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Mitochondrial dysfunction and nuclear-mitochondrial shuttling of TERT are involved in cell proliferation arrest induced by G-quadruplex ligands

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ABSTRACT

G-quadruplex ligands DODC and TMPyP4 have different binding modes to quadruplex structure and cause cell proliferation arrest. Here we showed that DODC was more efficient in cell growth inhibition than TMPyP4. Both G-quadruplex ligands induced nuclear-cytoplasmic shuttling and accumulation of TERT in mitochondria. This effect was not fully dependent on cellular oxidative stress. DODC induced robust cell apoptosis by perturbing mitochondrial function intensively. Overexpression of TERT could not counteract the effects of DODC on mitochondrial respiratory function. Taken together, our results suggest that interference of mitochondrial function by DODC is one of main targets for its anti-tumor ability.

1. Introduction

Telomeres are composed of tandem d(TTAGGG) repeats with a protracted single stranded overhang at the 3′ end and protect chromosome ends from being recognized as DNA breakage [1]. This guanine-rich region tends to form a 4-stranded DNAs by 4 guanines in a planar structure which termed as G-quadruplex [2]. Small molecules that stabilize the G-quadruplex conformation will interfere with the maintenance of telomere, leading to telomere shortening and cell senescence, and ultimately cell apoptosis [3].

G-quadruplex ligands are recognized as potential drugs for anticancer therapy and their anticancer functions are estimated in cell-based assays [4,5]. Some unexpected findings showed that G-quadruplex ligands induced cell senescence within a short time and even without telomere shortening [6,7]. These results are contradictive with the initial theory that G-quadruplex ligands inhibit cell proliferation by interfering telomere lengthening [8]. The molecular mechanisms of instant anti-proliferation effect of G-quadruplex ligands include inducing DNA damage response due to double-strand breakage and provoking telomere uncapping by competitive displacement of telomere binding protein [9,10].

Cancer cells generally are heterogenic in their telomere length and some have extremely short telomeres. It has been suggested that these cells with critical short telomere play a crucial role to the overall cell population [11]. Therefore, one cannot exclude a possibility that the instant effect of telomerase inhibition on critical short telomere was actually caused by G-quadruplex ligands. Recent studies showed that telomerase catalytic subunit TERT is translocated to mitochondria and plays a protective role under oxidative stress [12,13]. Whether G-quadruplex ligands interfere with cellular localization of telomerase has not been studied.

In this study, we showed that two G-quandruplex ligands, TMPyP4 and DODC, have similar effects on telomerase activity inhibition by cell-based assays although they have different binding mode and stabilization ability to G-quadruplex structures in vitro. G-quandruplex ligands induce the nuclear export of TERT within 6 h and nuclear-cytoplasmic shuttling is not dependent on cellular oxidative stress.

Mitochondria played an important role in cell apoptosis induced by G-quandruplex ligands. The strategy for testing the anti-tumor efficiency of G-quandruplex ligands should not be restricted to only evaluating the G-quadruplex structure stabilization binding ability.

Abbreviations: DODC, 3,3′-diethyloxadicarbocyanine iodide; TMPyP4, 5,10,15,20-tetra (*N*-methyl-4-pyridyl) porphine; ROS, reactive oxygen species; MMP, mitochondrial membrane potential; OCR, oxygen consumption rate

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2. Materials and methods

2.1. Cell culture and chemicals

Human cervical cancer cell line HeLa, human hepatoma cell line HepG2 and human glioma cell lines U251 were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) at 37 °C in a 5% CO_2 incubator with 100% humidity. Human hepatic L02 cell, a TERT-negative cell, was cultured in DMEM supplemented with 10% FBS under same condition. 3,3′-diethyloxadicarbocyanine iodide (DODC) and 5,10,15,20-tetra (*N*-methyl-4-pyridyl) porphine (TMPyP4) were purchased from Sigma–Aldrich. These drugs were prepared as 1000× stock solution in DMSO and were diluted in cell culture medium immediately before treatment.

2.2. Determination of cell proliferation

Cells were seeded in 96-well plates at 50% confluence and cultured for 16 h before the assay. MTT (Promega) was added to cells and incubated for 4 h at 37 °C. The reduction of MTT was analyzed in an ELISA Reader (BioTek) at 570 nm. Each treatment was determined in triplicate.

2.3. Assay for telomerase enzyme activity

Telomerase activity was measured by the telomerase repeat amplification protocol (TRAP) as described before [14]. Primer extension was carried out in the presence of an internal standard (IS). Cell lysate were diluted using lysis buffer to reach an approximate concentration of 1000 cells/μL. PCR products were resolved using 10% polyacrylamide gel and were stained with ethidium bromide to visualize the 6 bp ladder. The heat-inactivated cell lysate was used as negative control. Telomerase activity was taken as the integrated density of all PCR product bands by using the Quantity-One software for Bio-Rad Image analysis systems (Bio-Rad Laboratories), followed the previously described procedure [14].

2.4. Cell fractionation and immunoblotting

Whole cell lysates were prepared in RIPA-buffer. Mitochondria were prepared using Mitochondria Isolation Kit for Cultured Cells (Pierce) according to the manufacturer's protocol. Protein concentration was determined using the Bradford method. Equal amount of protein (20 μg) was subjected to 10% SDS–PAGE and transferred onto high-quality polyvinylidene difluoride (PVDF) membrane (Roche). The protein bands were probed with different primary antibodies including rabbit polyclonal antibody against human TERT (ab32020, Abcam), mouse monoclonal antibodies against mitochondria (ab3298, Abcam), tubulin (E12-043, Enogene) and β-actin (E12-041, Enogene), and were visualized by the Immobilon Western Chemiluminescent HRP Substrate (Millipore).

2.5. Immunocytochemistry

HeLa cells were grown on coverslips for 24 h. After treatment with TMPyP4 or DODC, cells were fixed in 4% parformaldehyde and permeabilized with 0.4% Triton X-100. All procedures were done at room temperature. Cells were washed twice in phosphate buffered saline (PBS) and were blocked in 1% BSA for 1 h at 37 °C, followed by an overnight incubation with antibody against TERT (ab32020, Abcam, 1:25). For fluorescence detection, cells were incubated with FITC-conjugated secondary antibody (KPL, 1:50). Nuclei were counterstained with 1 μg/ml DAPI (Roche). The stained cells were analyzed by laser scanning confocal microscopy (ZEISS, LSM 510 META).

2.6. Assay of cellular oxidative stress, mitochondrial membrane potential (MMP) and apoptosis

Cellular reactive oxygen species (ROS) was measured by staining with 5 μM DCF-DA (Sigma–Aldrich). To exclude potential artifacts of this probe, we also quantified the ROS level in HeLa cells treated with TMPyP4 using 1 μM DHE (Invitrogen). We did not analyze cells treated with DODC using DHE, simply because DODC is a dye with similar emission wavelength as this probe. We used 2 μg/mL JC-1 (Invitrogen) to quantify the MMP in HeLa cells with and without drug treatment. The fluorescence of cells was analyzed by flow cytometry (BD FACScan system, Vantage SE). Apoptosis of HeLa cells were determined by flow cytometry using an Annexin V-FITC Kit (Bender Medsystems, eBioscience) according to the manufacture.

2.7. Oxygen consumption

Cellular oxygen consumption was measured using intact cells and a Clark-type oxygen sensor (Hansatech instruments) at 25 °C. Equal number of HeLa cells (3×10^6) with different treatments was analyzed in growth culture medium in triplicate.

2.8. Plasmids and cell transfection

The cDNA of human TERT was amplified and cloned into the pCMV-myc vector (Clontech) and pCMV-myc-mito vector (Invitrogen). All constructs were confirmed by sequencing. HeLa cells were transiently transfected using FuGENE HD Transfection Reagent (Roche) according to the manufacture's instruction. Briefly, 1×10^6 cells were seeded in a 6-well plate 12 h before transfection. 2 μg of vector and 3 μL of transfection reagent were mixed in 100 μL Opti-MEM (Gibco) and added to cells drop by drop. After transfection for 48 h, cells were treated by TMPyP4 or DODC for 2 h before harvest.

2.9. Statistical analysis

All experiments were repeated for three times unless otherwise stated. Data are presented as mean ± S.D. of three independent experiments. Differences between cells treated with or without drug were analyzed using unpaired Student's *t*-test. A value of $P < 0.05$ was considered statistically significant (∗).

3. Results

3.1. DODC and TMPyP4 inhibit cell proliferation

The two G-quadruplex ligands, DODC and TMPyP4, can bind and stabilize the G-quadruplex structure which result in inhibition of telomerase activity in some types of cancer cells [15–17]. In particular, DODC had poor effects on telomerase activity inhibition as revealed by the cell-free TRAP and exonuclease I hydrolysis assay, whereas TMPyP4 showed good capability of stabilizing G-quadruplex structure formed by telomere sequences and G-rich region of the oncogene *c-MYC* promoter [18]. Due to the difference between DODC and TMPyP4, we suspect that TMPyP4 might be more efficient in inhibiting cell proliferation. As shown in Fig. 1A, the viability of three cancer cell lines (HeLa, HepG2 and U251) presented a dosage-dependent change when cells were treated with different concentrations of these drugs for 48 h. Unexpectedly, the half maximal inhibitory concentration (IC_{50}) of DODC (10 μM) was much lower than that of TMPyP4 (100 μM) for HeLa cells. Treatment of DODC and TMPyP4 in human hepatic L02 cells had similar effects as observed in these cancer cell lines (Supplementary Fig. 1).

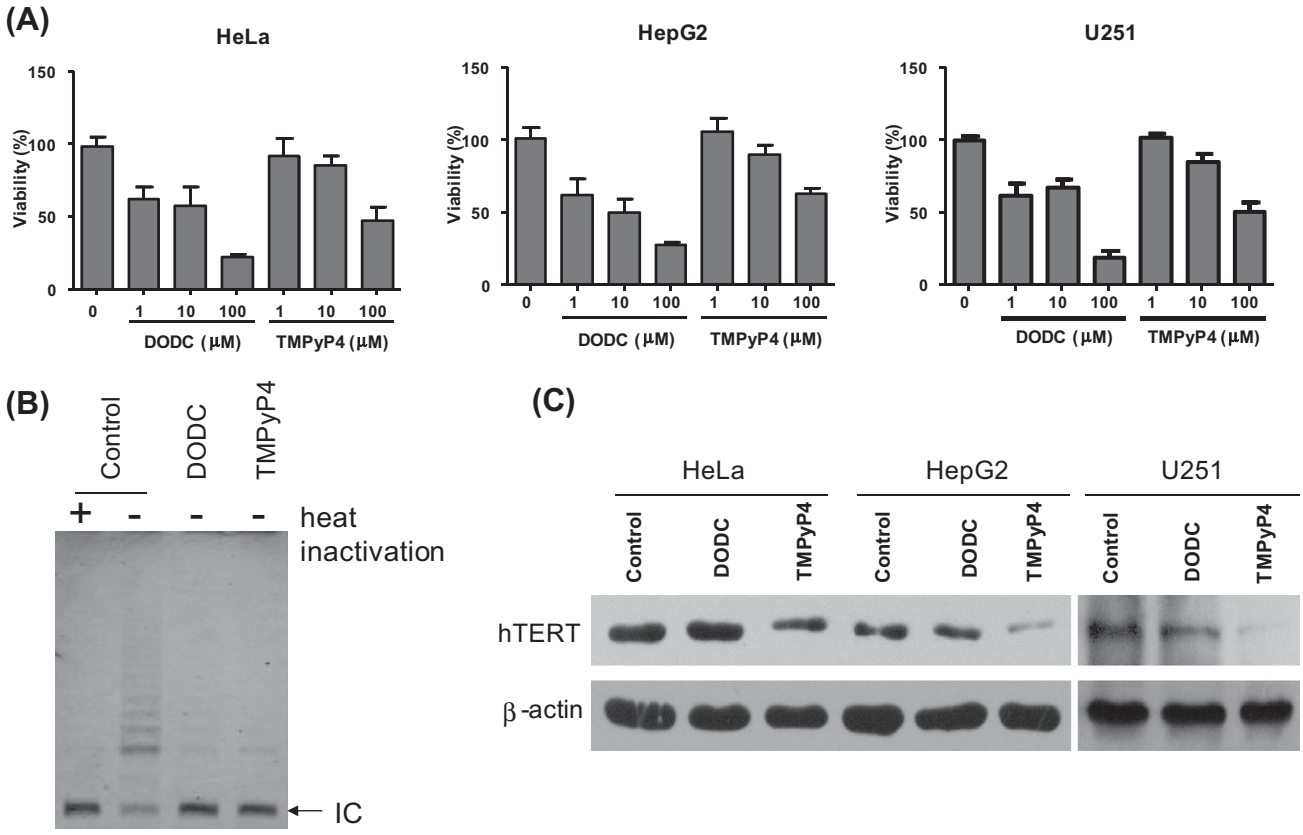

Fig. 1. Inhibition of cell proliferation, telomerase activity and expression by DODC and TMPyP4. (A) HeLa, HepG2 and U251 cells were incubated with 0, 1, 10, 100 μM DODC or TMPyP4 for 48 h. Cell viability was evaluated by the MTT assay and was normalized by the value without treatment of DODC or TMPyP4. (B) HeLa cells were treated with DODC (10 μM) or TMPyP4 (100 μM) for 48 h and telomerase activity were detected by the TRAP method. The bands indicated by the arrowhead referred to the 36 bp internal control (IC), as an indicator of PCR efficiency. (C) Immunoblotting analyses of hTERT and β-actin protein levels in HeLa, HepG2 and U251 cells treated with 10 μM DODC or 100 μM TMPyP4 for 48 h.

Evidently, the efficiency of DODC and TMPyP4 to induce cell growth arrest is not consistent with their capability of G-quadruplex structure stabilization and telomerase inhibition, suggesting that other mechanism may underlie the variance of their roles on cell growth inhibition.

3.2. DODC and TMPyP4 show different inhibitory effects on telomerase activity and TERT expression

The effects of DODC and TMPyP4 on the activity and expression of telomerase were examined using the TRAP method and immunoblotting. Analysis of HeLa cells treated with 10 μM DODC or 100 μM TMPyP4 for 48 h showed that both molecules inhibited telomerase activity at a similar level (Fig. 1B). Unexpectedly, TMPyP4 treatment had a stronger inhibitory effect on TERT protein expression than DODC in all three cell lines (Fig. 1C). Treatment with TMPyP4 caused reduction of both mRNA and protein expression of TERT in HeLa cells, whereas DODC had no apparent inhibition effect. Meanwhile, the *c-MYC* mRNA expression decreased remarkably in cells treated with TMPyP4 (Supplementary Fig. 2). These results suggested that different G-quadruplex ligands may have different inhibitory effects on telomerase activity and TERT expression via different regulatory pathways.

3.3. TERT shuttles from nucleus into cytoplasm and accumulates in mitochondria upon short-term treatment of DODC and TMPyP4

We tested whether different suppression effects of DODC and TMPyP4 on telomerase activity were caused by different pattern of translocation of telomerase within a cell. We treated HeLa cells with DODC (10 μM) or TMPyP4 (100 μM) for 2, 6 and 48 h, and stained with anti-hTERT antibody to examine the localization of telomerase by immunofluorescence assay. Telomerase was translocated from nucleus into cytoplasm at 2 h upon DODC and TMPyP4 treatment, and the cytoplasmic distribution of TERT was maintained at 6 h (Fig. 2).

To test whether the exported TERT from nucleus could be further transported into mitochondria, we exposed HeLa cells to DODC (10 μM) or TMPyP4 (100 μM) for different time points and quantified TERT protein level in mitochondrial fraction. As shown in Fig. 3, along with the treatment of DODC or TMPyP4, there was a gradual decrease of cellular TERT level, which was in accordance with the TERT protein level detected by immunofluorescence assay. Intriguingly, both drugs induced the translocation of TERT in mitochondria at 2 and 6 h, and DODC treatment had a stronger effect. To confirm this result was not caused by nuclear contamination in mitochondria fraction, we used tubulin as cytoplasmic marker and histone H2A as nuclear marker and found no trace of nuclear contamination (Fig 3B and supplementary Fig. 3).

3.4. Short-term treatment of DODC, but not TMPyP4, disrupts mitochondrial function and induces cell apoptosis

Recent studies reported that mitochondrial localization of TERT was induced by oxidative stress [12,13,19]. We measured cellular ROS level in cells treated with DODC and TMPyP4. After two hours treatment, the ROS level significantly increased in cells treated with DODC but not TMPyP4 (Fig. 4A). Therefore, the translocation

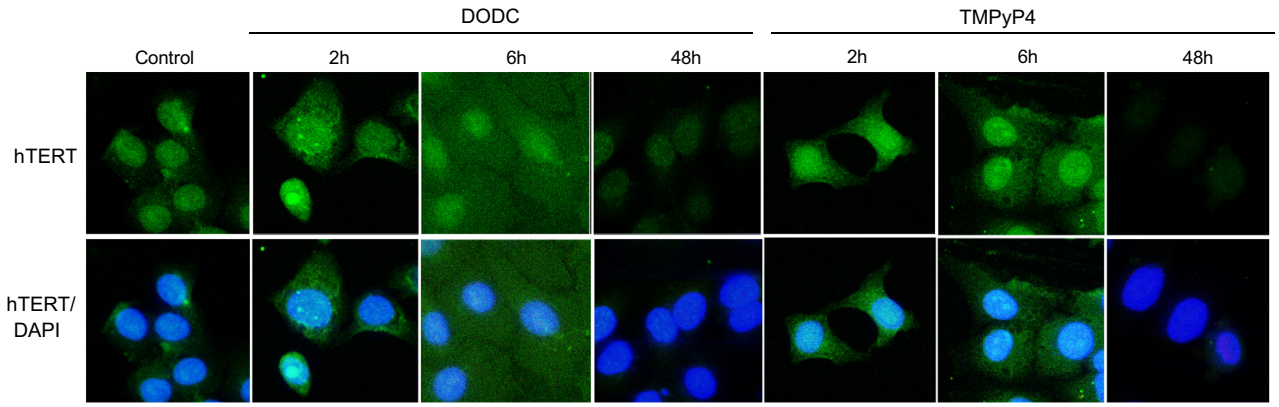

Fig. 2. Distribution of telomerase in cells treated by DODC or TMPyP4. HeLa cells were incubated with 10 μM DODC or 100 μM TMPyP4 for 2, 6 and 48 h. hTERT (green) and nucleus (blue) were stained by the immunofluorescence assay to visualize the cellular distribution of telomerase.

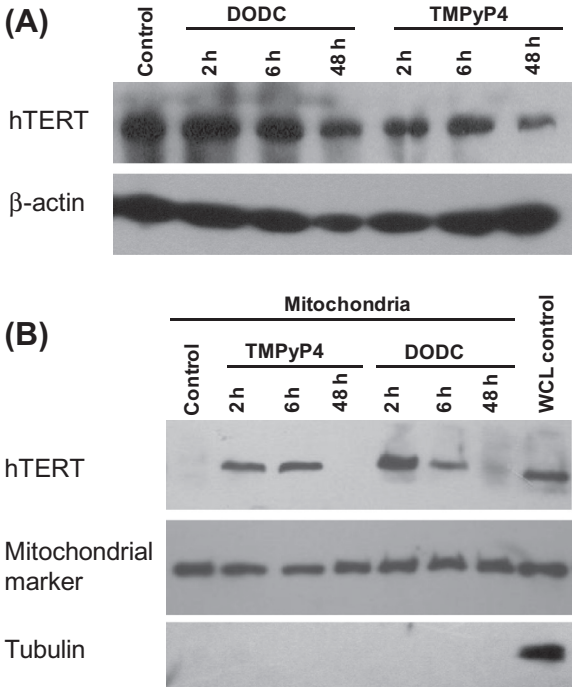

Fig. 3. Time course of telomerase expression in HeLa cells treated with DODC or TMPyP4. (A) Decreased level of hTERT was observed in whole cell lysate of HeLa cells treated with 10 μM DODC or 100 μM TMPyP4 for 2, 6 and 48 h. (B) Telomerase protein in mitochondrial fractions from treated HeLa cells. Equal protein loading was demonstrated with antibody against mitochondria (mitochondrial marker) and whole cell lysate (β-actin). Antibody against tubulin was used as a cytoplasmic marker to show the purity of mitochondria. WCL, whole cell lysate.

of telomerase from nucleus into mitochondria induced by these two drugs was not fully dependent on cellular oxidative stress. To validate the pattern as revealed by DCF-DA, we further quantified the ROS level in HeLa cells treated with TMPyP4 using DHE (Invitrogen) and observed no change of ROS (Supplementary Fig. 4), which was consistent with the above result.

In agreement with the rising of ROS, flow cytometric analysis indicated that the proportion of cells with low mitochondrial membrane potential (MMP) increased in cells treated with DODC (Fig. 4B). To further investigate if treatment of DODC and TMPyP4 altered mitochondrial function, we measured the oxygen consumption rate (OCR) in cells treated with both drugs using a Clark-type oxygen sensor. As shown in Fig. 4C, DODC treatment significantly reduced the OCR (up to 75% of the untreated cells), whereas TMPyP4 treatment had no apparent effect.

We further evaluated the effects of DODC and TMPyP4 on cell apoptosis within the time frame of mitochondrial localization of TERT. Fraction of apoptotic cells was significantly elevated in cells treated with DODC for 2 h but not for cells treated with TMPyP4 (Fig. 5).

3.5. Over-expression of TERT counteracts the elevated ROS but fails to improve the mitochondrial respiratory function decline induced by DODC

To investigate whether TERT overexpession would counteract the negative effects of DODC on mitochondria, we measured the change of cellular oxidative stress in TERT-overexpressing cells, with and without targeted export to mitochondria. We confirmed overexpression of exogenous TERT in HeLa cells by immunoblotting (Supplementary Fig. 5). The ROS level was measured in transfected cells after treatment with DODC or TMPyP4 for 2 h. We found that ectopic expression of TERT, regardless of its cellular localization, reduced the ROS level induced by DODC although the difference was not statistically significant. We observed similar salvaging effects in all three independent experiments. However, we discerned no alteration of the ROS level in transfected cells treated with TMPyP4 (Fig. 6A), a result consistent with the above observations (Fig. 4).

Since the decrease of ROS level might be resulted from the recovery of normal mitochondrial respiratory chain activity, we sought to determine whether the respiration rate of cells treated by DODC could be restored by overexpression of TERT. Our results showed that overexpression TERT could not rescue the decreased respiratory rate induced by DODC (Fig. 6B).

4. Discussion

G-quadruplex ligands are potential anticancer agents [4,5,20]. Recent studies showed that the instant anti-proliferation effect of G-quadruplex ligands was more complex than we had thought [6,7,21]. In this study, we aimed to examine whether G-quadruplex ligands such as DODC and TMPyP4 could interfere with telomerase expression and/or mitochondrial localization.

The two commercially available G-quadruplex ligands DODC and TMPyP4 had different G-quadruplexes binding affinity and specificity [18]. DODC, but not TMPyP4, was reported to have little inhibitory effect on telomerase activity as evaluated by exogenously adding DODC to cell-free TRAP reaction mixtures [18].

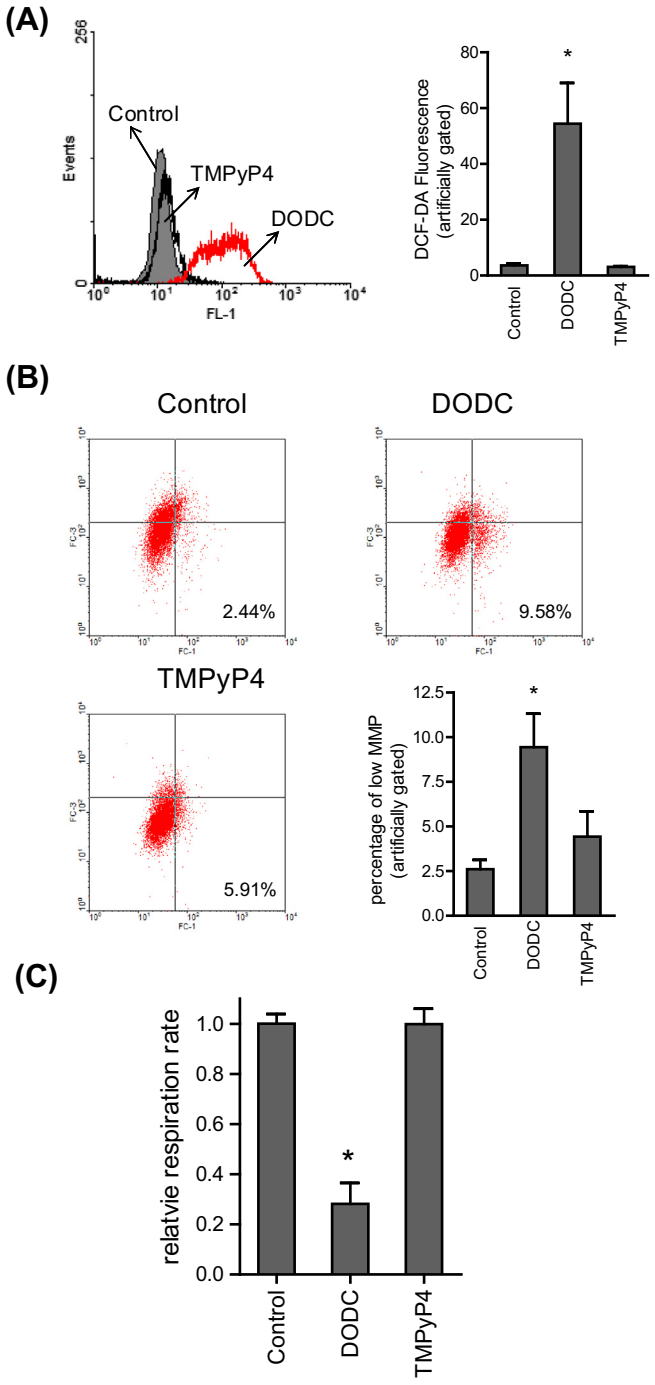

Fig. 4. ROS, MMP and OCR in cells treated by DODC or TMPyP4. HeLa cells were incubated with 10 μM DODC or 100 μM TMPyP4 for 2 h. Cellular level of ROS was measured by staining with DCF-DA (A) and change of MMP was determined by mitochondrial membrane sensitive dye JC-1 (B). (C) Relative oxygen consumption rates were measured in intact cells treated by DODC (10 μM) or TMPyP4 (100 μM) for 2 h. Cells without treatment were used for normalization. Bars represent mean ± S.D. of three independent experiments. $^{*}P < 0.05$ relative to control.

However, whether the antitumor ability of DODC and TMPyP4 is totally dependent on their stabilization with quadruplex structure has not been sufficiently studied. In our study, we found that both DODC and TMPyP4 led to reduced cell viability, and DODC had a stronger anti-proliferation ability than TMPyP4 in all three cancer cell lines (Fig. 1). Treatment with TMPyP4 and DODC decreased cell proliferation and increased cellular ROS level in L02 cells, which suggested that the effects of TMPyP4 and DODC on cell proliferation were TERT-independent (Supplementary Fig. 1). Intriguingly, at their IC_{50} for cell viability, there was a similar inhibitory effect on telomerase activity in HeLa cells treated with DODC or TMPyP4, although the mRNA and protein levels of TERT were reduced in cells treated with TMPyP4 but not in cells treated with DODC (Fig. 1 and Supplementary Fig. 2). The reduction of *TERT* mRNA level induced by TMPyP4 was probably caused by transcriptional inhibition through G-quadruplexes structure formed in the promoter region of the *TERT* and *c-MYC* genes [4,22] (Supplementary Fig. 2).

We further showed that both DODC and TMPyP4 induced the translocation of TERT from nucleus into mitochondria within 2 h of treatment and DODC had a stronger effect on the cytoplasmic translocation of telomerase (Figs. 2 and 3). Inactive type of hTERT that was localized in cytoplasm would lead to an increased degradation of wild-type hTERT [23]. Therefore, it is most likely that TMPyP4 inhibited telomerase activity mainly at the transcription/translation level whereas DODC mainly at the post-translation level. The short-term response of cells (apoptosis and death) treated by these ligands could not be merely explained by telomerase inhibition.

The translocation of telomerase from nucleus to mitochondria upon treatment of DODC and TMPyP4 is very intriguing. It links mitochondria with the anti-proliferation effect of G-quadruplex ligands. Hitherto, several studies characterized mitochondrial telomerase, albeit its reported role in mitochondria remains contradictory [12,19]. We found that the ROS level, MMP and OCR of cells treated with DODC presented significant difference with those of non-treated cells (Fig. 4). These findings suggested that the observed effect of DODC was most likely pertinent to mitochondria. In accordance to mitochondrial dysfunction induced by DODC, percentage of apoptotic cells elevated significantly in cells treated by this small molecule (Fig. 5). DODC was used as a carbocyanine membrane dye to probe the microenvironment of mitochondria and enhanced mitochondrial dysfunction induced by chemotherapy drugs [24]. Our results were consistent with this notion that DODC could disrupt the function of mitochondria [24]. In contrast, the basic structure of TMPyP4 is a porphyrin and has a large aromatic planar geometry, which means that all atoms lie in a single plane will stack with the plane formed by G-quadruplex DNA. Previous studies had reported that induction of apoptosis by TMPyP4 was associated with activation of DNA damage response and cell cycle regulatory factors, and TMPyP4 also inhibited the expression of crucial components in cell growth and proliferation, such as c-MYC [9]. We speculated that DNA damage and change of expression profile of key regulators controlling cell survival caused by TMPyP4 might be a driving force for telomerase delocalization from telomere and exporting from nucleus.

In an effort to confirm the mitochondrial protection role of TERT [16], ectopic TERT were used to test whether it could confer resistance to effect of DODC. Increased TERT expression reduced cellular ROS levels but had no effect on mitochondrial respiratory function during the short-term treatment of DODC (Fig. 6). These data were in agreement with previous reports linking TERT expression with reduced ROS in different types of cells [25].

TERT with disability to shuttle between nucleus and cytoplasm negatively impacted mitochondrial function [26,27]. Mice without TERT expression had a compromise of mitochondrial biogenesis and metabolism, suggesting depletion or down-regulation of TERT did not simply affect telomere maintenance [28]. All these studies showed that telomerase plays an important role in mitochondrial biology. Meanwhile, mitochondrial competence is important for the survival of cancer cells, and coincidental extinction of mitochondrial maintenance factor and telomerase activity enhanced antitumor effect [29]. Our results showed that DODC had a dual role on inhibiting telomerase activity and mitochondrial function.

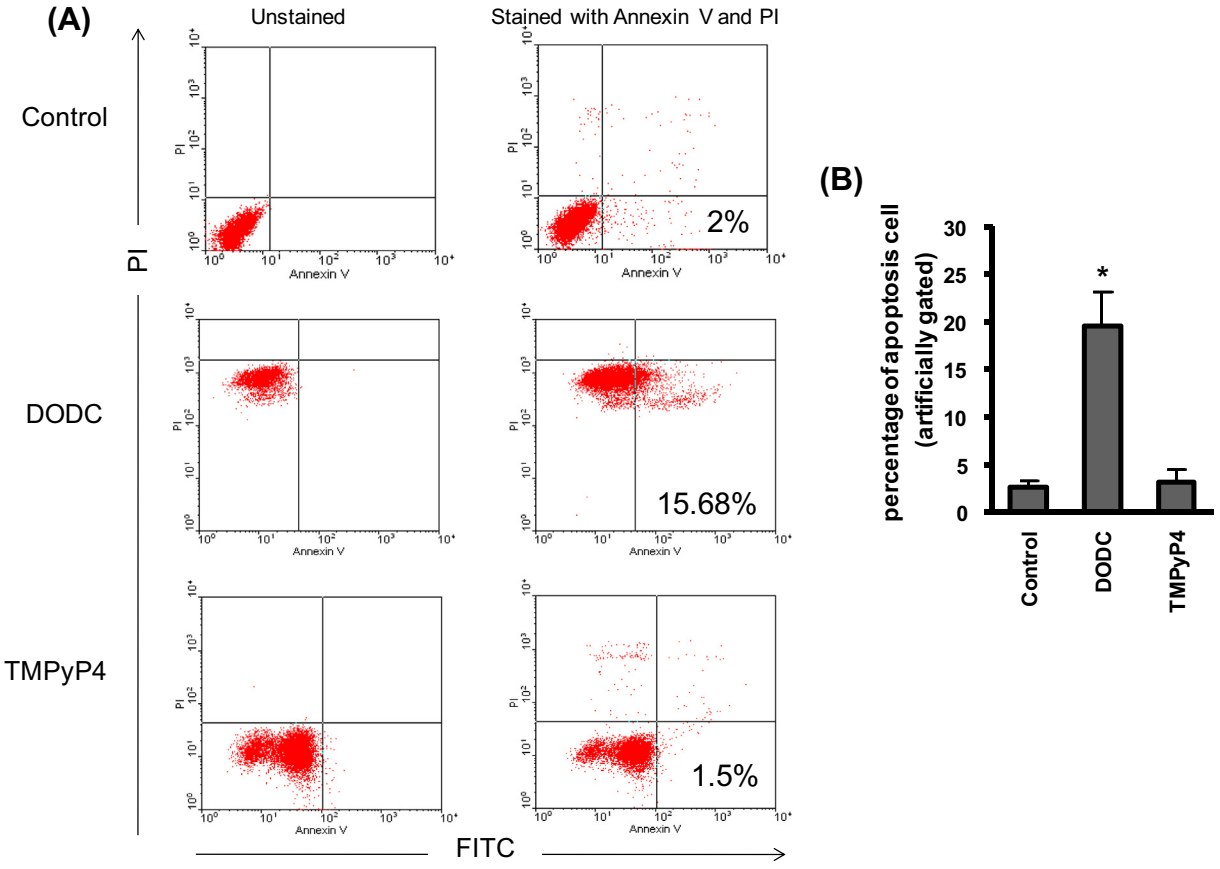

Fig. 5. Short-term treatment of DODC induced robust apoptosis. HeLa cells were incubated with 10 μM DODC or 100 μM TMPyP4 for 2 h, followed by staining with Annexin V and PI. Cells without Annexin V and PI staining were used as criteria of gating to exclude the interference of DODC and TMPyP4. (A) Representative images of the flow cytometric analysis. (B) Average of three independent analyses. Bars represent mean ± S.D. $^{*}P < 0.05$ relative to control.

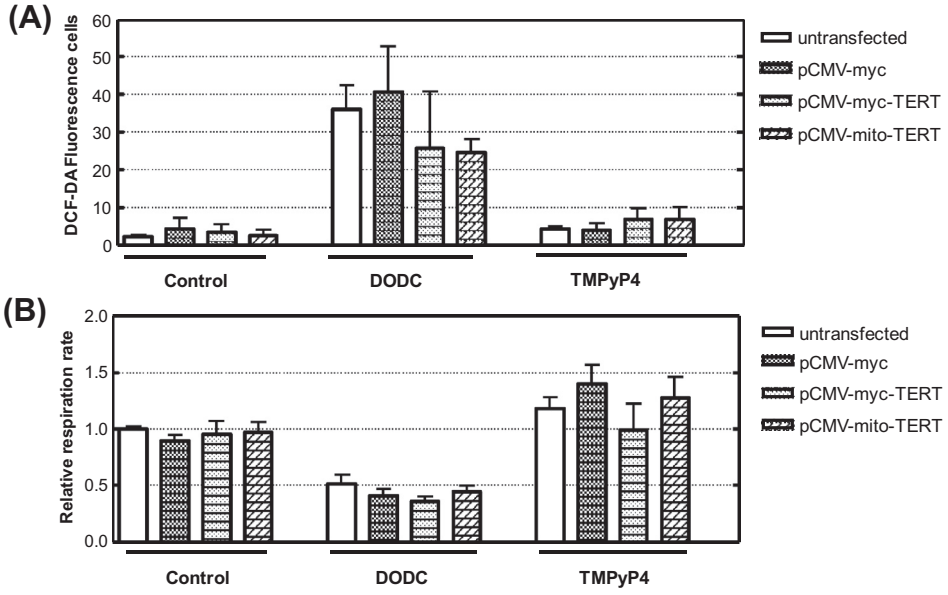

Fig. 6. Effects of ectopic telomerase on mitochondrial function in cells treated by DODC and TMPyP4. HeLa cells were transfected with an empty control vector (pCMV-myc), pCMV-myc-TERT, or pCMV-mito-TERT for 48 h, then treated with 10 μM DODC or 100 μM TMPyP4 for 2 h. Cellular ROS (A) and OCR (B) were determined in transfected cells as indicated. Data are mean ± S.D. of three independent experiments.

This is probably the reason why DODC displayed a stronger anti-proliferation ability than TMPyP4.

In short, we showed that treatment of DODC and TMPyP4 in cancer cell lines caused mitochondrial localization of TERT, but the underlying mechanism of this effect might be quite different. Our results showed that cytotoxic mechanism of G-quadruplex ligands, such as perturbation of mitochondrial function, should be considered besides the traditional concept of the G-quadruplex

stabilization. Further study is necessary to answer the question whether nuclear-cytoplasmic shuttling and mitochondrial localization of TERT induced by G-quadruplex ligands is a cause or consequence of drug effect and how this could contribute to a better understanding of TERT function in extra-nuclear compartments.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.04.010.

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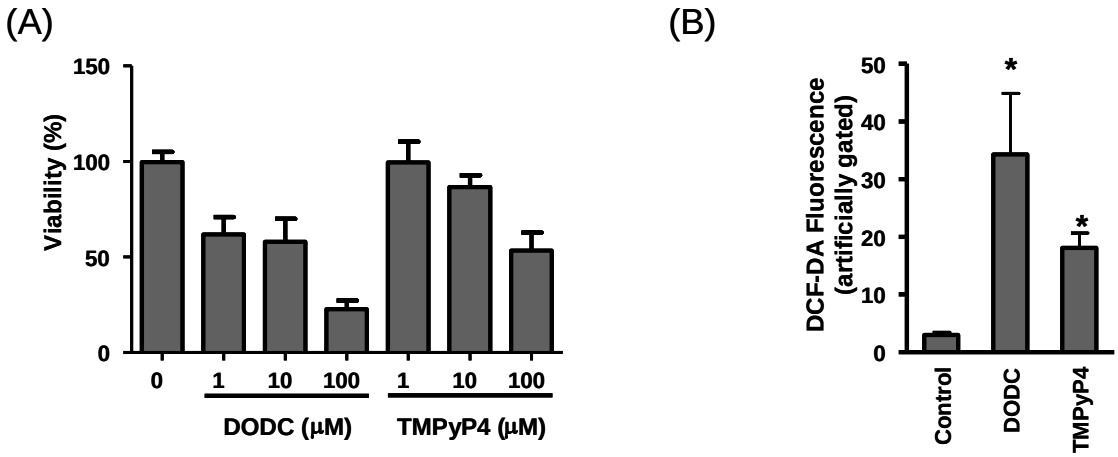
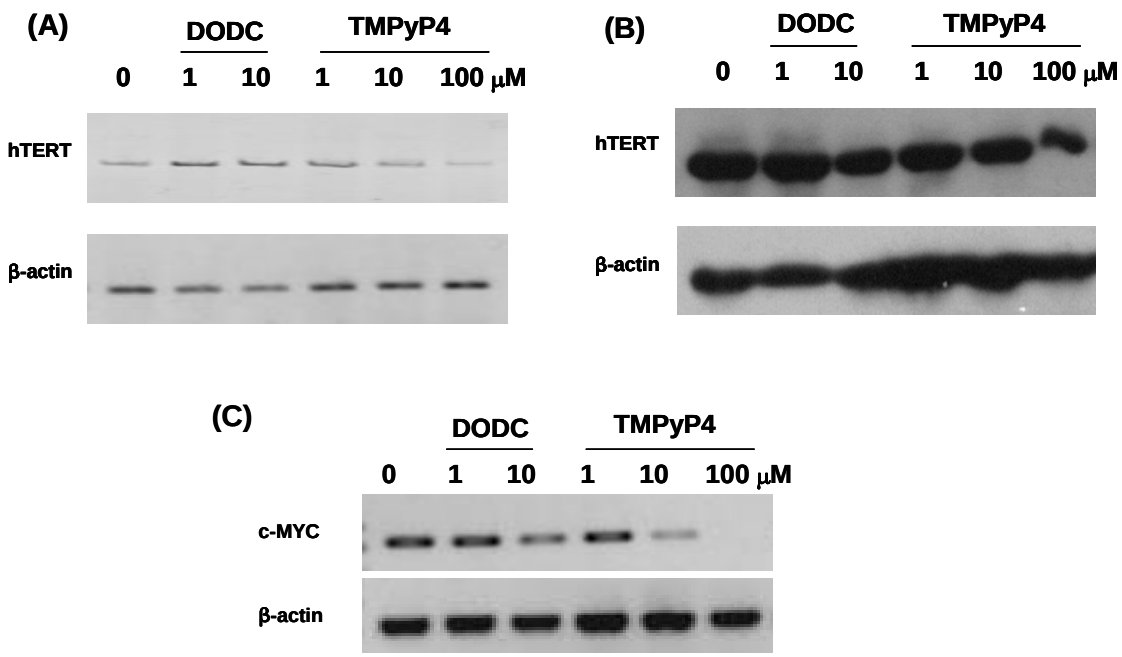
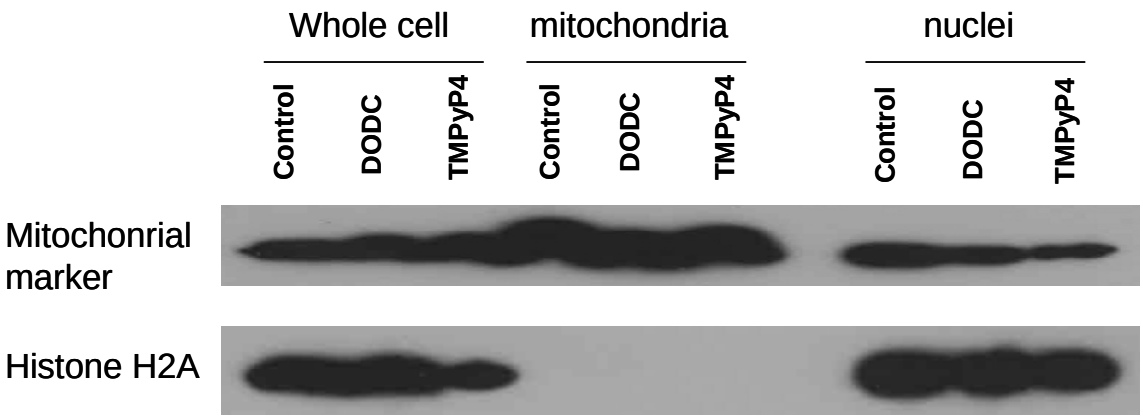
Appendix A. Supplementary data



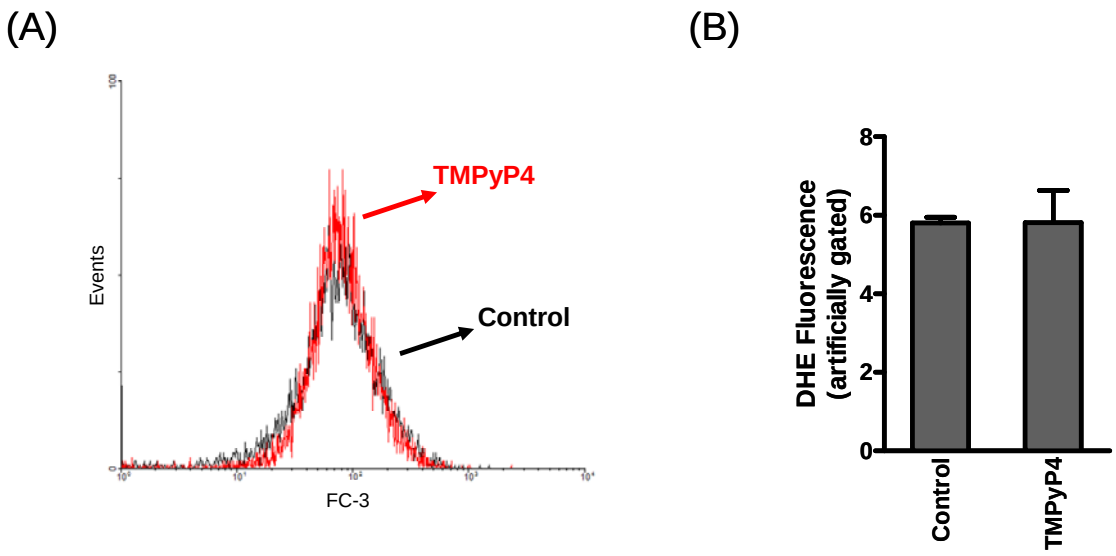
Supplementary Fig. 1. Effects of DODC and TMPyP4 on cell viability and reactive oxygen species (ROS) level of human hepatic L02 cell. Treatment of DODC or TMPyP4 at indicated concentrations for 48 h decreased the cell viability of L02 cells (A). The ROS level was increased in L02 cells after a treatment of 10 μM DODC or 100 μM TMPyP4 for 2 h, as represented by the intensity of DCF-DA fluorescence (B). Bars represent mean±SD of three independent experiments. * $P<0.05$ relative to control.



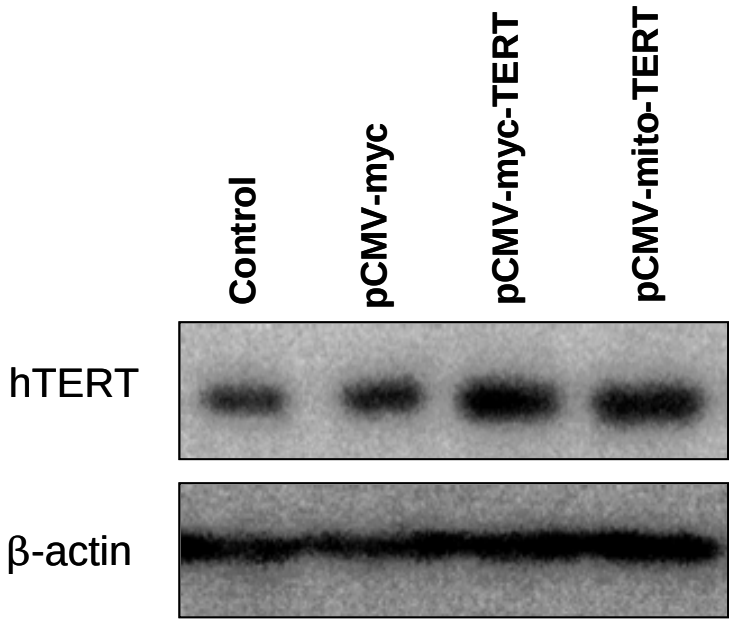
Supplementary Fig. 2. Expression of (A) *TERT* mRNA, (B) TERT protein, and (C) *c-MYC* mRNA in HeLa cells treated by DODC or TMPyP4 for 48 h at indicated concentrations.



Supplementary Fig. 3. The purity of mitochondria fraction of HeLa cells with and without DODC and TMPyP4 treatment. Mitochondria fraction was isolated by using Mitochondria Isolation Kit (Pierce) and potential nuclear contamination was evaluated by western blot for a nuclear marker histone H2A (Cell Signaling #2578). Whole cell lysate, mitochondria and nuclei were obtained from HeLa cells treated with 10 μM DODC or 100 μM TMPyP4 for 2h. The absence of histone H2A band in mitochondria fraction indicated no nuclear contamination. The observation of mitochondrial marker, as recognized by mouse monoclonal antibodies against mitochondria (ab3298, Abcam), in the nuclear fraction suggested the contamination of intact cell and/or mitochondria which could not be completely excluded by centrifugation.



Supplementary Fig. 4. ROS in HeLa cells treated by TMPyP4 probed by DHE. HeLa cells were incubated with 100 μM TMPyP4 for 2 h, and after stained with 1 μM DHE, cells were trypsinized and subjected to flow cytometric analysis (A). The ROS levels were represented by the intensity of DHE fluorescence. Bars represent mean ± SD of three independent experiments (B).



Supplementary Fig. 5. Overexpression of exogenous hTERT in HeLa cells. Cells were transfected with vectors pCMV-myc, pCMV-myc-TERT, or pCMV-mito-TERT for 48 h. Expression of hTERT was detected by immunoblotting. β-actin was used to show equal loading of total protein.