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ORIGINAL ARTICLE

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Repression of telomerase reverse transcriptase mRNA and hTERT promoter by gambogic acid in human gastric carcinoma cells

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Abstract *Objectives*: To investigate the effects and potential mechanisms of gambogic acid (GA), a naturally occurring anticancer agent, on the expression and regulation of telomerase in human gastric carcinoma cells. *Methods*: GA-induced inhibition of cell proliferation was evaluated by the commonly employed MTT assay on two human gastric carcinoma cell lines, MGC-803 and SGC-7901. Telomerase activity and hTERT mRNA expression were determined by telomeric repeat amplication protocol-polymerase chain reaction and reverse transcription-polymerase chain reaction, respectively. The hTERT promoter activity was measured by luciferase assay. The expression of c-MYC, an apoptotic gene that modulates the expression of hTERT promoter, was quantified by Western blotting. *Results*: The proliferation of human gastric carcinoma cell lines, MGC-803 and SGC-7901, was significantly inhibited with GA treatment. Both telomerase activity and hTERT mRNA expression were notably decreased in cells treated with GA. The activity of hTERT promoter and the expression of c-MYC were also remarkably decreased in GA-treated cells. *Conclusion*: This study demonstrated that GA treatment of human gastric carcinoma cell lines, MGC-803 and SGC-7901, significantly reduced the expression of c-MYC in a time- and concentration-dependent manner accompanied with the down-regulation of the hTERT transcription and the ultimate reduction in telomerase activity. Our results indicate that the hTERT is a target of c-MYC activity and identify a feasible mechanism of GA's potent anticancer activity.

Keywords Gambogic acid · Gastric carcinoma cells · Telomerase · hTERT · hTERT promoter · c-MYC

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Introduction

Gambogic acid (GA) is the major active ingredient of gamboges [1, 2], a class of brownish to orange resins exuded from *Garcinia hanburyi* tree (genus *Garcinia* of family Guttiferae) in Southeast Asia. Traditionally, GA has been used as a coloring material and folk medicines due to its unique color and broad spectrum of cytotoxic activities [3, 4]. The structure of GA (Fig. 1) has been established from both detailed NMR spectroscopic analysis [2] and X-ray crystallographic studies [5]; however, the molecular mechanism of its cytotoxic activity, especially its potent antitumor activity remains poorly understood and warrants further investigations [6, 7].

We have previously demonstrated that the potent anticancer activity (both in vitro and in vivo) of GA is mainly attributed to its activation of the impaired apoptotic pathways in cancerous cells via down-regulation of telomerase [7–9]. Here we report the direct interaction of GA with oncogene c-MYC, a ubiquitous transcription factor involved in the control of cell proliferation and differentiation, as the molecular mechanism of GA's inhibitory effect on telomerase activity. It was observed that GA treatment of two human gastric carcinoma cell lines, MGC-803 and SGC-7901, significantly reduced the expression of c-MYC in a time- and concentration-dependent manner accompanied with the down-regulation of the hTERT transcription and the ultimate reduction in telomerase activity in these cells. The results of this study indicate that the hTERT is the molecular target

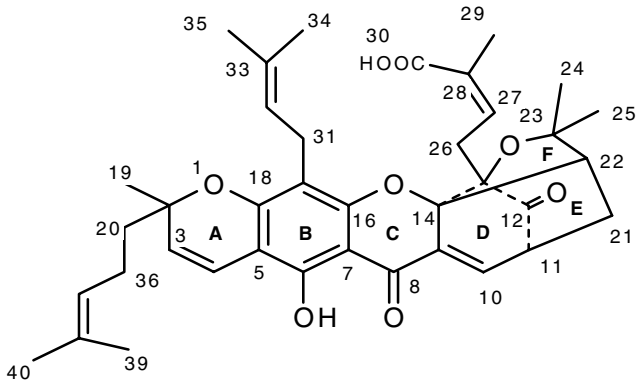

Fig. 1 Molecular structure and atom numbering scheme of gambogic acid ($C_{38}H_{44}O_8$, Mol. Wt.: 628.75)

of c-MYC activity and identify a plausible mechanism of GA's potent anticancer activity.

Materials and methods

Preparation of GA

The gamboge resin (lot # 20000526) of *G. hanburyi* was purchased from Jiangsu Provincial Medicinal Materials Company, China. GA was isolated and purified according to the methods previously described [9]. Preparations with GA content of 95% or higher were dissolved in RPMI-1640 medium (lot # 20010904, GIBCO, USA) and were used in all experiments unless otherwise indicated.

Reagents and antibodies

RPMI-1640 medium was purchased from GIBCO, USA. Heat-inactivated fetal bovine serum (FBS) was prepared by heating commercial FBS (from Sijiqing Company Ltd., China) at 56°C for 30 min in a water bath. Penicillin and streptomycin were supplied by Lukang Pharmaceutical Company Ltd., China. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, also known as MTT was obtained from Fluka, USA and was dissolved in 0.01 M PBS. Telomerase PCR enzyme-linked immunosorbent assay (ELISA) kit, TriPure isolation reagent, and DNase-free RNase were purchased from Roche, USA. RT-PCR kit was obtained from TaKaRa, Japan. Monoclonal antibody of c-MYC was purchased from Santa Cruz Biotech Ltd., USA.

Cell lines and cell culture

Human gastric carcinoma cell lines, MGC-803 and SGC-7901, were purchased from Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. The cells were cultured at 37°C in a Hirasawa incubator (Hirasawa, Japan) under a humidified atmosphere of 95% air + 5% CO_2 with RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 100 U/ml benzylpenicillin, and 100 μg/ml streptomycin.

Cell growth inhibition assay

Human gastric carcinoma cell lines MGC-803 and SGC-7901 were cultured in RPMI 1640 media as described above till mid-log phase. Cells were harvested by centrifugation at 250 *g* for 5 min and resuspended in RPMI-1640 + 10% FBS to make a stock cell suspension containing 2×10^5 cells/ml. Hundred microliters of this stock cell suspension was then added to the wells of a 96-well plate. GA was weighed and diluted with culture media to make a 10 mM stock solution. This stock solution is further diluted with culture media to make a series of secondary stock solutions so that addition of 1 μl of the secondary solutions will result in seven to eight different final concentrations (0.45–10.0 μM) of GA. Five experiments were performed in a parallel manner for each concentration point, and the results were reported as mean ± SD. Controls were performed in which only culture media was added into wells containing cells. Media was then added to bring the total volume of each well to 200 μl. The cells were then incubated at 37°C in a 5% CO_2, 95% air atmosphere. After 24, 48, and 72 h of incubation, the culture medium was removed and the cells washed twice with PBS. Then 20 μl of 5 mg/ml MTT was added to each well. The cells were further incubated at 37°C for 4 h. The supernatant was discarded and 100 μl of dimethyl sulfoxide (DMSO) was added to each well. The mixture was shaken on a micro-vibrator for 5 min and the absorbance was measured with an Enzyme Immunoassay Instrument at 570 nm that serves as a measure of cell viability [10–13]. The inhibition ratio (I%) was calculated by the following equation:

$$I\% = \left(\frac{A_{\text{control}} - A_{\text{treated}}}{A_{\text{control}}}\right) \times 100.$$

The IC_{50} was taken as the GA concentration that caused 50% inhibition of cell proliferation and was calculated by the Logit method.

Measurement of telomerase activity by TRAP-ELISA

A commercial telomerase polymerase chain reaction (PCR)-ELISA kit (Roche, Switzerland) was used to determine telomerase activity in control and GA-treated MGC-803 and SGC-7901 cells according to the manufacturer's instructions. The telomerase PCR-ELISA kit is a method derived from the telomeric repeat amplification protocol (TRAP) originally designed for measuring telomerase activity in proliferating cells [14, 15].

Briefly, MGC-803 and SGC-7901 cells were exposed to either different concentrations of GA for a given period of time (48 h) or a fixed concentration of IC_{50} (determined from MTT assay) for GA over different periods of time (24, 48, and 72 h). At the end of the specified incubation time, cells were harvested by centrifugation at 3,000 *g* for 10 min at 4°C. Cell pellets were washed with PBS and homogenized in appropriate amount of ice-cold lysis buffer [10 mM Tris–HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM β-mercaptoethanol, 0.5% 3-(3-cholamidopropyl) dimethylamino-1-propanesulfonate, and 10% glycerol] to make a cell suspension of 1×10^6 cells/ml. After 30 min of incubation on ice, the lysate was centrifuged at 16,000 *g* for 20 min at 4°C and the supernatants were kept at −80°C till needed. PCR amplication was carried out following standard protocols with slight modifications. Typically, 2 μl of the supernatant and 25 μl of reaction mixture containing 20 mM Tris–HCl (pH 8.0), 1.5 mM $MgCl_2$, 60 mM KCl, 0.005% Tween 20, 1 mM EGTA, 50 mM deoxynucleotide triphosphates (dNTPs), 0.2 μg of TS primer, 0.2 μg of CX primer [14], 1 μg of T4g 32 protein (Boehringer Mannheim), and 2.5 U of Taq DNA polymerase were transferred into an assay tube. Sterile water was then added to bring the final volume to 50 μl. After 30 min incubation at 25°C, the reaction mixture was heated at 90°C for 3 min and then subjected to 30 cycles of PCR including denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 90 s. The amplification product (5 μl) from the PCR reaction was mixed with 20 μl of denaturation reagent and incubated at room temperature for 10 min. An aliquot of 225 μl of hybridization buffer was then added to each well. After thorough mixing, 100 μl of the mixture from each well was transferred into the precoated microtiter plate (MTP) modules and incubated at 37°C for 2 h with shaking. Anti-digoxin-peroxidase (DIC-POD) working solution (100 μl) was then added and incubated at room temperature for another 30 min in the same shaker. Then the solution was removed completely from each well and the precipitate rinsed five times with 250 μl of washing buffer for a minimum of 30 s. After removal of the washing buffer, 100 μl of tetramethylbenzidine dihydrochloride (TMB) substrate solution was added to each well and the mixture was incubated at room temperature for 20 min with gentle shaking for color development. Finally, 100 μl of quenching reagent was added to each well to stop color development and the amount of TRAP products was determined by measuring the absorbance difference at 450 and 690 nm [15, 16].

Semi-quantitative determination of hTERT mRNA by RT-PCR

Total cellular RNA was extracted from GA (at IC_{50}) treated MGC-803 and SGC-7901 cells using the TriPure Solution following the manufacture's instructions. The purity of the RNA extracted was determined by the ratio of A_{260}/A_{280} using a BioPhotometer (Eppendorf, Germany). Reverse transcription-polymerase chain reaction (RT-PCR) was performed following the protocol supplied with TaKaRa kit [15, 17, 18]. Typically, 300 ng of total RNA was reverse-transcribed in a 20 μl reaction mixture containing 1× reverse transcriptase reaction buffer, 200 μM dNTPs, 10 ng random hexamer primer, 40 U of RNase and 100 U of Moloney murine leukemia virus (MMLV) reverse transcriptase for 50 min at 42°C, and then heated for 10 min at 72°C. After heat inactivation at 94°C for 4 min, 2 μl of the reverse-transcribed cDNA were subjected to 35 cycles of PCR in 48 μl of 1× reaction buffer [10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl], 200 μM dNTPs, 2.5 U of Taq DNA polymerase, and 0.2 μM of specific primers. Each PCR cycle consisted of denaturation at 94°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 30 s. The amplified PCR products were separated by electrophoresis on a 2% agarose gel containing ethidium bromide and quantitated by relative intensities of the bands as compared to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using Gel Base/Gel Blot/Gel Excel/Gel Sequence analysis software (UVP, UK). A value of 100% was given to the relative intensity of untreated cells (control). The sequences of PCR primers were as follows: the hTERT, 5′-AGA GTG TCT GGA GCA AGT TGC-3′ (forward) and 5′-CGT AGT CCA TGT TCA CAA TCG-3′ (reverse).

Detection of hTERT promoter by luciferase assay

pGL3-hTERT was synthesized by Sangon Corp (Shanghai, China), The expression of hTERT mRNA was analyzed by RT-PCR amplification using 5′-CGGAAGAGTGTCTGGAGCAA-3′ as the forward primer and 5′-GGATGAAGCGGAGTCTGGA-3′ as the reverse primer according to the hTERT's complete cDNA sequence (GeneBank Accession no. AF015950).

Luciferase reporter vector was constructed by inserting the hTERT promoter's 258 bp core sequence (−212 to +46 bp) into luciferase reporter vector pGL3-Basic, a promoter- and enhancerless vector (Promega) in sense orientation relative to the luciferase coding sequence at MluI and BglII sites [19].

Cells were seeded on 12-well plates at a density of 2×10^5 cells/well, cultured overnight, and transfected with 200 ng luciferase reporter plasmids pGL3-basic, pGL3-control, pGL3-hTP, respectively, using the Effectene Transfection Reagent (QIAGEN, Valencia, CA, USA). After transfection, cells were treated with different concentrations of GA (0.8/1.4, 1.4/1.5, 1.2/2.0, 1.4/2.5, and 1.6/3.0 μM) for 24 h.

Luciferase assay was performed using Dual-Luciferase Reporter Assay System on MiniLumat LB-9506 luminometer (Berthold Technologies, Bad Wildbad, Germany). The control plasmid PRL-TK was co-transfected with the luciferase reporter plasmids mentioned

above to standardize transcription efficiency. The experiments were independently repeated three times, and the experimental data were shown as average values with standard deviations included.

Detection of c-MYC protein by Western blotting

MGC-803 cells and SGC-7901 cells were harvested by two centrifugations at 3,000 *g* for 10 min at 4°C. The cell pellets were resuspended in appropriate amount of precooled PBS to make a suspension with cell density of 1×10^6 cells/ml. The cells were lysed with 1% Triton X-100 and incubated with primary antibody, anti-c-MYC (C-33) antibody (Santa Cruz Biotech, diluted 1:500), for 30 min at room temperature. The reaction mixture was then washed with PBS buffer three times for 15 min each and incubated at room temperature for 30min with goat antimouse IgG antibody (Santa Cruz Biotech, diluted 1:2,000) in a darkroom. After ext ensive washing (three times with PBS, 15 min each), the membranes were incubated with secondary antibody (antirabbit IgG-horseradish peroxidease-conjungated antibody, 1:10,000 dilution) and the proteins were visualized using an enhanced chemiluminescence detection system (Amersham) [20, 21]

Statistical analysis

All data were expressed as mean ± SD and statistically compared by one-way ANOVA with Dunnett's test or unpaired Student's *t* test in different experiments; $P < 0.05$ was taken as statistically significant.

Results

Inhibition of cell proliferation

Two human gastric carcinoma cell lines, MGC-803 and SGC-7901, were cultured in the absence and presence of varying concentrations of GA. The effects of GA on cell growth were assessed by the commonly used MTT assay. As shown in Fig. 2, GA-exhibited potent cytotoxic activity against the proliferation of both cell lines studied compared with negative controls at the same exposure time ($P < 0.01$, unpaired *t* test). The results in Fig. 2 also demonstrated that the efficiency of cell growth inhibition increased dramatically as the concentration of GA was increased. For a contact time of 48 h, the predicted IC_{50} of GA for the two cell lines, MGC-803 and SGC-7901, was 1.4 and 2.0 μM, respectively. It was noted that the efficiency of growth inhibition was also correlated with exposure time at a given GA concentration. However, the dependence on exposure time was only significant when GA concentration was below IC_{50}. When GA was applied at higher concentrations, the effect of contact time became less significant.

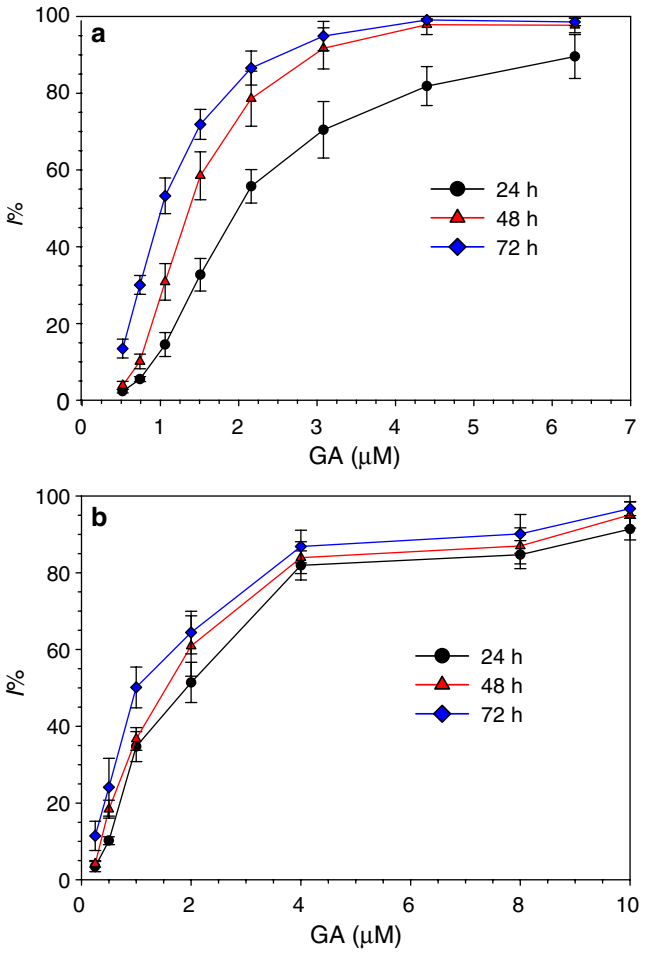

Fig. 2 Effects of GA on the proliferation of human gastric carcinoma cell lines MGC-803 (**a**) and SGC-7901 (**b**). The results shown were the mean of five parallel experiments for each concentration point

Repression of telomerase activity

Telomerase activity in GA-treated MGC-803 and SGC-7901 cells was measured by the telomerase PCR-ELISA kit [14] and was expressed by the difference between absorption at 450 and 600 nm ($A_{450}-A_{690}$). As shown in Fig. 3, GA treatment resulted in the significant decrease in telomerase activity in the two cell lines studied. When GA was applied at concentrations corresponding to the IC_{50} values for MGC-803 and SGC-7901 cells, the detected telomerase activity progressively decreased as exposure time increased. For example, When MGC-803 cells were exposed to 1.4 μM GA for 24, 48, and 72 h, the detected telomerase activity steadily decreased from 2.11 in untreated controls to 1.78, 1.22, and 0.54, respectively (Fig. 3a). Compared to the untreated controls, telomerase activity in MGC-803 cells was reduced to 60.2, 23.3, and 8% upon treatment with 1.4 μM GA for 24, 48, and 72 h, respectively (Fig. 4a). Therefore, a contact time of 72 h is necessary to reduce telomerase activity to a level comparable to that in negative controls

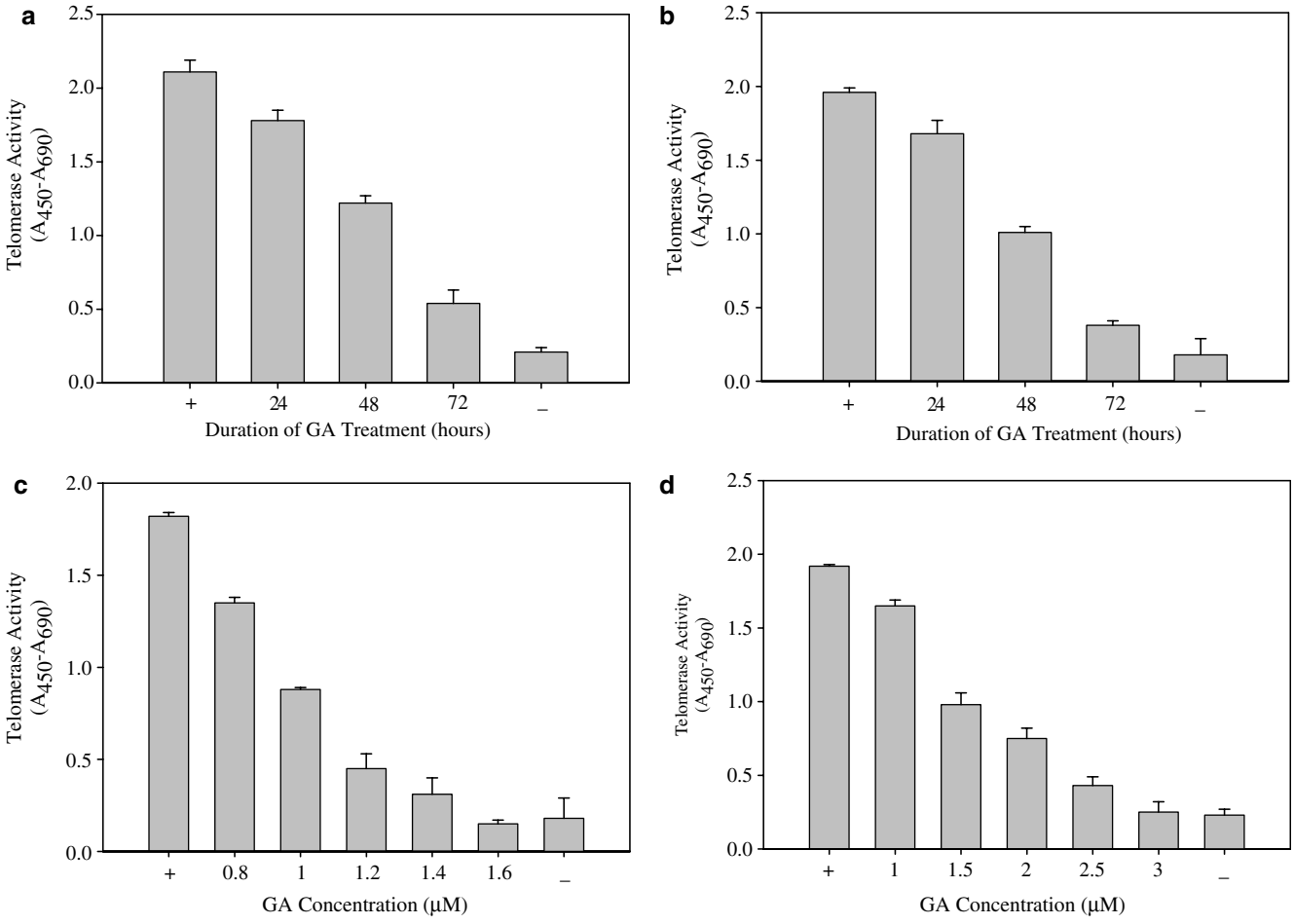

Fig. 3 Effects of GA treatment on telomerase activity in human gastric carcinoma cell lines MGC-803 and SGC-7901. Telomerase was extracted from MGC-803 and SGC-7901 cells treated with 1.4 μM (MGC-803) or 2.0 μM (SGC-7901) GA for different period of time (**a**, **b**) or at various concentrations for a period of 48 h (**c**, **d**). Telomerase activity was determined using the TRAP-ELISA method as described in Materials and methods. Absorbance shown was the mean ± SD of data from at least three independent experiments. Negative control represents assays without telomerase extract added (−), while positive control denotes assay using extract from untreated cells (+). **a** MGC-803 cells treated with 1.4 μM GA for 24, 48 and 72 h, respectively. **b** SGC-7901 cells treated with 2.0 μM GA for 24, 48 and 72 h, respectively. **c** MGC-803 cells treated with various concentrations (0.8, 1.0, 1.2, 1.4, 1.6 μM) of GA for 48 h. **d** SGC-7901 cells treated with various concentrations (1.0, 1.5, 2.0, 2.5, 3.0 μM) of GA for 48 h

(0.21) where no telomerase extract was added in the assay. The average absorbance of 0.21 U indicates the absence of telomerase activity in our negative controls [16]. Similar results were observed for SGC-7901 cells as shown in Fig. 3b. After exposure to 2.0 μM GA for 24, 48, and 72 h, the detected telomerase activity in SGC-7901 cells was gradually decreased from 1.96 in untreated controls to 1.68, 1.01, and 0.38, respectively (Fig. 3b). Judged against the untreated controls, telomerase activity in SGC-7901 cells was decreased to 68.1, 34.2, and 9% upon treatment with 2.0 μM GA for 24, 48, and 72 h, respectively (Fig. 4b).

On the other hand, when MGC-803 cells were exposed to varying concentrations (0.8, 1.0, 1.2, 1.4, and 1.6 μM) of GA for a given period of time (48 h), the observed telomerase activity showed a similar progressive decrease from 1.82 in untreated controls to 1.35, 0.88, 0.45, 0.31, and 0.15, respectively (Fig. 3c). Taking the telomerase activity in untreated controls as 100, telomerase activity in MGC-803 cells was decreased to 74, 48, 25, 17, and 9% upon 48 h of treatment with 0.8, 1.0, 1.2, 1.4, and 1.6 μM, respectively (Fig. 4c). Therefore, treatment with higher concentrations of GA is more efficient in the suppression of telomerase activity. When GA is applied at a dose of 1.6 μM, a contact time of 48 h is sufficient to completely inhibit telomerase activity as judged against negative controls (0.18) where no telomerase extract was added in the activity assay.

Similarly, when SGC-7901 cells were exposed to varying concentrations (1.0, 1.5, 2.0, 2.5, and 3.0 μM) of GA for a given period of time (48 h), the observed telomerase activity showed an essentially similar gradual decrease from 1.92 in untreated controls to 1.65, 0.98, 0.75, 0.43, and 0.25, respectively (Fig. 3d). Assuming the telomerase activity in untreated controls as 100, telomerase activity in SGC-7901 cells was decreased to 86,

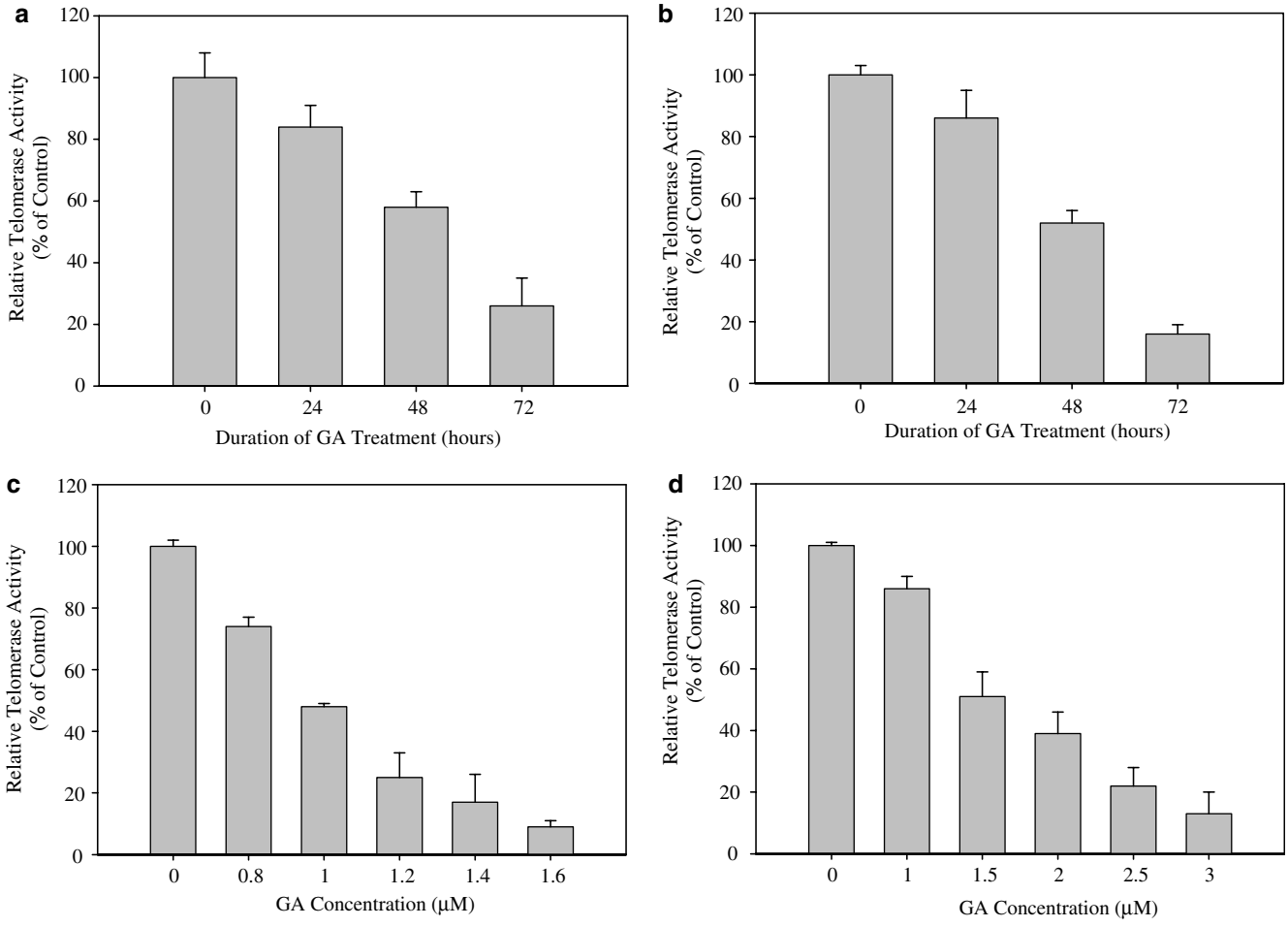

Fig. 4 Semi-quantitative analysis of relative telomerase activity in GA-treated MGC-803 cells/SGC-7901 cells. Telomerase was extracted from cells treated with 1.4 μM (MGC-803) or 2.0 μM (SGC-7901) GA for different period of time (**a** for MGC-803 and **b** for SGC-7901) or at various concentrations for a period of 48 h (**c** for MGC-803 and **d** for SGC-7901). Telomerase activity was determined using the TRAP-ELISA method as described in Materials and methods. The data shown are representatives of at least three independent experiments. Negative control represents assay without telomerase extract added, while positive control denotes assay using extract from untreated cells. Telomerase activity in untreated cells was taken as 100%

51, 39, 22, and 13% upon 48 h of treatment with 1.0, 1.5, 2.0, 2.5, and 3.0 μM, respectively (Fig. 4d). This is in complete agreement with the results obtained from MGC-803 cells. Obviously, treatment with higher concentrations of GA is more efficient in the suppression of telomerase activity. When GA is applied at a dose of 3.0 μM, a contact time of 48 h is sufficient to completely inhibit telomerase activity as compared with negative controls (0.23) where no telomerase extract was added in the activity assay.

Down-regulation of hTERT mRNA

RT-PCR experiments were performed to examine whether the down-regulation of telomerase activity in GA-treated MGC-803 and SGC-7901 cells was due to a suppressed expression of hTERT, the catalytic subunit of human telomerase. It was noted that treatment significantly reduced the expression of hTERT mRNA as evaluated by the quantitation of the amplification products using a Gel Base/Gel Blot/Gel Excel/Gel Sequence analysis software (Fig. 5). Our data indicated that the reduced telomerase activity in GA-treated MGC-803 and SGC-7901 cells was indeed caused by the suppressed expression of the hTERT gene.

Repression of transcriptional activity of hTERT promoter

The hTERT promoter activities of pGL3-hTERT in both MGC-803 and SGC-7901 cells were measured by Dual-Luciferase reporter assay. As shown in Fig. 6, when MGC-803 cells were treated with GA (1.0 μM), the activity of luciferase in pGL3-hTERT was dramatically decreased as compared to that of pGL3-control and pGL3-basic (Fig. 6a). Similarly, when SGC-7901 cells were treated with GA (2.0 μM), the activity of luciferase in pGL3-hTERT was significantly decreased

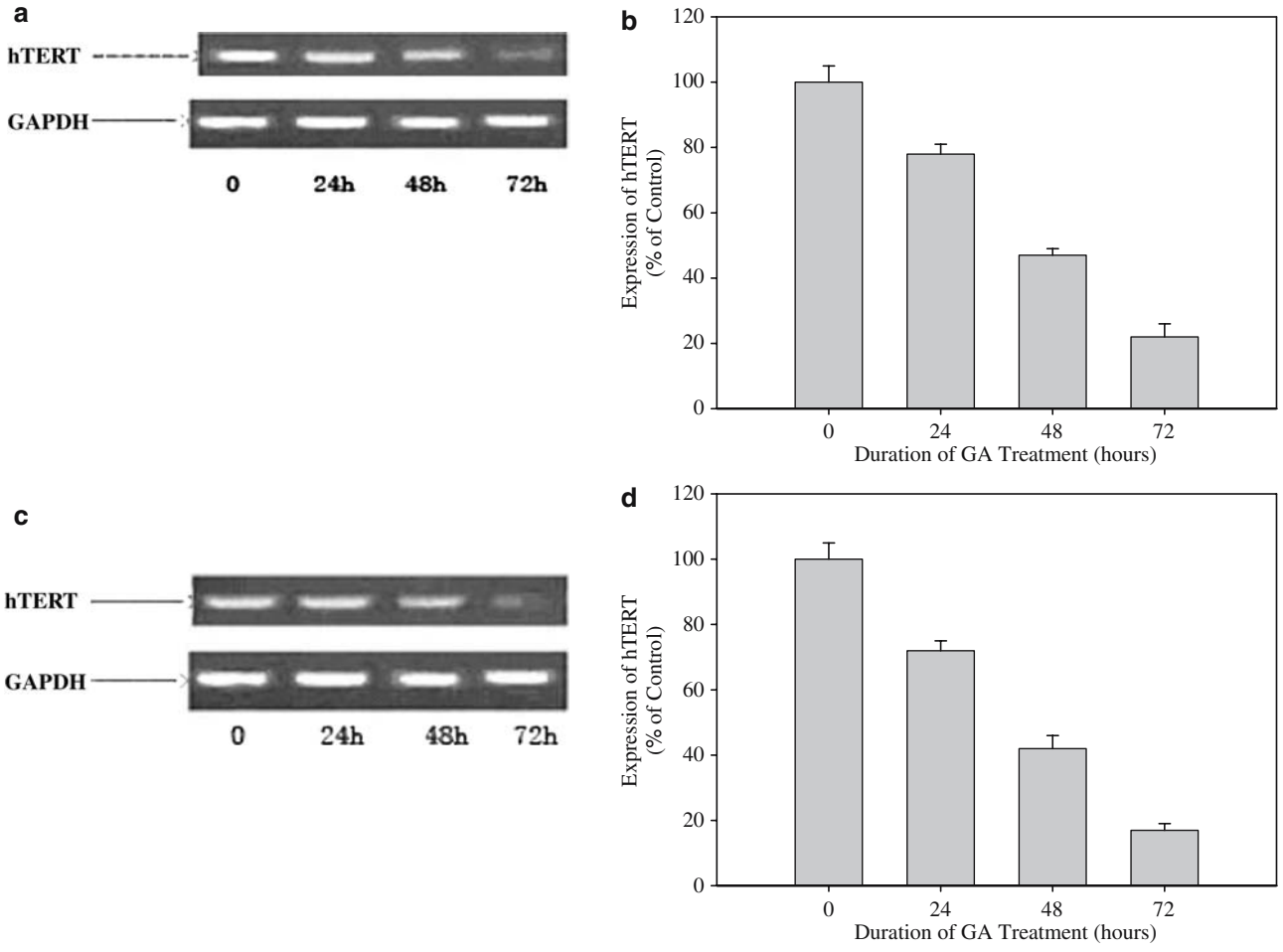

Fig. 5 Expression of hTERT mRNA in MGC-803 (**a**) and SGC-7901 (**c**) cells treated with GA (1.4 μM for MGC-803 and 2.0 μM for SGC-7901) as a function of contact time. The hTERT mRNA was co-amplified with GAPDH by RT-PCR. Amplified DNA was electrophoresed through 2% agarose gels and visualized by staining with ethidium bromide. The data presented are representatives of at least three independent experiments. DNA quantification was performed using the Gel Base/Gel Blot/Gel Excel/Gel Sequence analysis software (**b**, **d**). Data were obtained from three independent experiments and expressed as a ratio of AUPhTERT/AUPGAPDH. *AUP* area under peak. The *numbers in parentheses* represent the treatment time

as compared to that of pGL3-control and pGL3-basic (Fig. 6b).

Inhibition of c-MYC protein

To examine whether expression of c-MYC protein was also inhibited by GA treatment, Western blot experiments were carried out by exposing MGC-803 and SGC-7901 cells to GA at concentrations corresponding to their respective IC_{50} for a period of 24, 48, and 72 h. As shown in Fig. 7, more than 96–98% of c-MYC protein was expressed in cells not treated with GA as indicated by the intense 67 kDa band in both the control group and the group of 24 h treatment. However, with the prolongation of GA treatment, significant decrease in c-MYC protein expression was observed as judged by the obvious intensity decrease of the 67 kDa band. It is worth mentioning that increasing the treatment from 24 to 48 h dramatically reduced the expression of c-MYC protein from 67 to 37% in MGC-803 cells (Fig. 7a) and from 71 to 33% in SGC-7901 cells (Fig. 7b), indicating the importance of treatment time to the suppression of c-MYC protein expression.

Discussion

Our previous studies have firmly demonstrated that GA, the major active component extracted from the gamboge resin of *G. hanburyi*, has potent cytotoxic activities against several human carcinoma cell lines both in vitro and in vivo [7–9]. However, the molecular mechanism of GA's antitumor activity remains controversial and warrants further investigations. In this study, we demonstrated the direct interaction of GA with oncogene c-MYC as the molecular mechanism of GA's potent antitumor activity in two human gastric carcinoma cell

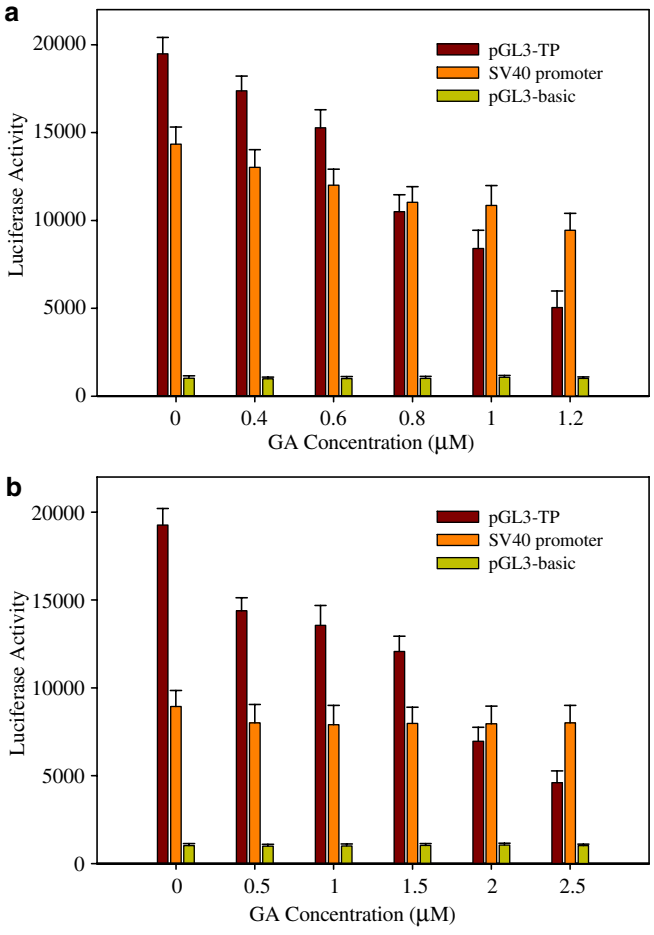

Fig. 6 Specific inhibition of hTERT promoter in GA-treated MGC-803 (**a**) and SGC-7901 (**b**) cells. Dual luciferase activity was used to examine the transfection effect with pGL3-hTERT, pGL3-control, and pGL3-basic plasmid. Data were obtained from three independent experiments. When MGC-803 cells were treated with GA (1.4 μM) the value of luciferase in pGL3-hTERT was significantly decreased; however the value of pGL3-control and pGL3-basic was expressed as usual (**a**). Similarly, when SGC-7901 cells were treated with GA (2.0 μM), the activity of luciferase in pGL3-hTERT was also dramatically decreased (**b**)

lines, corroborating with our previous results using human hepatoma cell line, SMMC-7721 [22]. MTT assay showed that GA displayed significant inhibition on the proliferation of both MMC-803 and SGC-7901 cells in a dose- and time-dependent manner with a median inhibitory concentration (IC_{50}) of 1.4 (for MMC-803) and 2.0 μM (for SGC-7901) after a contact time of 48 h. Extending the contact time to 72 h increased the inhibition rate to 89.45 and 81.12% for MMC-803 and SGC-7901 cells, respectively (Fig. 2). Our in vivo studies have showed that GA did not affect body weight, white blood cell number in rats inoculated with these carcinoma cell lines (Q.L. Guo et al., unpublished results), suggesting that GA is capable of discriminating transformed (targets) from normal cells. TRAP experiments showed that GA-treated MMC-803 and SGC-7901 cells experienced a profound down-regulation of telomerase activity due to a suppressed expression of the hTERT mRNA as indicated by our RT-PCR experiments. Most significantly, the present study revealed for the first time that GA treatment notably reduced the expression of c-MYC protein in human gastric carcinoma cell lines. It seems that GA's potent anticancer activity is achieved through a series of strictly ordered molecular events. GA first interacts with c-MYC on the promotor region of the hTERT. This interaction impairs the expression of the hTERT mRNA that in turn decreases the synthesis of telomerase and thus the observed reduction in telomerase activity, a biomarker of cell immortalization and malignant transformation.

The role of telomerase, a ribonucleoprotein responsible for maintaining the ends of chromosomes, has been the subject of intense investigation in recent years due to its potential role in aging, cancer, and immortalization [23–25]. Studies of the telomerase enzyme complex have revealed the presence of two major subunits that contribute to the catalytic activity of the enzyme—an RNA component (hTER) that serves as the template for the polymerase activity and a catalytic subunit that displays reverse transcriptase activity (hTERT). Although both hTER and hTERT are necessary for reconstitution of telomerase activity in vitro [18, 26, 27], they have different distribution within cells. While hTER is widely expressed in embryonic and somatic tissues, hTERT is tightly regulated and is not detectable in most somatic cells. The hTERT mRNA expression temporally parallels changes in telomerase activity during cellular differentiation and neoplastic transformation [28, 29]. Further support for the essential role of hTERT comes from recent studies showing that ectopic expression of hTERT is sufficient for restoring telomerase activity in a number of telomerase-negative cell lines, including foreskin fibroblasts, mammary epithelial cells, retinal pigment epithelial cells, and umbilical endothelial cells [25, 27, 30]. It has also been reported that the activated oncogene c-MYC plays a key role in the modulation of hTERT [29, 31, 32]. Because c-MYC is amplified in many different types of human cancers, it is possible that c-MYC is a critical factor in regulating cell proliferation and oncogenesis.

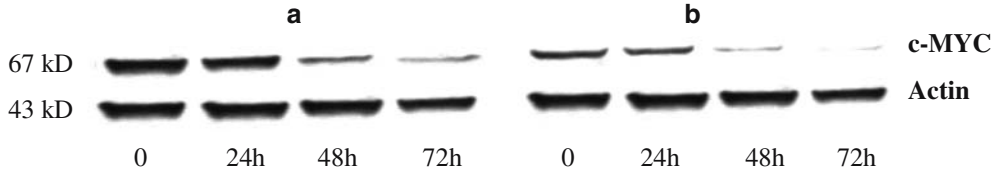

Fig. 7 Western blotting analysis of the expression of c-MYC protein in GA-treated MGC-803 (**a**) and SGC-7901 (**b**) cells as a function of contact time

Based on the fact that the hTERT promoter contains several c-MYC binding sites, we postulated that the c-MYC binding region of the hTERT might be the direct target of GA. The significantly decreased telomerase activity in both MGC-803 and SGC-7901 cells upon GA treatment proved this hypothesis. Telomerase activity was essentially negligible in both MGC-803 and SGC-7901 cells treated with GA at concentrations corresponding to their respective IC_{50} for a period of 48 h, probably due to the dramatic decrease in hTERT mRNA synthesis that was caused by the reduction of c-MYC expression in tumor cells. Although activation of the hTERT and telomerase has been observed in many human carcinomas, the mechanism of their regulation remains controversial [30, 33]. One plausible theory suggests the formation of a heterodimeric complex between c-MYC oncoprotein and the Max protein is responsible for the activation of gene transcription [34]. This c-MYC/Max dimer recognizes and binds the consensus sequence, 50-CACGTG-30, known as an "E-box" [17]. Furthermore, a related sequence, 50-CA(C/T)GCG-30, also binds c-MYC/Max heterodimer providing additional mechanisms of regulation through binding at these noncanonical sites [35]. Of the 29 potential binding sites for c-MYC complexes that have been identified in the region of the hTERT gene, the main focus has been on the two E-boxes (50-CACGTG-30) located centrally within the hTERT core promoter. Direct activation of the hTERT by c-MYC at these E-boxes has been suggested by gene reporter analyses using the luciferase gene and mobility shift assay [19, 36]. Our luciferase assay results presented in this report indicated that GA could specially inhibit the activity of pGL3-hTERT in the two human gastric carcinoma cell lines studied. Further evidence for the direct c-MYC activation of the hTERT was provided by studies demonstrating that c-MYC-induced upregulation of the hTERT occurs in the absence of new protein synthesis [30, 37, 38]. Our Western blotting experiments demonstrated that the expression of c-MYC protein correlated perfectly with the synthesis of hTERT and telomerase activity. The c-MYC protein can be detected when cells were treated with GA for 24 h; however it nearly vanished when the contact time was extended to 48 h. Considering the fact that the two E-boxes in the promoter region of the hTERT are the direct binding sites of c-MYC, it is concluded that c-MYC binds within E-boxes in the promoter region of the hTERT. This binding directly affects the expression of the hTERT mRNA, which in turn modulates the activity of telomerase in the two gastric carcinoma cell lines employed in this study.

In conclusion, the results reported in this paper demonstrated that GA-inhibited telomerase activity by down-regulating the expression of the hTERT gene (a target of c-MYC activity) and identify a plausible mechanism for GA's potent anticancer activity in cultured human gastric carcinoma cell lines.

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