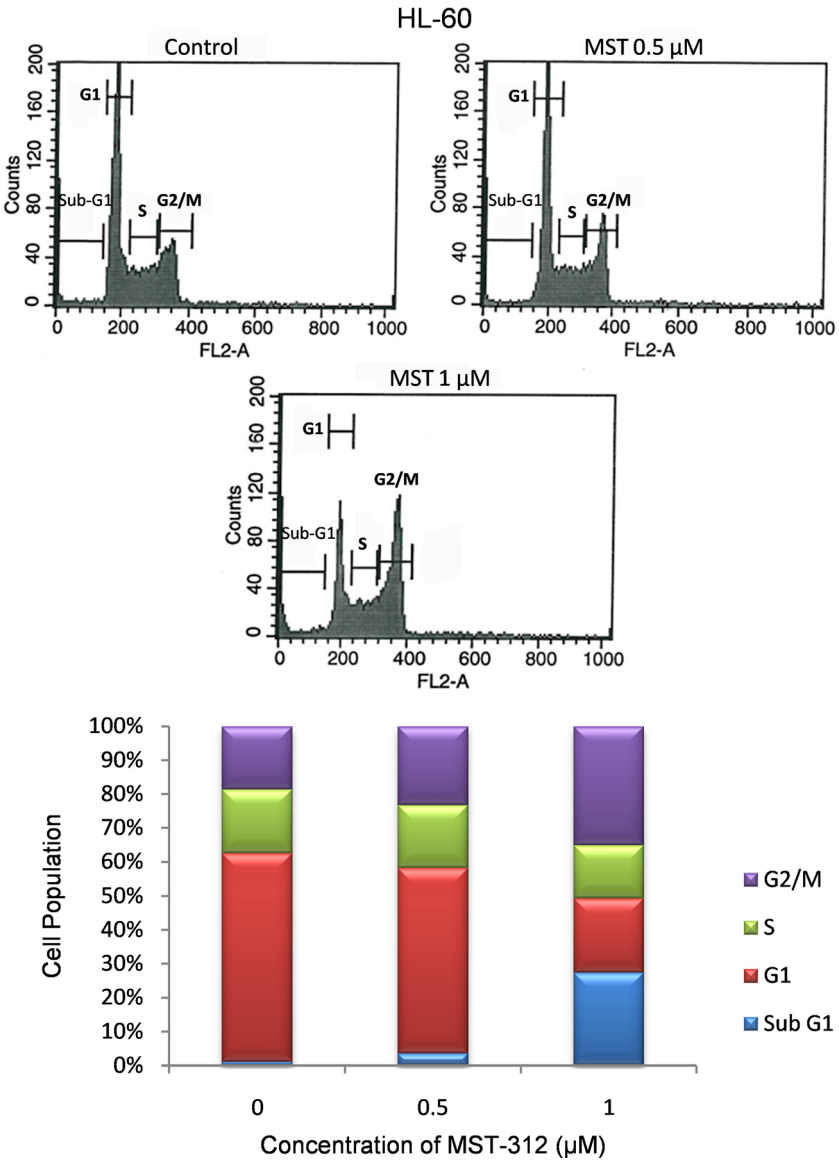

Fig. 3 G2/M cell cycle arrest and increased apoptotic sub-G1 population in HL-60 cells treated with MST-312. HL-60 cells were treated with various concentrations of MST-312 for 36 h. The changes in cell cycle phase distribution were assessed by cell cycle analysis. One representative experiment of three performed is presented

that MST-312 arrests APL cells at G2/M phase, thereby inhibiting cell proliferation.

MST-312 promotes apoptosis in APL cells but not in normal human PBMCs

In order to determine the effect of MST-312 on cell apoptosis, APL cells were analyzed for Annexin-V binding and Annexin-V combined with propiduium iodide (PI) by FITC Annexin V Apoptosis Detection Kit II. As shown in Figs. 4 and 5, short-term incubation of APL cells with MST-312 substantially increased percentages of Annexin-V and Annexin-V/PI double-positive cells in comparison to the untreated cells, indicating acute apoptotic effect of MST-312 on APL cells. The apoptotic effect was dose-dependent, with approximately 34 % (total percentages of Annexin-V and Annexin-V/PI double-positive cells) increase in apoptosis for NB4 cells and 25 % increase for HL-60 cells after short-term (36 h) exposure to l µM MST-312. This concentration of MST-312 was used in the following experiments.

To investigate the effect of MST-312 on cell death of normal cells, human PBMCs were isolated, treated, and analyzed by FITC Annexin-V Apoptosis Detection Kit II. As presented in Fig. 6, we did not observe any apoptotic effect of MST-312 on PBMCs using concentrations up to 2 µM in 36 h incubation.

MST-312 induces caspase activation in APL cells

To further assessment of apoptosis, we examined the effect of MST-312 on caspase activation. APL cells were treated with MST-312 for 36 h and then the

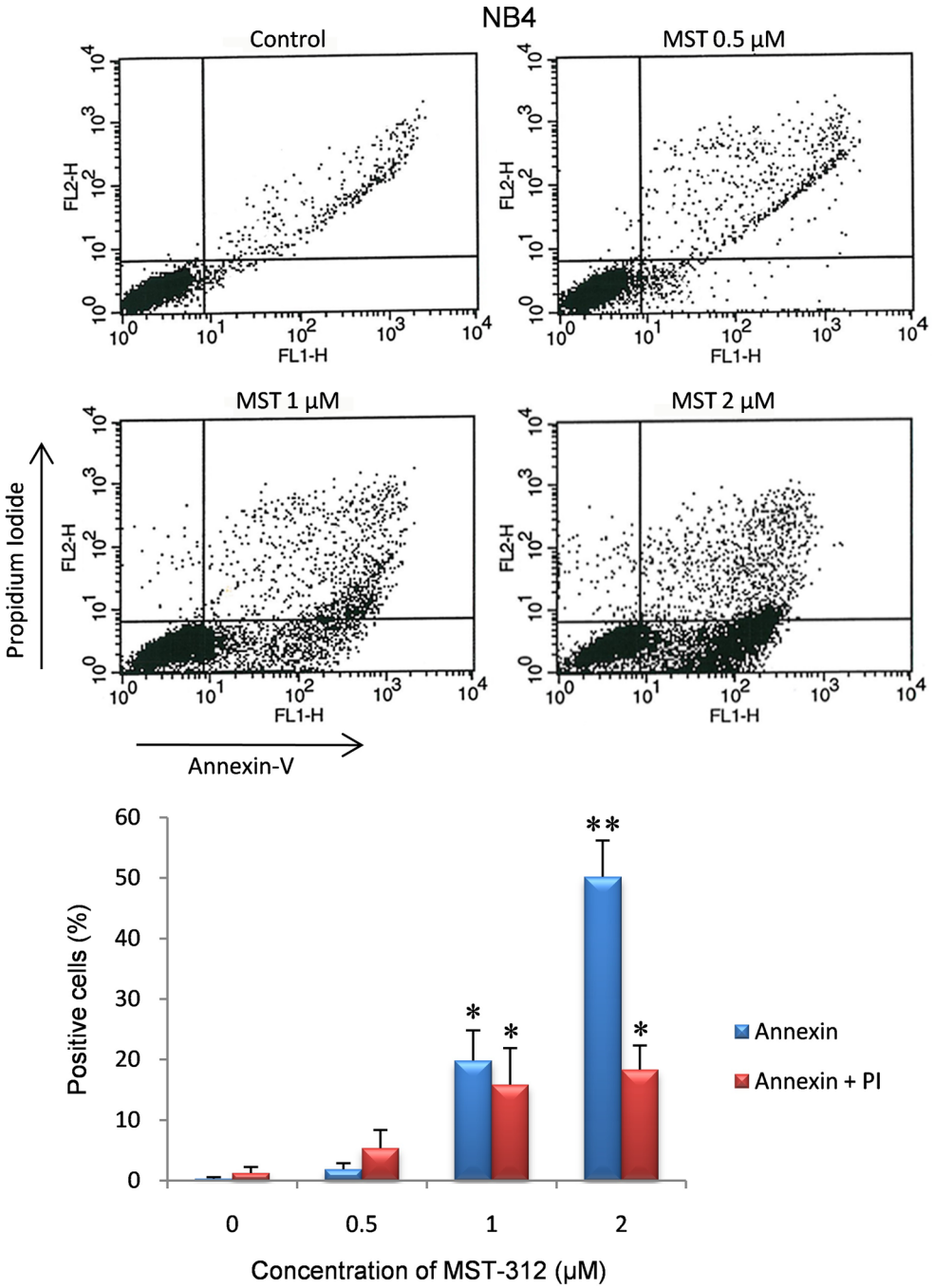

Fig. 4 Short-term (acute) apoptotic effect of MST-312 on NB4 cells. NB4 cells were treated with various concentrations of MST-312 for 36 h. Then, cells were analyzed for Annexin-V and Annexin-V plus PI uptake by flow cytometry. One representative experiment of three performed is shown (n=3, *P<0.05; **P<0.01, relative to cells untreated with MST-312)

expression of cleaved products of caspase-3, caspase-9, and poly(ADP-ribose) polymerase (PARP) were assessed using Western blot analysis. PARP is one of proteins undergoing cleavage following caspase-3 activation. As presented in Fig. 7, activation of caspase-3 and caspase-9 and cleavage of PARP were observed in response to MST-312. These findings indicated that MST-312 induces apoptosis of APL cells in caspase-mediated manner.

MST-312-induced telomerase inhibition is coupled with suppression of the NF-κB activity

The effect of MST-312 on telomerase activity of APL cells was assessed by TRAP assay. As shown in Fig. 8a, approximately 42 % reduction in telomerase activity for NB4 cells and about 34 % reduction for HL-60 cells were observed after 36 h exposure to 1.0 μM of MST-312. To ascertain the mechanisms by which MST-312-induced telomerase inhibition

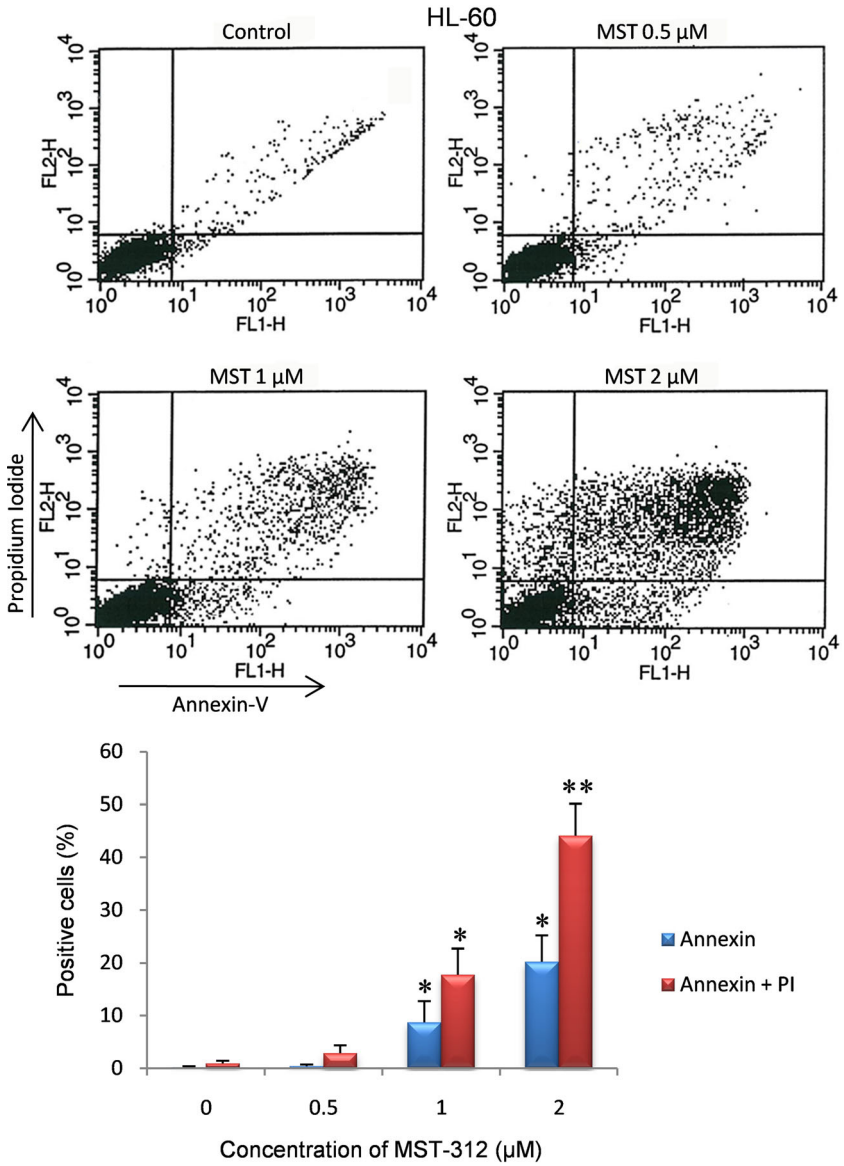

Fig. 5 MST-312 induces acute apoptosis in HL-60 cells. HL-60 cells were treated with different concentrations of MST-312 for 36 h. Then, cells were analyzed for Annexin-V and Annexin-V plus PI uptake by flow cytometry. One representative experiment among three independent assays is shown (n=3, *P<0.05; **P<0.01, relative to cells untreated with MST-312)

exerts short-term (acute) growth arrest and apoptosis, we investigate the effect of MST-312 on NF-κB activity. Most stimuli activate NF-κB by IKK-mediated IκBα phosphorylation and its degradation by the ubiquitin–proteasome pathway allowing nuclear translocation of NF-kB and its subsequent binding to DNA. Therefore, in order to investigate the NF-κB activity, APL cells were treated with 1.0 μM of MST-312, harvested after 36 h and then subjected to Western blotting by the antibodies directed toward IκBα and phospho-IκB (at Ser32). Western blot results revealed decreased expression of phospho-IκB and increased expression of IκBα in treated APL cells compared with untreated cells, indicating MST-312-induced suppression of NF-κB activity in APL cells (Fig. 8c). In addition, to verify whether NF-κB DNA-binding activity is also repressed by MST-312, APL cells were subjected to NF-κB DNA-binding assay. As presented in Fig. 8d, the DNA binding of NF-κB decreased within 36 h following the MST-312 treatment. Taken together, these findings suggest that MST-312-induced telomerase inhibition is accompanied with suppression of NF-κB activity.

MST-312-induced telomerase inhibition is accompanied with downregulation of NF-κB-regulated anti-apoptotic genes

With consideration of MST-312-induced suppression of NF-κB activity, we then examined whether MST-312 affected anti-apoptotic genes regulated by NF-κB. To this end, the APL cells were treated with MST-312, harvested for 36 h after treatment and then analyzed for the expression of survivin, Bcl-2 and Mcl-1 anti-apoptotic genes using quantitative real-time PCR. As shown in Fig. 9, significant decreased expression of these

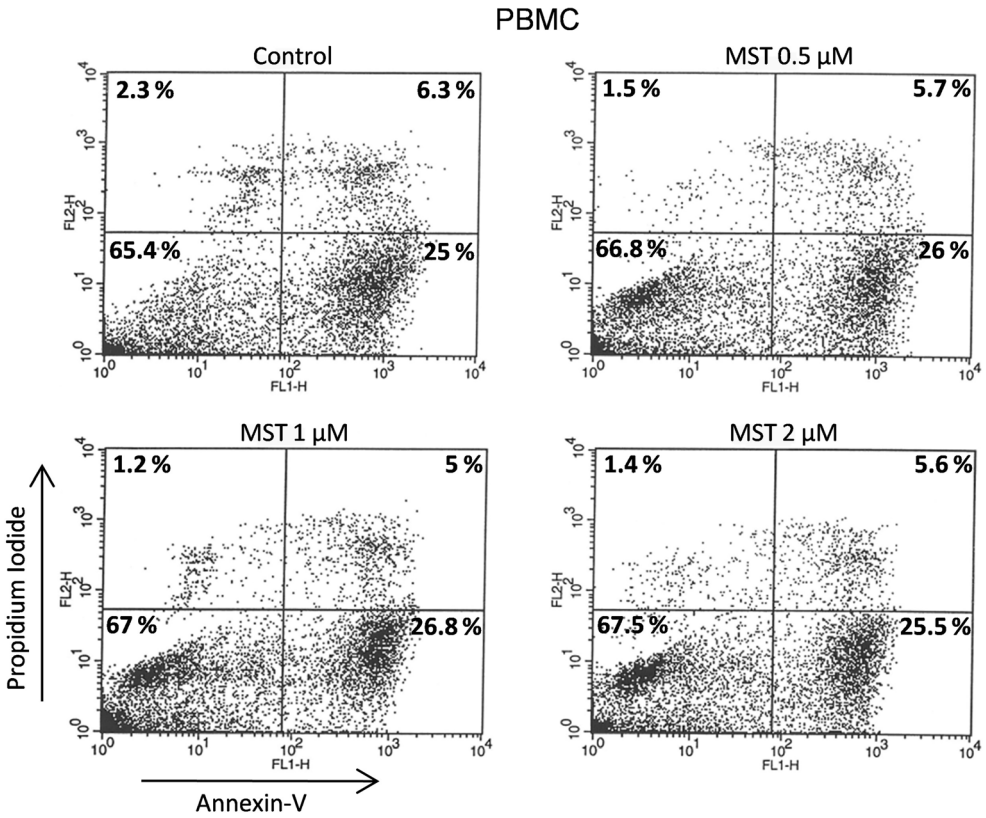

Fig. 6 MST-312 does not induce cell death in normal human PBMCs. PBMCs were treated with various concentrations of MST-312 for 36 h. Then, cells were analyzed for Annexin-V and Annexin-V plus PI uptake by flow cytometry. One representative experiment of three performed is shown

anti-apoptotic genes was seen in APL cells treated with MST-312, compared with untreated cells. This finding revealed MST-312-induced downregulation of anti-apoptotic NF-κB target genes in APL cells, thereby promoting apoptosis.

MST-312 downregulates the expression of c-Myc and hTERT genes

Telomerase regulation occurs mainly at the level of transcription of hTERT [35]. A growing body of data indicates that various transcription factors act on the hTERT promoter, resulting in regulation of its expression [36]. Among the transcription factors, c-Myc is thought to be a strong regulator of hTERT gene transcription [37]. On the other hand, both c-Myc and hTERT are target genes of NF-κB [38–40]. With consideration of inhibitory effect of MST-312 on NF-κB activity, we wished to investigate the transcriptional alterations of hTERT and c-Myc following MST-312 treatment. As presented in Fig. 10, there was a significant decrease in hTERT and c-Myc gene expression following 1.0 μM MST-312 treatment for 36 h. Taken together, MST-312-induced downregulation of c-Myc and hTERT and subsequent decreased telomerase activity may enhance the telomerase inhibitory effect of MST-312.

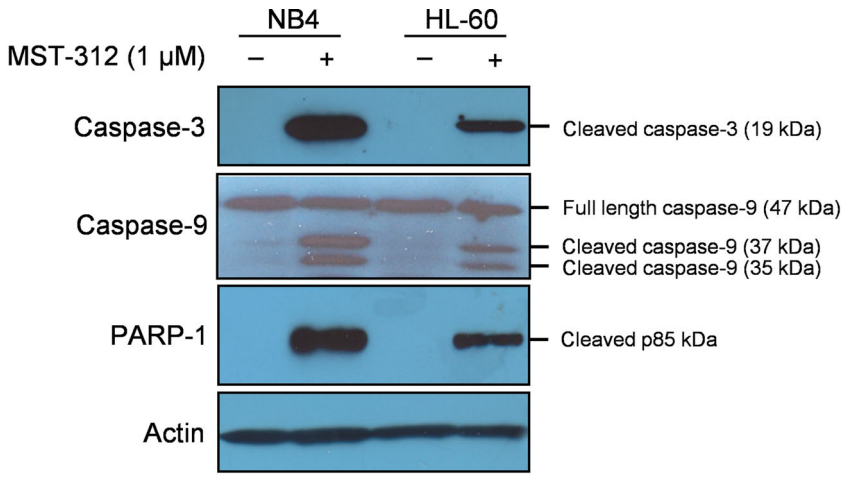

Fig. 7 MST-312-induced caspase activation in APL cells. APL (NB4 and HL-60) cells were treated with 1 μM concentration of MST-312 for 36 h. Then, equal amounts of cell lysates (80 μg) were subjected to electrophoresis and analyzed by Western blot for cleavage of caspase-3, caspase-9, and PARP. Actin serves as loading control. The immunoblot is representative of three independent experiments

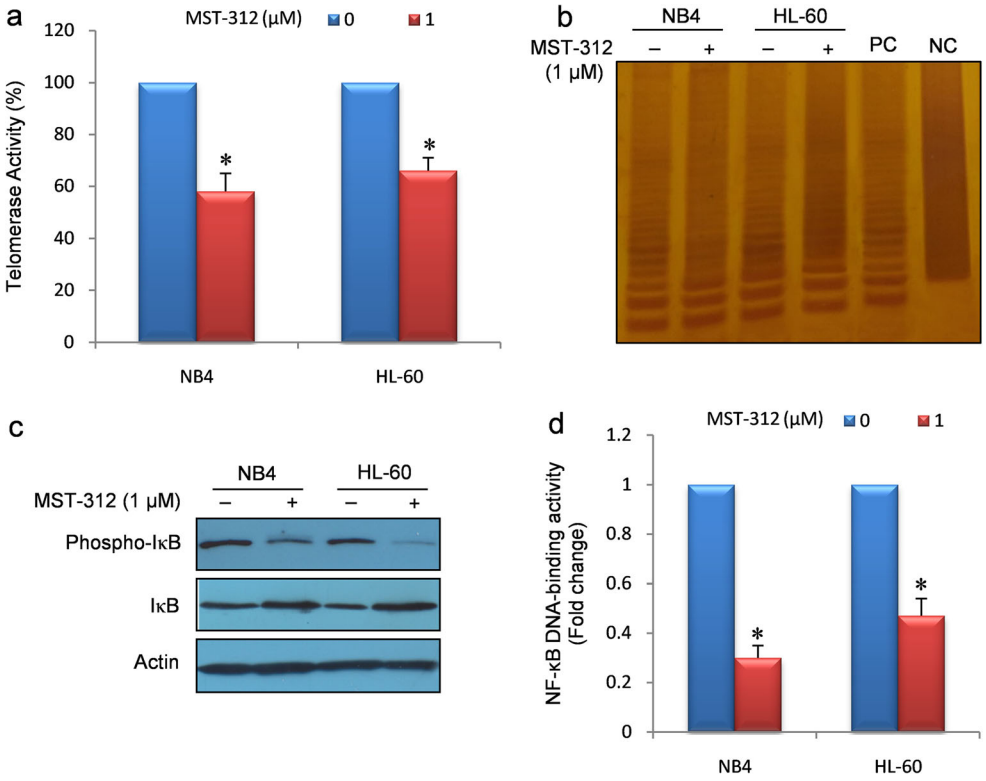

Fig. 8 MST-312-induced telomerase inhibition is coupled with suppression of NF-κB activity. **a** APL (NB4 and HL-60) cells were treated with 1 μM MST-312. After 36 h, cells were harvested and telomerase activity was measured by PCR/ELISA-based TRAP assay using TeloTAGGG Telomerase PCR ELISA kit, according to the manufacturer's instructions. Percentage of telomerase inhibition was calculated by comparing telomerase activity of MST-312-treated cells with telomerase activity of untreated cells. **b** PCR products of TRAP assay were run through 12 % polyacrylamide gel electrphoresis (*PAGE*) and the ladder was visualized by staining with silver nitrate. An extract of the telomerase-positive cell line 293 as positive control (*PC*) and a cell extract treated with DNase-free RNase as negative control (*NC*) were included in experiment. **c** APL cells were treated with 1 μM concentration of MST-312. After 36 h, cells were harvested and subjected to immunoblot analysis using IκB antibody and phospho-specific antibody against IκB phosphorylated at Ser32. As a loading control, actin level was determined. One representative experiment of three is shown. **d** APL cells were treated with 1 μM MST-312 for 36 h. Then, cells were harvested and the nuclear fraction was separated using Nuclear extraction kit. NF-κB DNA binding activity was quantified by enzyme-linked immunosorbent assay using the TransAM NF-B p65 transcription factor assay kit, according to the manufacturer's instructions. Values represented as mean±S.E. (n=3, *P<0.05, relative to cells untreated with MST-312)

Discussion

Since telomerase is constitutively expressed in the vast majority of human cancers, telomerase-targeted therapy has been considered as one of the most promising approaches for cancer treatment [26]. In this setting, various types of anti-telomerase drugs are now in phase I and II clinical trials [41, 42]. MST-312 is a telomerase inhibitor that has emerged as a promising candidate for telomerase-targeted therapy [32]. Previous studies in brain and lung tumors have indicated that there are two different effects for MST-312, depending on the time of exposure [34, 43]. Acute (short-term) effect, which occurs following short-term (72 h) exposure with MST-312, leads to DNA damage, ATM-dependent G2/M cell cycle

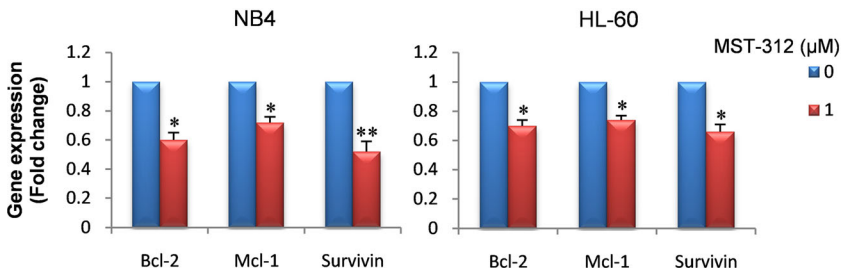

Fig. 9 MST-312 decreases the expression of antiapoptotic genes regulated by NF-κB. APL (NB4 and HL-60) cells were treated with 1 μM MST-312 for 36 h. Subsequently, RNA was isolated and cDNA synthesis was performed. The result of Sybr-green real-time PCR using specific primers has been shown. All values were normalized to GAPDH (n=3, *P<0.05; **P<0.01, relative to cells untreated with MST-312)

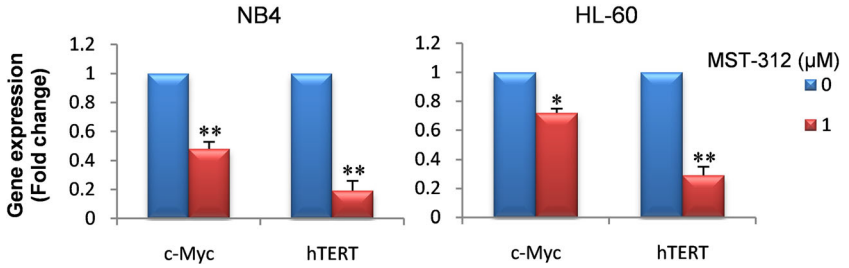

Fig. 10 MST-312 downregulates c-Myc and hTERT expression. After treatment of APL (NB4 and HL-60) cells with 1 μM MST-312 for 36 h, RNA was extracted and cDNA synthesis was done. The result of Sybr-green real-time PCR using specific primers has been shown. All values were normalized to GAPDH (n=3, *P<0.05; **P<0.01, relative to cells untreated with MST-312)

arrest, and reduced cell viability. This effect is independent of telomere erosion and mediated by uncoupling of the telomerase complex from the telomere DNA which is then recognized as DNA-double strand breaks by the ATM-pathway. Long-term (more than 1.5 months) treatment with MST-312 leads to a long-term effect, with significant telomere shortening. Consistent with previous findings, we also indicated that short-term treatment with MST-312 induced G2/M cell cycle arrest with decreased cell viability in APL cells (Figs. 1, 2, and 3). In addition, we observed increased apoptotic cells (Sub-G1 population) following acute telomerase inhibition (Figs. 2 and 3). Furthermore, our apoptosis analysis confirmed short-term apoptotic effect of MST-312 on APL cells (Figs. 4 and 5). However, no such effects in terms of cytotoxicity and apoptosis were observed in normal human PBMCs (Fig. 6), suggesting tumor-selective growth inhibitory effect of MST-312. Similar to MST-312, telomerase inhibition using anti-sense oligonucleotides [44, 45] or G-quadruplex compounds [46] also exerts short-term cytotoxic effects on tumor cells, independent of critical telomere erosion. Since the acute effects of telomerase inhibition are unrelated to telomere attrition, this suggests telomerase might have functions in addition to telomere maintenance [47].

Increasing evidence has highlighted non-canonical (non-telomeric) activities of telomerase that are independent of its conventional function in telomere maintenance [10–12]. These activities included regulation of cell cycle and gene expression [12–15], inhibition of apoptosis [16–18], and modulation of cellular signaling such as NF-κB [19, 20]. To clarify the mechanisms by which telomerase inhibition exerts acute growth arrest and apoptosis, the effect of telomerase inhibitor on the NF-κB signaling, a critical pro-survival pathway, was examined. We demonstrated here for the first time that the telomerase inhibitor MST-312 has an anti-NF-κB activity on APL cells. Our Western blot analysis showed suppression of NF-κB activity following short-term administration of MST-312, through decrease of IκB phosphorylation and its subsequent degradation (Fig. 8c). In addition, we verified that NF-κB DNA-binding activity also is repressed by MST-312 (Fig. 8d), resulting in supression of genes regulated by NF-κB such as antiapoptotic (survivin, Bcl-2, Mcl-1), proliferative (c-Myc), and telomerase-related (hTERT) genes (Figs. 9 and 10). Repression of NF-κB signaling and subsequent downregulation of the pro-survival genes may be a possible explanation for MST-312-induced acute growth arrest and apoptosis.

Deregulation and overexpression of both NF-κB and telomerase has been frequently found in human malignancies. Earlier studies have implied upregulation of hTERT expression by NF-κB [38, 39]. On the other hand, recent study performed by Ghosh et al. suggested that hTERT directly regulates NF-κB-dependent gene expression by binding to p65 and recruiting to a subset of NF-κB promoters, such as those of IL-6, TNF-α, and IL-8 [19]. Importantly, Ding et al. recently showed that hTERT regulates the expression of NF-κB target genes independent of its telomerase activity [48]. Interestingly, it has been shown that MST-312 has no effect on NF-κB signaling in telomerase-negative cells indicating the telomerase-specific effect of this compound [19]. Thus, the most straightforward interpretation of our results is that telomerase-dependent NF-kB suppression induced by MST-312 may contribute in apoptosis of APL cells.

In conclusion, we demonstrated that the telomerase inhibitor MST-312 causes acute growth arrest and apoptosis in APL cells through suppression of NF-κB activity. In addition, our findings reinforce the notion that telomerase served as a modulator of the NF-κB signaling pathway, in a manner independent of its activity in telomere maintenance. Therefore, telomerase-targeted therapy by MST-312 which targets both telomeric (telomere maintenance) and non-telomeric (NF-κB activation) activity of telomerase, may provide a promising approach for treatment of APL and other telomerase-positive cancers.

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Conflicts of interest None

References

1. Stein EM, Tallman MS. Acute promyelocytic leukemia in children and adolescents. Acta Haematol. 2014;132(3-4):307–12.

2. Chen Z, Wang Z-Y, Chen S-J. Acute promyelocytic leukemia: cellular and molecular basis of differentiation and apoptosis. Pharmacol Ther. 1997;76(1):141–9.
3. Testa U, Riccioni R. Deregulation of apoptosis in acute myeloid leukemia. Haematologica. 2007;92(1):81–94.
4. Fulda S. Evasion of apoptosis as a cellular stress response in cancer. International journal of cell biology. 2010;2010.
5. Hassan M, Watari H, AbuAlmaaty A, Ohba Y, Sakuragi N. Apoptosis and molecular targeting therapy in cancer. BioMed research international. 2014;2014.
6. Verdun RE, Karlseder J. Replication and protection of telomeres. Nature. 2007;447(7147):924–31.
7. Shay JW, Wright WE. Senescence and immortalization: role of telomeres and telomerase. Carcinogenesis. 2005;26(5):867–74.
8. Harley CB. Telomerase and cancer therapeutics. Nat Rev Cancer. 2008;8(3):167–79.
9. Low KC, Tergaonkar V. Telomerase: central regulator of all of the hallmarks of cancer. Trends Biochem Sci. 2013;38(9):426–34.
10. Chang S, DePinho RA. Telomerase extracurricular activities. Proc Natl Acad Sci. 2002;99(20):12520–2.
11. Martínez P, Blasco MA. Telomeric and extra-telomeric roles for telomerase and the telomere-binding proteins. Nat Rev Cancer. 2011;11(3):161–76.
12. Smith LL, Coller HA, Roberts JM. Telomerase modulates expression of growth-controlling genes and enhances cell proliferation. Nat Cell Biol. 2003;5(5):474–9.
13. Xiang H, Wang J, Mao Y, Liu M, Reddy VN, Li D. Human telomerase accelerates growth of lens epithelial cells through regulation of the genes mediating RB/E2F pathway. Oncogene. 2002;21(23):3784–91.
14. Kirkpatrick KL, Newbold RF, Mokbel K. The mRNA expression of hTERT in human breast carcinomas correlates with VEGF expression. J Carcinogen. 2004;3(1):1.
15. Sharma GG, Gupta A, Wang H, Scherthan H, Dhar S, Gandhi V, et al. hTERT associates with human telomeres and enhances genomic stability and DNA repair. Oncogene. 2003;22(1):131–46.
16. Lee J, Sung Y, Cheong C, Choi Y, Jeon H, Sun W, et al. TERT promotes cellular and organismal survival independently of telomerase activity. Oncogene. 2008;27(26):3754–60.
17. Massard C, Zermati Y, Pauleau A, Larochette N, Metivier D, Sabatier L, et al. hTERT: a novel endogenous inhibitor of the mitochondrial cell death pathway. Oncogene. 2006;25(33):4505–14.
18. Rahman R, Latonen L, Wiman KG. hTERT antagonizes p53-induced apoptosis independently of telomerase activity. Oncogene. 2004;24(8):1320–7.
19. Ghosh A, Saginc G, Leow SC, Khattar E, Shin EM, Yan TD, et al. Telomerase directly regulates NF-κB-dependent transcription. Nat Cell Biol. 2012;14(12):1270–81.
20. Park J-I, Venteicher AS, Hong JY, Choi J, Jun S, Shkreli M, et al. Telomerase modulates Wnt signalling by association with target gene chromatin. Nature. 2009;460(7251):66–72.
21. Jost PJ, Ruland J. Aberrant NF-κB signaling in lymphoma: mechanisms, consequences, and therapeutic implications. Blood. 2007;109(7):2700–7.
22. Cilloni D, Martinelli G, Messa F, Baccarani M, Saglio G. Nuclear factor κB as a target for new drug development in myeloid malignancies. Haematologica. 2007;92(9):1224–9.
23. Baud V, Karin M. Is NF-κB a good target for cancer therapy? Hopes and pitfalls. Nat Rev Drug Discov. 2009;8(1):33–40.
24. Dutta J, Fan Y, Gupta N, Fan G, Gelinas C. Current insights into the regulation of programmed cell death by NF-κB. Oncogene. 2006;25(51):6800–16.
25. Nagel D, Vincendeau M, Eitelhuber A, Krappmann D. Mechanisms and consequences of constitutive NF-κB activation in B-cell lymphoid malignancies. Oncogene. 2014.
26. Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, et al. Specific association of human telomerase activity with immortal cells and cancer. Science. 1994;266(5193):2011–5.
27. Zhang X, Mar V, Zhou W, Harrington L, Robinson MO. Telomere shortening and apoptosis in telomerase-inhibited human tumor cells. Genes Dev. 1999;13(18):2388–99.
28. Herbert B-S, Pitts A, Baker S, Hamilton S, Wright W, Shay J, et al. Inhibition of human telomerase in immortal human cells leads to progressive telomere shortening and cell death. Proc Natl Acad Sci. 1999;96(25):14276–81.
29. Röth A, Dürig J, Himmelreich H, Bug S, Siebert R, Dührsen U, et al. Short telomeres and high telomerase activity in T-cell prolymphocytic leukemia. Leukemia. 2007;21(12):2456–62.
30. Blackburn EH. Telomere states and cell fates. Nature. 2000;408(6808):53–6.
31. Ghaffari S, Shayan-Asl N, Jamialahmadi A, Alimoghaddam K, Ghavamzadeh A. Telomerase activity and telomere length in patients with acute promyelocytic leukemia: indicative of proliferative activity, disease progression, and overall survival. Annals of Oncology. 2008:mdn394.
32. Seimiya H, Oh-hara T, Suzuki T, Naasani I, Shimazaki T, Tsuchiya K, et al. Telomere shortening and growth inhibition of human cancer cells by novel synthetic telomerase inhibitors MST-312, MST-295, and MST-199. Mol Cancer Ther. 2002;1(9):657–65. **Supported in part by a grant-in-aid for scientific research on priority areas from the Ministry of Education, Culture, Sports, Science and Technology, Japan.**
33. Gurung RL, Lim HK, Venkatesan S, Lee PSW, Hande MP. Targeting DNA-PKcs and telomerase in brain tumour cells. Mol Cancer. 2014;13(1):232.
34. Serrano D, Bleau A-M, Fernandez-Garcia I, Fernandez-Marcelo T, Iniesta P, Ortiz-de-Solorzano C, et al. Inhibition of telomerase activity preferentially targets aldehyde dehydrogenase-positive cancer stem-like cells in lung cancer. Mol Cancer. 2011;10(96).
35. Collins K. Physiological assembly and activity of human telomerase complexes. Mech Ageing Dev. 2008;129(1):91–8.
36. Wojtyla A, Gladych M, Rubis B. Human telomerase activity regulation. Mol Biol Rep. 2011;38(5):3339–49.
37. Cerni C. Telomeres, telomerase, and myc. An update. Mutation Res/Rev Mutation Res. 2000;462(1):31–47.
38. Zuo Q-P, Liu S-K, Li Z-J, Li B, Zhou Y-L, Guo R, et al. NF-kappaB p65 modulates the telomerase reverse transcriptase in the $HepG_2$ hepatoma cell line. Eur J Pharmacol. 2011;672(1):113–20.
39. Yin L, Hubbard AK, Giardina C. NF-κB regulates transcription of the mouse telomerase catalytic subunit. J Biol Chem. 2000;275(47):36671–5.
40. Wang JC, Bennett MR. Nuclear factor-κB–mediated regulation of telomerase the Myc link. Arterioscler Thromb Vasc Biol. 2010;30(12):2327–8.
41. Olaussen KA, Dubrana K, Domont J, Spano J-P, Sabatier L, Soria J-C. Telomeres and telomerase as targets for anticancer drug development. Crit Rev Oncol/Hematol. 2006;57(3):191–214.
42. Shay JW. Telomerase as a target for cancer therapeutics. Gene-based therapies for cancer: Springer; 2010. p. 231-49.
43. Wong VC, Ma J, Hawkins CE. Telomerase inhibition induces acute ATM-dependent growth arrest in human astrocytomas. Cancer Lett. 2009;274(1):151–9.
44. Gellert GC, Dikmen ZG, Wright WE, Gryaznov S, Shay JW. Effects of a novel telomerase inhibitor, GRN163L, in human breast cancer. Breast Cancer Res Treat. 2006;96(1):73–81.
45. Kraemer K, Fuessel S, Schmidt U, Kotzsch M, Schwenzer B, Wirth MP, et al. Antisense-mediated hTERT inhibition specifically reduces the growth of human bladder cancer cells. Clin Cancer Res. 2003;9(10):3794–800.
46. Mikami-Terao Y, Akiyama M, Yuza Y, Yanagisawa T, Yamada O, Yamada H. Antitumor activity of G-quadruplex–interactive agent

TMPyP4 in K562 leukemic cells. Cancer Lett. 2008;261(2):226–34.

47. Calado RT, Chen J. Telomerase: not just for the elongation of telomeres. Bioessays. 2006;28(2):109–12.

48. Ding D, Xi P, Zhou J, Wang M, Cong Y-S. Human telomerase reverse transcriptase regulates MMP expression independently of telomerase activity via NF-κB-dependent transcription. FASEB J. 2013;27(11):4375–83.