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Identification of small molecule inhibitors of telomerase activity through transcriptional regulation of hTERT and calcium induction pathway in human lung adenocarcinoma A549 cells

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ABSTRACT

High telomerase activity (TA) is detected in most cancer cells; and thus, TA inhibition by drug or dietary food components is a new strategy for cancer prevention. In this report, we examined the effects of fourteen natural or synthetic compounds on TA in human lung adenocarcinoma A549 cells. The results demonstrated that some of the tested compounds inhibited TA, being 2′-hydroxy-2,3,4′,6′-tetramethoxychalcone (HTMC) was the most potent among tested. In A549 cells, HTMC also inhibited the cell proliferation, decreased the expression of human telomerase reverse transcriptase (hTERT) and sequentially reduced the hTERT promoter. In soft agar assay HTMC treatment reduced the colony formation of A549 cells. Cellular fractionation and immunofluorescence assay demonstrated that there was no translocation of hTERT from nuclei to cytoplasm. Further studies revealed that the release of Ca^{2+} was the underlying mechanism of suppressed TA and hTERT transcription in A549 cells exposed to HTMC. These in vitro data support the development of HTMC as a therapeutic agent for cancer complications.

1. Introduction

Telomerase as an enzyme is responsible for the renewal of the chromosomal ends, the so-called telomeres.[1] Telomeres are essential units that prevent the loss of genetic information. In normal somatic cells, which show little or no telomerase activity (TA) to synthesize new telomeres.[1] In contrast, most cancer cells have mechanisms that compensate for telomere shortening through the activation of telomerase, allowing them to stably maintain their telomeres and grow indefinitely.[2] These observations suggest that telomerase reactivation is a rate-limiting step in cellular immortality and carcinogenesis, and telomerase repression can act as a tumor-suppressive mechanism.[2] The key advantages of targeting telomerase in comparison with most other cancer targets are its relative universality, criticality and specificity for cancer cells, including the putative cancer stem cell.[2] Telomere length in human is primarily controlled by three major components including human telomerase RNA component (hTR), telomerase-associated protein 1 (TP1), and human telomerase reverse transcriptase (hTERT).[2] Of these, we are primarily interested in identification of compound that induced down regulation of TA via suppression of hTERT expression, because the hTERT is a core telomerase protein and its expression limited to germinal and cancer cells.[2] It is well known that the expression of dominant-negative hTERT or its siRNA reduces the lifespan of human cancer cells by completely inactivating telomerase.[2] Therefore, it is thought that suppression of hTERT expression is an ideal strategy for the development of anticancer chemotherapeutics.[1,3]

The inhibition of TA has been approached through different strategies, ranging from anti-sense-based inhibitors to random screening of synthetic and natural products.[1] Among the latter, several compounds have been reported include rubromycins,[4] alterperylenol,[5] epigallocatechin gallate,[6] anthraquinone derivatives,[7–9] G-quadruplex ligands.[10–12] In our previous studies, we found that loss of telomerase activity can be potentially favorable prognostic marker in lung carcinomas.[13–16] We are currently undertaking the therapeutic assessment of medicinal mushroom *Antrodia camphorata*.[17–20] In parallel, we sought to identify potent TA inhibitor compounds to expand the scope of bioactive agents and to gain insight into their anticancer mechanism of action. To this end, here, we report the screening of 14 natural or synthetic compounds on TA in human lung adenocarcinoma cell line A549 cells.

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2. Results

2.1. Inhibition of telomerase activity (TA) in A549 cells

In an effort to identify potential candidate to inhibit TA, we have selected 14 natural or synthetic compounds (Fig. 1), based on their growth inhibitory potential against tumor cell proliferation and inflammatory mediators production in different type of cell lines as we reported previously.[21–26] The effects of these compounds (Fig. 1) on TA were examined in A549 cells with a random concentration of 25 μM, using modified telomeric repeat amplification protocol (TRAP) assay. We found that among tested, 2′-hydroxychalcone derivatives, 2′-hydroxy-2,3,4′,6′-tetramethoxychalcone (HTMC) followed by compound **6** potentially inhibited TA with respect to control (Fig. 2). Among the tested 3′,4′,5′-trimethoxychalcone derivatives, compounds **07–01**, **07–02**, **07–03**, and isoflavonoid, isobonducellin (**37**) have moderate effect on TA (Fig. 2). In contrast, the flavonol glycosides, kaempferitrin (**20**) and kaempferol 3-*O*-β-D-glucopyranosyl-(1→4)-α-L-rhamnopyranosyl-7-*O*-α-L-rhamnopyranoside (**21**); flavanones, 5,7-dimethoxyflavonone (**31**) and 7-*O*-methylglabranin (**32**); lignan, phyllanthin (**38**); phenolic acid, 4-methoxygallic acid (**40**); and 3′,4′,5′-trimethoxychalcone derivative **07–04** have no effect on TA at the tested concentration 25 μM (Fig. 2). Furthermore, the potent compound HTMC also inhibited the A549 cells TA in a dose (6.25, 12.5, 25.0 and 50.0 μM) and time (6 h, 12 h, and 24 h) dependent manner (see Supplementary data). Among the tested, HTMC was almost completely inhibited the TA of A549 cells at concentration of 25 μM (Fig. 2). Subsequently, HTMC was used to examine the TA associated cellular and molecular events in A549 cells.

We next examined the HTMC effect on A549 cell proliferation by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium] assay. The results demonstrated that HTMC treated for 24 h, A549 cells proliferation was decreased in a concentration-dependent manner (data not shown). Particularly, treated with 50 μM HTMC for 24 h reduced the A549 cells proliferation by 59%.

2.2. Effect of HTMC on the expression of hTERT in A549 cells

Although active telomerase requires the co-expression of hTR and hTERT components, the expression of hTERT correlates closely with TA in most cancers.[2] Therefore, we attempted to elucidate whether the above observed HTMC inhibitory effect on TA was due to the repression of hTERT expression in A549 cells. We used RT-PCR, real-time RT-PCR (Taqman assay) and Western blot analysis and examined the changes in hTERT expression following A549 cells treated with HTMC for 6 h. As indicated in Figure 3A, in HTMC treated A549 cells the mRNA expression levels of hTERT decreased in a dose-dependent manner. In contrast, hTR and GADD153 mRNA expressions were not altered, demonstrated the specificity for hTERT mRNA (Fig. 3A). To quantify the degree of the decreased hTERT mRNA, real-time PCR was performed using total RNA from A549 cells treated with vehicle or HTMC in the concentration range from 6.25 μM to 50 μM. As shown in Figure 3B, hTERT mRNA was decreased in a dose-dependent manner and reached to 92% after 50 μM HTMC treatment for 6 h. Furthermore, the expression levels of hTERT were also shown to undergo a dose-dependent decrease in whole cell extraction fraction following treatment with HTMC as confirmed by Western blot analysis (Fig. 3C).

2.3. Effect of HTMC on the hTERT promoter activity

The hTERT promoter activity has been closely associated with TA and hTERT expression in cancer cells but not in telomerase-negative normal cells.[2] To determine whether the decreased levels of hTERT in A549 cells was the result of decreased hTERT promoter activity, we dissected the full-length hTERT fragment (−548 to +50) into five regions p548 (−548 to +50), p212 (−212 to +50), p196 (−196 to +50), p177 (−177 to +50) and p95 (−95 to +50) (Fig. 4A). After transfection, A549 cells were treated with 25 μM HTMC for 24 h and luciferase reporter activity was determined by transient transfection assay. A plasmid expressing the bacterial β-galactosidase gene was co-transfected in each experiment to serve as internal control of transfection efficiency. The luciferase activity in HTMC treated cells was clearly down-regulated in the constructs p548, p212, p196, p177, and p95 in the range approximately from 25% to 70% as compared with untreated cells (Fig. 4B).

2.4. Effect of HTMC on the anchorage-independence of A549 cells

We next examined the effect of HTMC on the colony formation efficiency of A549 cells using anchorage-independence cell growth assay. A549 cells are highly tumorigenic and readily form colonies in soft agar medium. In this report, the results of soft agar assay demonstrated that colonies formation efficiency of HTMC treated A549 cells was drastically reduced as compared with untreated cells. Additionally, the colonies observed in the HTMC-treated cultures were smaller and more densely packed than the untreated controls (Fig. 5A). The number of colonies formed in agar was reduced by 23% when A549 cells were stimulated with 6.25 μM HTMC. Stimulation of A549 cells with 12.5 and 25.0 μM HTMC decreased the number of colonies by 96% and 99%, respectively (Fig. 5B) as compared with control cells. In general, increased the concentration of HTMC pretreatment concomitantly reduced the colony formation efficiency of A549 cells.

2.5. HTMC suppressed TA and hTERT expression in A549 cells through calcium induction

Previous studies have shown that accumulation of wild-type proteins in endoplasmic reticulum leads to release of Ca^{2+} from organelles that can be blocked by Ca^{2+} chelators.[2,14] To evaluate the signaling pathways involved in HTMC telomerase regulation, the intracellular calcium levels were analyzed in HTMC treated A549 cells. As shown in Figure 6A, HTMC treated A549 cells released the Ca^{2+} in a concentration-dependent manner, which was detected by calcium-sensitive indicator Fluo-3 AM. This was evidenced by increased green staining in A549 cells after treatment with HTMC for 24 h (Fig. 6A). These results suggested that HTMC induced the release of Ca^{2+} in A549 cells. To examine the specific role of HTMC in the released Ca^{2+}, A549 cells were pretreated with calcium chelator BAPTA-AM for 1 h and then with HTMC for 24 h. Analysis of calcium fluorescence demonstrated that the influx of calcium release was decreased, indicated that HTMC significantly contributed in the released Ca^{2+} in A549 cells (Fig. 6A). In addition, the specific role of Ca^{2+} released by HTMC on the regulation of TA was also examined. For this, A549 cells were treated with 25 μM HTMC, or pretreated for 1 h with various cancer cells growth inhibitors such as MEK inhibitor U0126 (10 μM), or ERK inhibitor PD98059 (20 μM), or MAPK inhibitor SB203580 (50 μM), or intracellular calcium chelator BAPTA-AM (20 μM), or 26S proteasome inhibitor MG132 (20 μM), or nuclear export inhibitor leptomycin B (20 nM), or PI3 K inhibitor LY294002 (50 μM) and then treated with 25 μM HTMC for 24 h and assayed for TA using TRAP method. Similar to the previous results, HTMC inhibited the TA; however, the concomitant addition of 20 μM BAPTA-AM completely abolished the ability of HTMC to inhibit TA (Fig. 6B). Using TRAP and RT-PCR assays, we found that BAPTA-AM completely abolished the ability of HTMC to repress TA and hTERT mRNA levels in

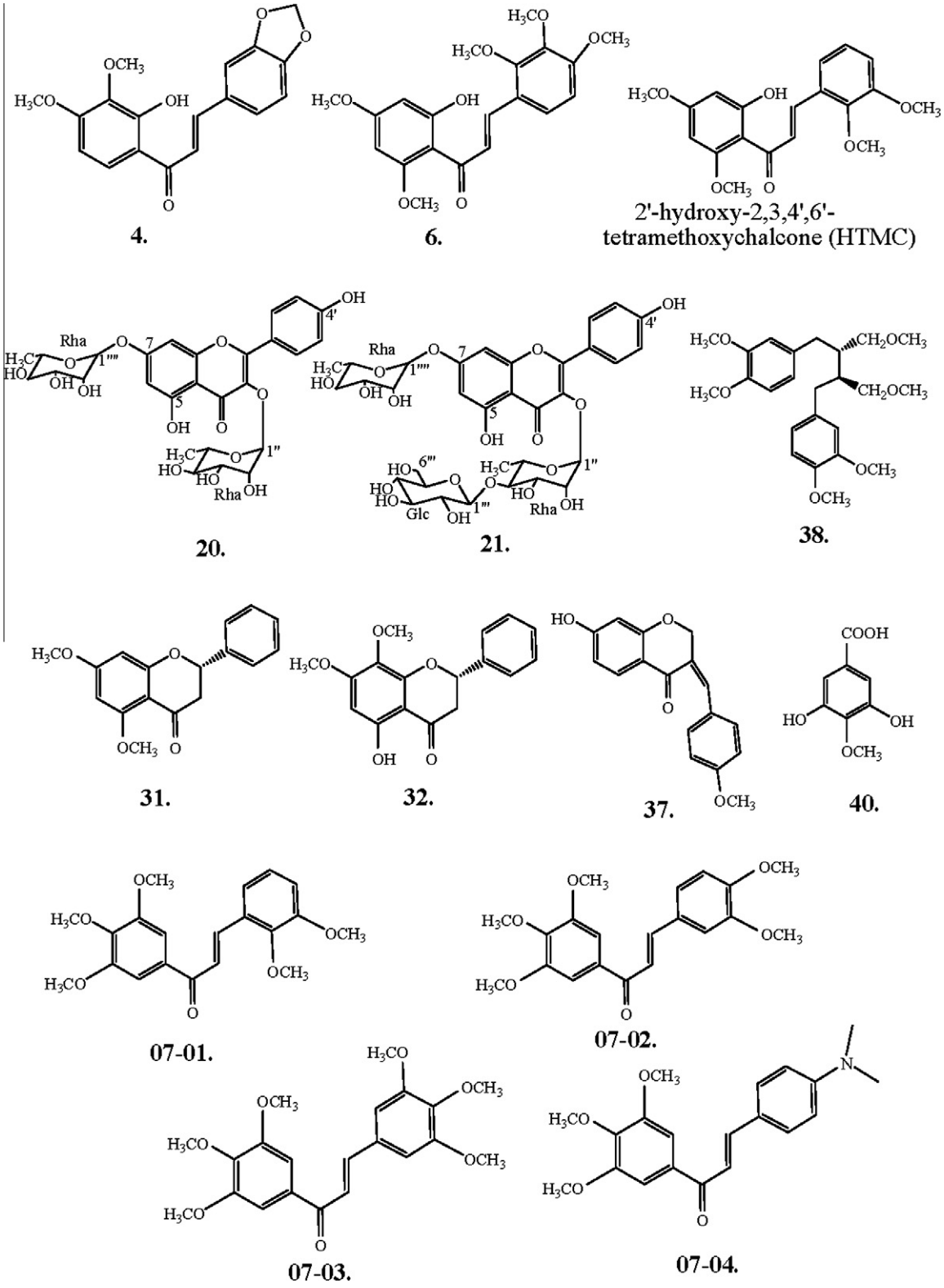

Figure 1. Chemical structures of natural or synthetic compounds tested on telomerase activity in human lung cancer cell line A549 cells.

A549 cells (Fig. 6C and D). In the absence of HTMC treatment, incubation of A549 cells with BAPTA-AM alone did not significantly change the levels of TA and hTERT as compared with controls (Fig. 6C and D). Furthermore, immunofluorescence staining of 25 μM HTMC treated A549 cells demonstrated a decreased expression of hTERT, and there was no translocation of hTERT from nuclei to cytoplasm (Fig. 6E). However, A549 cells pretreated for 1 h with intracellular calcium chelator BAPTA-AM and then treated with 25 μM HTMC for 24 h, partly restored the expression of hTERT in the nuclei (Fig. 6E). Taken together, these results suggested that HTMC suppressed telomerase activity and hTERT expression by inducing calcium release in A549 cells.

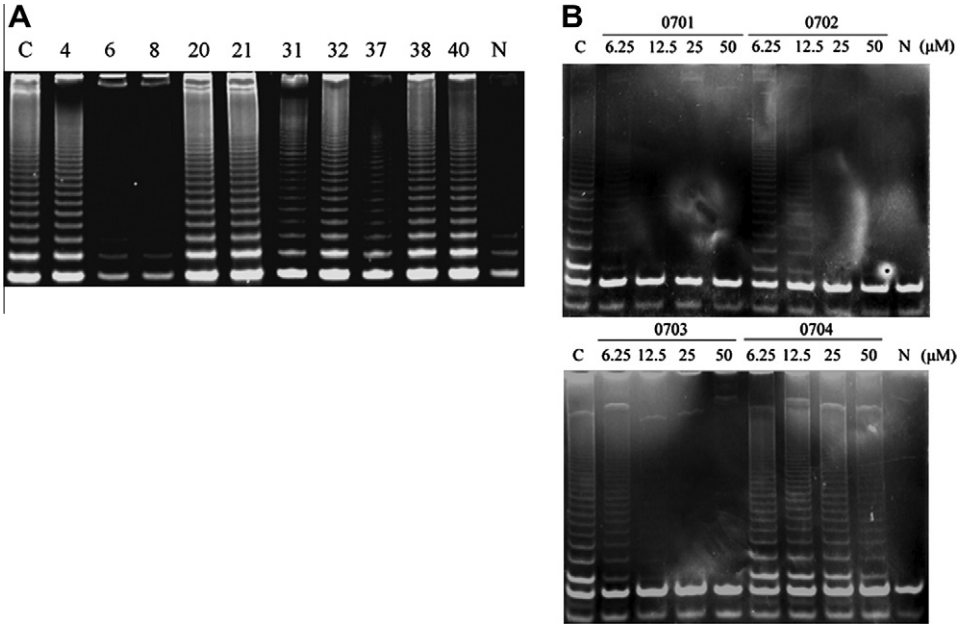

Figure 2. Effect of various natural and synthetic compounds on the level of telomerase activity in human lung A549 cells. After (24 h) plating, cells were exposed to 25 μM of each tested compound for 24 h. Cell pellets were collected and subjected to TRAP assay. The telomerase activity in untreated A549 cells was as control (C). N, negative control used lysis buffer only.

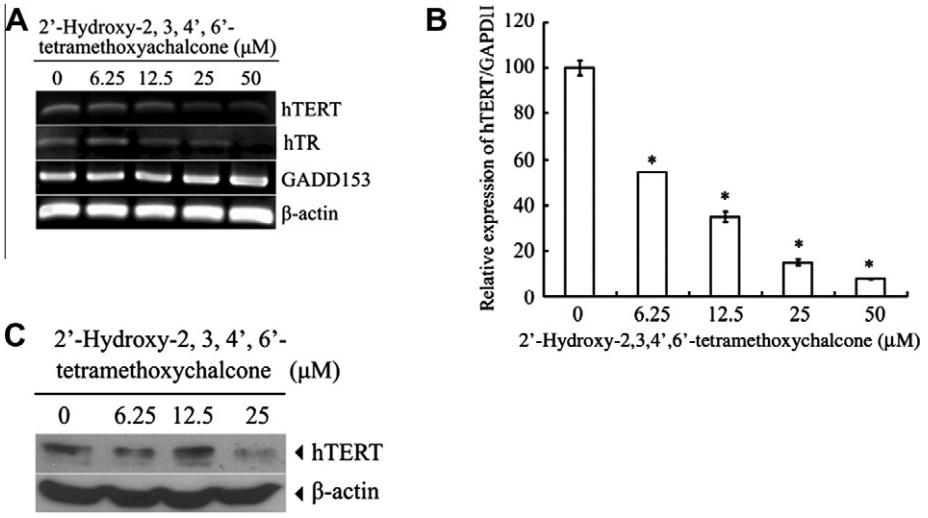

Figure 3. Effect of 2′-hydroxy-2,3,4′,6′-tetramethoxychalcone (HTMC) on the expression of hTERT, hTR and GADD153 mRNA and protein level in A549 cells. (A) After 6 h of incubation with HTMC, total RNA was isolated, and RT-PCR was performed using indicated primers, the amplified products were run in 1.5% agarose gel and visualized by ethidium bromide staining. β-Actin was used as an house-keeping control gene. (B) A549 cells were treated with indicated concentrations of HTMC for 6 h, relative hTERT mRNA level was determined by real-time RT-PCR (Taqman Assay) and normalized to GAPDH mRNA. Representative data were from three independent experiments and * indicates p <0.05 as compared with untreated cells. (C) Western blot analysis of hTERT subunit A549 cells treated with indicated concentrations of HTMC for 24 h.

3. Discussion

The unique role of telomerase in particular in cancer cells introduced a new area of research directed to the goal of telomerase inhibition as smart approach for cancer therapy.[1] Numerous studies using various active ingredients were performed to inhibit TA. Beside the G-quadruplex ligands,[10–12] small synthetic and nucleic acids based telomerase inhibitors, there is a multiplicity of compounds that can cause a decrease in TA.[1] Differentiating agents like all-trans retinoic acids, adriamycin and doxorubicin reduced TA and hTERT mRNA expression.[1] Another family of telomerase inhibitors is the isothiazolone derivatives which could reduce the enzyme activity by interfering with a cysteine in its active center.[1] Other putative inhibitors derived from natural sources are curcumin, epigallocatechin gallate (EGCG), retinoic acids and a herbal complex based on plant extracts from *Hoelen*, *Angelicae*, *Scutellariae*, *Glycyrrhizae radix* and other herbs.[1] Previous studies indicate that EGCG reduce telomerase activity by decrease in hTERT promoter methylation and increase in hTERT repressor E2F-1 binding at the promoter.[27] Our previous report also demonstrated that curcumin inhibit telomerase activity and its transcription factors including NFkB, AP1 and Sp1.[16] Multiple mechanisms and several transcription factors regulate telomerase ability at DNA and protein level. In this report, our efforts attempted to identify potential compound that can modulates TA by up-regulating calcium concentration and repress hTERT expression at transcriptional level.

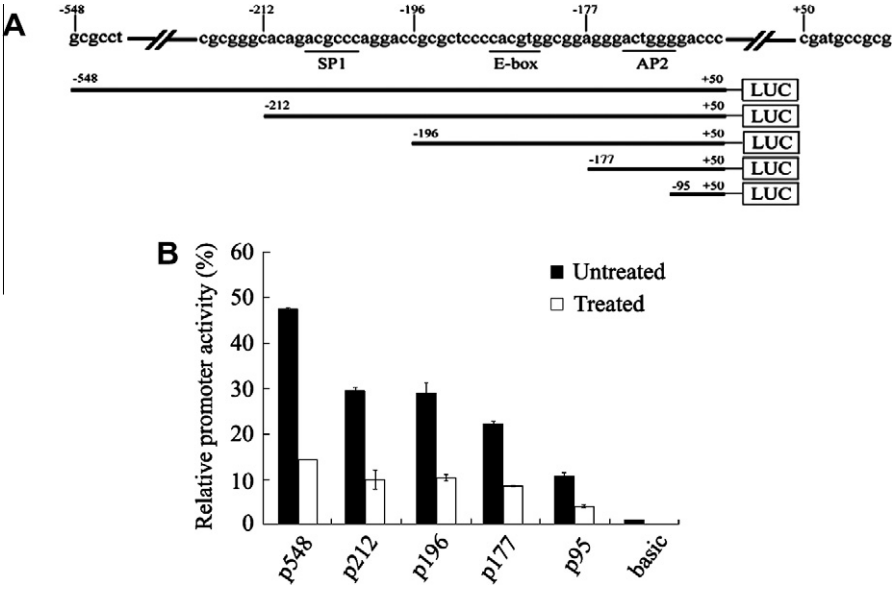

Figure 4. Effect of 2′-hydroxy-2,3,4′,6′-tetramethoxychalcone (HTMC) on hTERT promoter activity. A549 cells were transfected with luciferase reporter plasmid containing hTERT promoter deletion mutants p548 (−548 to +50), p212 (−212 to +50), p196 (−196 to +50), and p177 (−177 to +50), and were left untreated or treated with HTMC for 24 h followed by measurement of hTERT activity by luciferase reporter assay.

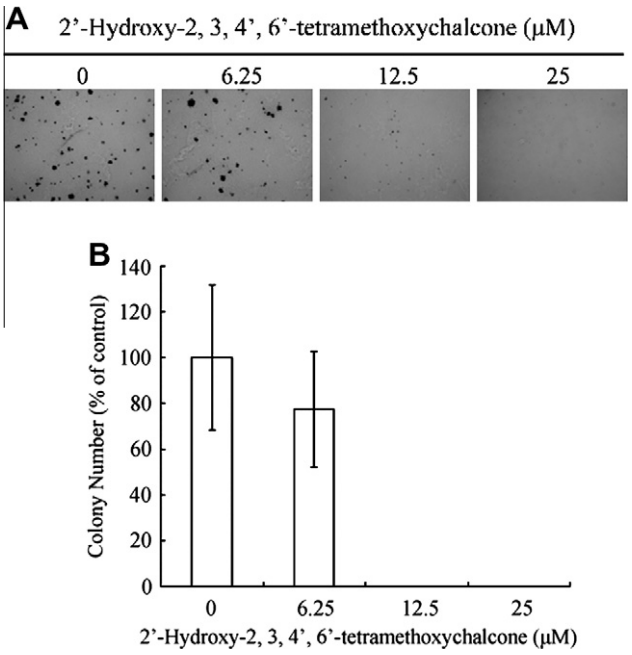

Figure 5. Effect of HTMC on anchorage-independent growth. (A) A549 cells were treated with different concentrations of 2′-hydroxy-2,3,4′,6′-tetramethoxychalcone in 0.35% agarose containing 10% FBS-DMEM over 0.5% agarose containing 10% FBS-DMEM, cell colonies were counted after 21-day incubation at 37 °C in 5% CO_2 under microscopy. (B) Size >30 μM were counted as a colony. Each bar represents mean ± SD of triplicate experiments.

We found that compound HTMC was the most potent among tested, and caused a dose-dependent decrease in TA and hTERT mRNA expression without a marked alteration of hTR or GADD153 as supported by both RT-PCR and Western blot experiments (Figs. 2 and 3). It was interesting to note that HTMC, a natural chalcone from a medicinal plant *Caesalpinia pulcherrima*,[28] has significant cytotoxic activity against Jurkat (human lymphocytic) and U937 (human monocytic) cells,[21] however, the molecular mechanisms of its anti-proliferative action on malignant cell growth are not clear. To the best of our knowledge, this was the first report to evaluate the compounds tested here for their TA inhibitory assay.

hTERT is a structurally and functionally important component of telomerase, which exerts its function of elongating telomere end repeats in the nuclei. Telomerase inhibition is sequence-dependent and the primary mode of regulation of hTERT is proposed to be transcriptional.[29] However, recent studies indicate that another possible mechanism is the posttranscriptional regulation which involved the translocation of hTERT from nuclei into cytoplasm.[29] As shown in Figure 6E, immunofluorescence staining of HTMC treated A549 cells demonstrated that the expression of hTERT protein decreased. In addition, although hTERT is detected in both nuclei and cytoplasm, the majority of the signal is localized in the nuclei, as the production of hTERT takes place in cytoplasm, therefore a weaker hTERT staining can also be detected. These results indicated that HTMC regulation of hTERT was transcriptional in A549 cells. In addition, the −548 to +50 bp region proximal to the transcription initiation site of the hTERT promoter is responsible for most of its transcriptional activity. This region has several binding sites include two typical E-boxes and several GC-boxes for the transcription factors c-myc and Sp1, respectively.[2] The core promoter p548, which is necessary for hTERT expression also contains Sp1/Sp3 binding sites.[2] Thus, investigation of mechanisms that modulate hTERT expression in tumor and normal cells has become an area of intense interest in the study of human cancer.[30] In this report, the data from the luciferase assays showed that HTMC decreased the hTERT promoter activity sequentially (Fig. 4). These results further indicated that HTMC treatment in A549 cells induced the down regulation of TA with suppression of hTERT expression at transcriptional levels. As the exact hTERT promoter-binding site of HTMC unknown, further studies were needed to elucidate the effects of HTMC on the hTERT promoter activity.

Anchorage independence is an important feature that allows tumor cells to survive under certain inappropriate host conditions, such as during invasion and metastasis.[31] In this report, A549 cells lost their ability to form colonies in the presence of HTMC for three weeks treatment (Fig. 5). One possible explanation to the observed effects was that HTMC-induced telomerase inhibition may cause a chronic DNA damage response of an uncapped telomere or a few chromosome ends thus reduced the colony formation efficiency of the A549 cells.[31]

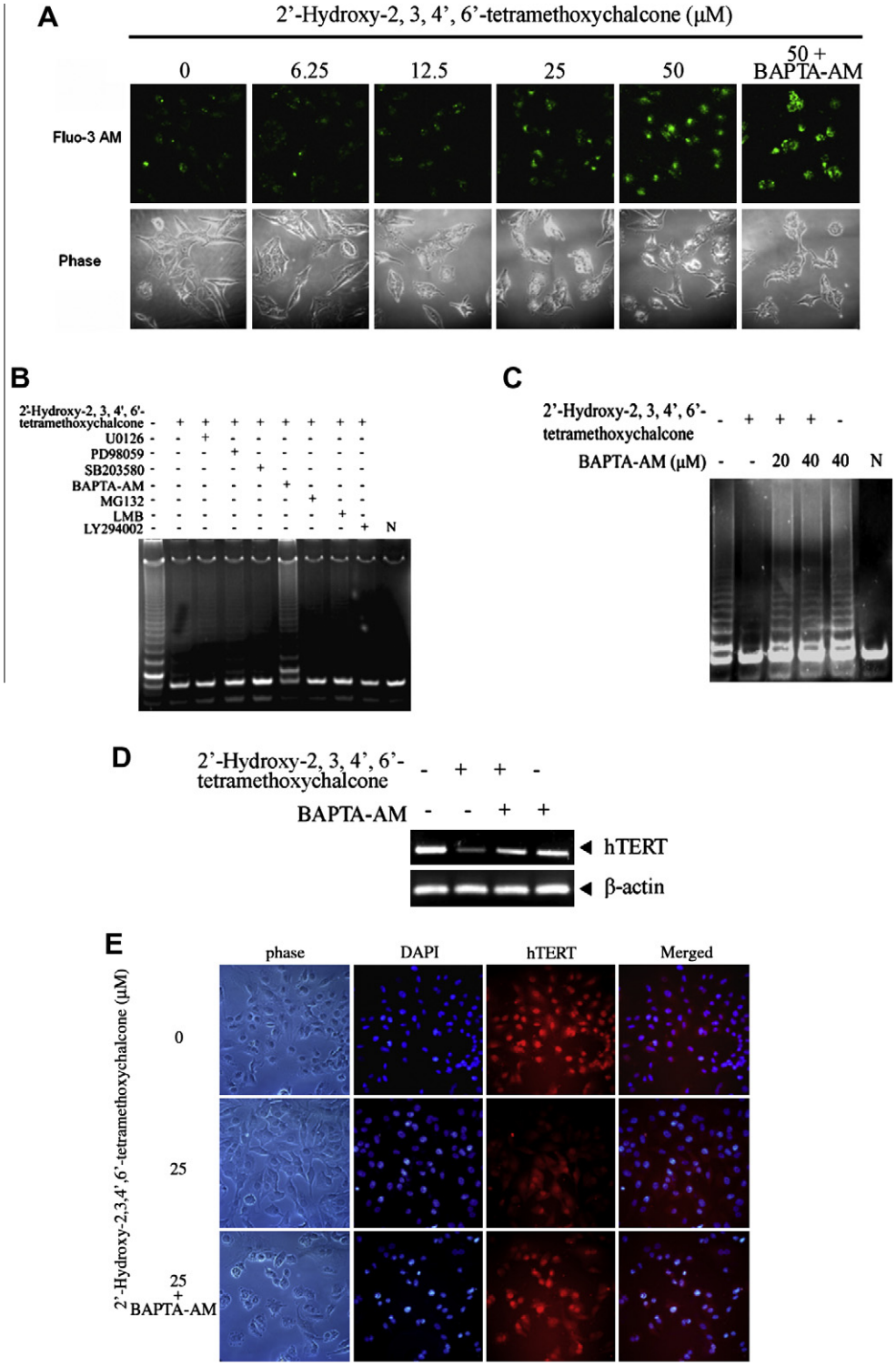

Figure 6. Involvement of intracellular calcium ion in 2′-hydroxy-2,3,4′,6′-tetramethoxychalcone (HTMC)-induced inhibition of telomerase activity in A549 cells. (A) A549 cells were treated with HTMC (0, 6.25, 12.5, 25.0 and 50.0 μM) for 24 h, and were treated with Fluo-3 AM for Ca^{2+} staining. (B) 1 h prior to incubation with HTMC, A549 cells were pretreated with U0126 (10 μM), SB 203580 (50 μM), BAPTA-AM (20 μM), MG132 (20 μM), Leptomycin B (20 nM), LY294002 (50 μM). (C) Intracellular calcium chelator BAPTA-AM (20 and 40 μM) was added to A549 cells 1 h prior to incubation with HTMC, after 24 h cells were harvested and subjected to TRAP assay. (D) Effect of BAPTA-AM on HTMC-induced suppression of hTERT mRNA level in A549 cells. BAPTA-AM (20 μM) was added to cells 1 h prior to incubation with HTMC and after 24 h total RNA was isolated and RT-PCR was performed using indicated primers. The amplified products were run in 1.5% agarose gel and visualized by ethidium bromide staining. β-Actin was used as an house-keeping control gene. (E) Effect of HTMC on the distribution of hTERT protein in A549 cells. Immunofluorescence staining for hTERT using specific anti-hTERT antibodies and TRITC goat anti-rabbit IgG (H+L) conjugate secondary antibodies in culture A549 cells after treatment with HTMC (25 μM) for 24 h.

Calcium is an intracellular second messenger, which regulates a variety of biological processes.[32] In most cases calcium does not act directly, but uses calcium-binding proteins as mediators of its signals then restored to basal level once signal is transmitted. Calcium binds to these calcium-binding proteins and induces conformational changes, thus exposing initially inaccessible protein binding

sites and allowing their interaction with specific target proteins.[32] Previously Rosenberger et al. (2007) suggest that calcium play a functional role in the inhibition of TA in epidermal keratinocytes.[33] These authors also reported that calcium-mediated inhibition of telomerase activity by S100A8/S100A9 complex, in which calcium is bound to S100A9 and subsequently releases S100A8 from the complex, immediately S100A8 binds to telomerase, thus reducing telomerase activity.[33] In our study TRAP assay indicated that telomerase activity is reduced in a dose-dependent and time-dependent manner (see Supplementary data Fig. 1). TA was significantly reduced at 25 μM HTMC, further analysis with this concentration at different treatment periods we found that telomerase activity declined with longer treatment periods (see Supplementary data Fig. 1), this suggested once HTMC was taken in by cells, its telomerase inhibiting effect was a continuous process, besides its ability of reducing hTERT expression in RNA and protein level (as shown in Fig. 3), it may also caused a direct inhibitory effect by up-regulating calcium concentration. Therefore an increased calcium concentration can still be observed after 24 h treatment of HTMC. On the other hand, as shown in Figure 6A, treatment with HTMC increased calcium concentration in the nuclei, this result suggested that calcium might play a role in the mechanism of telomerase inhibition by HTMC. Previous report indicate that a direct inhibition of telomerase activity by adding calcium chloride in the cell-free extract by TRAP assay,[33] and supported our finding that focal calcium stimulation in nuclei by HTMC may inhibited TA via similar manner. BAPTA-AM is a cell-membrane permeable calcium chelator, which widely used as an intracellular calcium sponge. As shown in Figure 6A, calcium concentration was increased within or around the nuclei area, whereas in A549 cells pretreated with BAPTA-AM, calcium stimulations were seen in condensed, scattered dots outside the nuclei. We hypothesize that calcium induced by HTMC was chelated by BAPTA-AM which resulted restored the TA in cells treated with HTMC. These results further supported by the data from Figure 6B and C, in which, along with various other inhibitors, TA was only restored in cells treated with BAPTA-AM, and supported our hypothesis that calcium participated in the mechanism of TA inhibition by HTMC. Taken together, these results demonstrated that HTMC inhibited telomerase activity through transcriptional regulation of hTERT and induction of Ca^{2+}-dependent pathway.

4. Conclusions

In summary, this report identified a potent telomerase inhibitor HTMC. In addition, HTMC decreased the expression of hTERT, and sequentially reduced the hTERT promoter in A549 cells. HTMC treatment also reduced the colony formation efficiency of A549 cells. Additionally, the results of this study demonstrated that the release of Ca^{2+} was the underlying mechanism of suppressed telomerase activity and hTERT transcription. Therefore, the potential of HTMC to inhibit telomerase activity and its associated molecular events, and the chemical nature of this low-molecular-mass compound that translates to lower cost of synthesis compared to nucleotide-based drugs suggested that it has potential to develop new therapeutic drug for cancer therapy.

5. Experimental

5.1. Reagents and materials

Specialized chemicals used were obtained from Sigma Chemical Company (Saint Louis, MO). The preparation and structure identification details of tested compounds are reported in our previous communications.[21–26] Antibodies against hTERT, hTR, GADD153, and substrate antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against β-actin was obtained from Sigma. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) was purchased from Promega (Madison, WI). Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Cell Signaling, MA) and enhanced chemiluminescence reagent (ECL) and Hyperfilm-ECL were purchased from Amersham (Little Chalfont). All other reagents used were of the analytical grade and obtained from Sigma.

5.2. Cell line and culture

Human lung adenocarcinoma cell line A549 was obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained at 37 °C in a 5% CO_2-humidified atmosphere on Dulbecco's modified Eagle's medium (DMEM) (Gibco, Rockville, MD) and Basal medium Eagle (BME) (Sigma, Saint Louis, MI) medium containing 10% fetal bovine serum (FBS) and 100 ng/mL each of penicillin and streptomycin (Life Technologies, Inc., Rockville, MD).

5.3. Telomere repeat amplification protocol (TRAP) assay

The TRAP assay was performed as we described previously.[13]

5.4. Cell proliferation assay

Cells were seeded at 1×10^7 cells/mL and then treated with the indicated concentrations of HTMC. After 24 h incubation, the cell number and viability were determined by MTS assay.[13]

5.5. Isolation of RNA and RT-PCR

The detailed steps of RNA isolation and RT-PCR were performed by the method as we described previously.[13] The primer sequences and PCR conditions for hTERT, hTR, GADD153 and β-actin were also performed as we described previously.[13] The products were visualized via electrophoresis on 1.5% agarose gel and stained with ethidium bromide. We confirmed the quality of cellular mRNA according to the intensity of β-actin.

5.6. Reporter gene assay

The hTERT promoter deletion mutants p548 (−548 to +50), p212 (−212 to +50), p196 (−196 to +50), and p177 (−177 to +50), cloned upstream of the firefly luciferase reporter in the pGL3-Basic vector (Promega Corp.) by following the protocol described previously.[13] For luciferase assay, cells (7.5×10^4) were seeded onto 24-well plates, cultured overnight, and transfected with the plasmids described above (1 μg/well) with DEAE-dextran (Amersham-Pharmacia plc, Little Chalfont, Bucks, UK). After 24 h incubation, the medium was carefully removed and fresh medium containing various concentrations of tested compound was added to the wells. The cells were treated continuously with HTMC for 24 h. Cells were collected and transcriptional activity was assayed with Luciferase Assay System (Promega). A plasmid expressing the bacterial β-galactosidase gene was co-transfected in each experiment to serve as internal control of transfection efficiency.

5.7. Western blot analysis

Anti-hTERT (ab32020, abcam) and anti-β-actin (AC-40, Sigma) were used for the detection of hTERT, and β-actin, respectively. The complete protocol for Western blot analysis has been described in our previous report.[13] Blots were developed with an enhanced luminol chemiluminescence (ECL) reagent (NEN, Boston).

5.8. Soft agar colony formation assay

Anchorage-independent growth assay of A549 cells was performed by the method as we described previously.[15]

5.9. Fluorescence immunocytochemistry and confocal microscopy

A549 cells were seeded (1×10^4 cells per chamber) onto an eight-chamber slide (Nunc 177402, Naperville, IL). Cells were rendered quiescent, followed by treatment with HTMC for the indicated concentration. Cells were fixed in 4% paraformaldehyde for 20 min and permeabilized with 0.3% Triton X-100 in PBS for 20 min. Washed cells was incubated in 1% BSA in PBS for 1 h. For co-immunostaining, cells were first incubated with an antibody against hTERT (rabbit, 1:250; 1 h, room temperature; Rockland, Gilbertsville, PA), and tetramethylrhodamine isothiocyanate goat anti-rabbit IgG (H+L) conjugate was used as a secondary antibody (1:100, 1 h; ZyMax™ Grade, Invitrogen, Carlsboad, CA). Slides were mounted with mount medium and dried at room temperature. Nuclei were counterstained with 0.2 μg of DAPI (4′,6-diamidino-2-phenylindole) per mL. Computer-assisted image analysis of fluorescence was performed using a confocal microscopy scanning laser microscope (Leica TCS, wavelength excitation 488 nm, emission 525 nm for FITC; 540/570 nm for TRITC).

5.10. Intracellular free calcium measurement

A549 cells were grown on poly-L-lysine coated glass coverslips at 5×10^4 cells in 35 mm dish, and then treated with 25 μM HTMC for 24 h. Other cells were pretreated for 1 h with MEK inhibitor U0126 (10 μM), or ERK inhibitor PD98059 (20 μM), or MAPK inhibitor SB203580 (50 μM), or intracellular calcium chelator BAPTA-AM (20 μM), or 26S proteasome inhibitor MG132 (20 μM), or nuclear export inhibitor leptomycin B (20 nM), or PI3 K inhibitor LY294002 (50 μM) and then treated with 25 μM HTMC for 24 h. Cells were rinsed twice with HBSS (20 mM HEPES, 10 mM glucose, 150 mM NaCl, 1.2 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2$, pH 7.4). They were then loaded with 3 μM Fura-3/AM dissolved in HBSS from a working solution and 0.02% pluronic acid F-127, at 37 °C for 60 min and then rinsed twice with HBSS. Next, the cells were incubated in HBSS for an additional 30 min to allow complete de-esterification of the dye. Calcium imaging was accomplished using an LSM 410 invert confocal laser scanning microscope (Carl Zeiss Jena, Germany). Excitation was done by the 488 nm line of an argon laser, emission was collected using a 505–550 band pass filter, and pinhole was set at 1.87 airy units. Confocal imaging was performed with a resolution of 512×512 pixel at 256 intensity. The frame rate was 1 frame/min. Several cells were viewed together through 20× plan-Neofluar Zeiss objective (0.5 NA) using a factor-2 computer zoomed image. Detailed images were also collected using a 40× plan-Neofluar Zeiss objective (1.3 NA).

5.11. Statistics

The data are presented as mean ± standard deviation of triplicate experiments. Statistical comparisons were made by means of one-way analysis of variance (ANOVA), followed by a Duncan multiple-comparison test. The symbol (*) indicates p <0.05 when compared with untreated controls.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.08.021.

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