

Gambogic Acid Inhibits Proliferation of Human Lung Carcinoma SPC-A1 Cells *in Vivo* and *in Vitro* and Represses Telomerase Activity and Telomerase Reverse Transcriptase mRNA Expression in the Cells

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We determined the *in vivo* and *in vitro* antitumor activities of gambogic acid (GA) and one of the possible mechanisms for its inhibitory activities. *In vivo* antitumor activity of GA was evaluated by the relative tumor growth ratio (T/C) in nude mice, and *in vitro* inhibition of SPC-A1 cells was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and trypan blue exclusion assay. Telomere repeats amplification protocol (TRAP)-polymerase chain reaction (PCR)-enzyme-linked immunosorbent assay (ELISA) and RT-PCR were used to quantitatively detect telomerase activity and the expression of human telomerase reverse transcriptase (hTERT) mRNA, respectively. Results from our *in vivo* study showed that transplantable tumor growth remained suppressed for up to 21 d with minimal animal weight loss in nude mice treated with gambogic acid (*i.v.*). Proliferation of SPC-A1 cells cultured *in vitro* was significantly inhibited ($p < 0.01$), showing time-dependent and dose-dependent inhibition. Telomerase activity and hTERT mRNA expression were both decreased significantly, when cells were exposed to gambogic acid for 24, 48 and 72 h (for 24 h $p < 0.05$, and for 48, 72 h, $p < 0.01$). These results suggest that gambogic acid could inhibit the growth of SPC-A1 cells and its tumor xenografts, and when treated with gambogic acid for a period of time, telomerase activity and expression of hTERT mRNA in the tumor cells were both inhibited significantly. It is safe, at least in part, to conclude that the down-regulating telomerase activity of GA by modifying partly the expression of hTERT mRNA in SPC-A1 cells may be one possible mechanism for the inhibitory activity of GA in the cells.

Key words gambogic acid; SPC-A1 cell; antitumor effect; telomerase; human telomerase reverse transcriptase (hTERT) mRNA

Gambogic acid (GA, C₃₈H₄₄O₈) is the main active factor of gamboge, a brownish or orange resin obtained from *Garcinia hanburyi* Hook F. (genus *Garcinia* of family Guttiferae). It was reported in traditional Chinese medical documents that gamboge is cold, acidic, unsmooth, and poisonous. Gamboge can also detoxify, kill parasites and be used as a hemostatic agent, but its antitumor activity was first discovered by Chinese pharmacologists and clinical therapists after 1949. The investigations on gamboge were undertaken mainly in the 1970–80s, during which time the Cooperation of Gamboge Antitumor Investigation (CGAI) was founded. CGAI has separated and identified the chemical structure of GA (Fig. 1), and studied the antitumor activity of the crude extract of gamboge in ethanol *in vitro* and *in vivo*, as well as its absorption, distribution and excretion in mice.^{2–6} They also studied clinically the antitumor activity and toxicity of the crude extract.^{7,8} Besides GA, the crude extract also consisted of other compounds such as organic acids, terpenes and alkaloids. CGAI was unable to determine the content of GA in the crude extract. As a result, the work of CGAI could not be continued. In the 1990s, Kong *et al.* investigated gamboge

activity and the components using different extraction methods.⁹ In 1996, Asano *et al.* reported that they had extracted 11 compounds from gamboge.¹⁰ Recently, they have modified the carboxyl of GA to acyl-amine and discovered some more active compounds. However, there is no report on the mechanism of the antitumor activity of GA.

Observations from our previous studies *in vivo* have indicated that GA inhibited the growth of Heps, S180, EAC and EC in Kunming species mice, and Lewis lung carcinoma in C57BL/6 mice (unpublished observations and ref. 11). Results from our previous study *in vitro* also showed that GA had a significant inhibitory effect on cultured human hepatoma SMMC-7721 cells, BEL-7402 cells, human gastric carcinoma MGC-803 cells, and SGC-7901 cells, and the induction of apoptosis has been proven to be one possible mechanism for the inhibition of GA on the growth of these tumor cells (unpublished observations and ref. 12). The objective of this study was to determine other possible mechanisms for the inhibitory activity of GA by systematically studying the growth inhibitory effect of GA in human lung carcinoma SPC-A1 cells and its transplantable tumor in nude mice, and then investigating the changes in telomerase activity and human telomerase reverse transcriptase (hTERT) mRNA expression in the cells before and after GA treatment.

MATERIALS AND METHODS

Plant Material The gamboge resin of *G. hanburyi* was purchased from Jiangsu Provincial Medicinal Materials Company, China, and the lot number was 20000526. The person who identified the collected substance was Dr. Feng Feng, Department of Natural Medicinal Chemistry of China

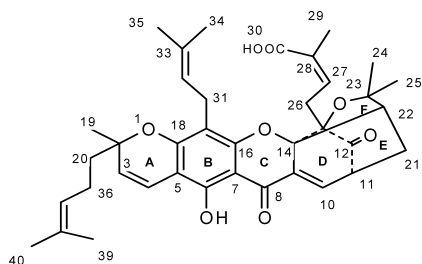


Fig. 1. Chemical Structure of Gambogic Acid (GA)

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Pharmaceutical University. A specimen of the gamboge resin is stored at the Department of Physiology of China Pharmaceutical University, and the specimen number is also recorded and permanently stored at the department.

Medicine GA was provided by the Department of Physiology, China Pharmaceutical University. Hydroxycamptothecine (OPT), an inhibitor of DNA topoisomerase I which was used as the reference drug, was purchased from Feiyun Pharmaceutical Company, Huangshi, China. Both were dissolved in 0.9% NaCl before use in the *in vivo* experiments, and in RPMI-1640 medium in the *in vitro* experiments.

Animals Male and female BALB/cA nude mice, 35–40 d old and weighing 18–22 g, were supplied by Shanghai Institute of Materia Medica, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences. The mice were raised in air-conditioned rooms under controlled lighting (12 h lighting/d) and were fed standard laboratory food and water *ad libitum*. The animal care and surgery protocols were designed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health, U.S.A.

Cells and Reagents Human lung carcinoma SPC-A1 cells, provided by the Cell Bank of the Shanghai Institute of Biochemistry & Cell Biology, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, were maintained in RPMI-1640 medium (Gibco, U.S.A.) supplemented with 10% heat-inactivated fetal bovine serum (Sijiqing, Hangzhou, China), benzylpenicillin 100 KIU/l, streptomycin 100 mg/l, pH 7.4 in a humidified atmosphere of 95% air + 5% CO₂ at 37 °C. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and RobusT II reverse transcription-polymerase chain reaction (RT-PCR) Kit were obtained from Sigma and RobusT, respectively. The Telomerase PCR-enzyme-linked immunosorbent assay (ELISA) kit, TriPure Isolation Reagent, and DNase-free RNase were purchased from Roche.

Measurement of the Relative Growth Ratio T/C (%) in Nude Mice¹³⁾ Transplantable tumors (induced by 5 × 10⁶ SPC-A1 cells injected s.c. into nude mice) were chopped into fragments (about 1.5 mm³), each of which was transplanted (s.c.) into the right axillary fossa of 24 nude mice. When a tumor had increased to 100–300 mm³, the mice were equally randomized into 4 groups (negative control, 3 mg/kg OPT, 4 and 8 mg/kg GA). Tumor size was measured twice a week in two perpendicular dimensions with Vernier Calipers and converted to tumor volume (TV) using the formula: $(a \times b^2)/2$, where *a* and *b* refer to the longer and shorter dimensions, respectively. The body weights of the animals were measured twice a week at the same times as the tumor dimension measurement and mortality was monitored daily. Medicines were administered (i.v.) three times a week for 3 weeks. Relative tumor volume (RTV) was calculated by the formula: V_t/V_0 (*V*₀ was TV at day 0 when the mice were divided; *V*_{*t*} was TV of each measurement). The evaluation index for inhibition was the relative tumor growth ratio $T/C = T_{RTV}/C_{RTV} \times 100\%$, where *T*_{RTV} and *C*_{RTV} represented RTV of the treated and control groups, respectively.

Determination of Growth Curve by Trypan Blue Exclusion Assay¹⁴⁾ The logarithmically growing cells were dispersed with 0.02% ethylenediaminetetraacetic acid (EDTA) to prepare the 3 × 10⁴/ml cell suspension and then seeded into

40-well micro-culture plates (180 μl/well). When the cells had adhered, different concentrations of GA were given (20 μl/well). The control group was only given the same volume of dilute solution without any drugs. After incubation for 12, 24, 36, 48, 60 and 72 h, the suspended and adherent cells were both collected to prepare cells suspension, and then 40 μl of the suspension from each well was transferred to a cytometric plate, plus 10 μl of 0.4% trypan blue. Live cells that could exclude the dye solution were counted manually under an optical microscope at ×100, and then the growth curves for SPC-A1 cells were drawn.

Measurement of Proliferation of SPC-A1 Cells by MTT Assay The growth inhibitory effect of GA on SPC-A1 cells was measured by MTT assay.^{15,16)} The logarithmically growing cells were digested with 0.02% EDTA to prepare a 3 × 10⁴/ml cell suspension. The cell suspension was partitioned into the wells of 40-well plates with 100 μl/well and cultured. When the cells had adhered, different concentrations of GA were added to the wells (100 μl/well). The control group was given the same volume of dilute solution without any drugs. After 24, 48 and 72 h culture, 5 g/l MTT solution (20 μl/well) was added to the wells. After another 4 h culture, the supernatant was discarded and 0.04 mol/l isopropanol hychloride was added (100 μl/well). The suspension was then shaken on a micro-vibrator for 5 min and the absorbance (*A*) was read at a wavelength of 570 nm on an enzyme immunoassay instrument. The cell inhibitory ratio for the cell growth was calculated using the following formula:

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

The efficacy of the inhibitory effect of GA on the growth of SPC-A1 cells was expressed as IC₅₀ (the drug concentration that reduces the absorbance value by 50% in treated cells, with respect to untreated cells) that was calculated by the Logit method.

Morphological Observation by Electron Microscopy GA (1.25 μg/ml) was given, when the cells had grown to monolayer. The control was only given dilute solution. After cultivation for 48 h, the cells were collected and fixed with 3% glutaraldehyde and washed with 0.1 mol/l phosphate buffered saline (PBS). The cells were fixed with 1% osmic acid, dehydrated in an ethanol gradient, embedded in EPO812, sliced into ultrathin sections, and then stained with uranium acetate and lead citrate.¹⁷⁾ The morphological changes were observed under an electron microscope (HI-TACHI, Japan).

Quantitative Measurement of Telomerase Activity by Telomere Repeats Amplification Protocol (TRAP)-PCR-ELISA Cell extracts were prepared according to Telomerase PCR-ELISA kit product profile after the cells were exposed to 1.25 μg/ml GA for 24, 48 and 72 h. Briefly, the cells were collected and washed at 3000 × *g* for 10 min at 4 °C with PBS and resuspended in pre-cooled lysis reagent at a concentration of 2.5 × 10⁵ cells/ml. After 30 min incubation on ice, the lysates were centrifuged at 16000 × *g* for 20 min at 4 °C and the supernatant was stored at −80 °C for further use. Three microliters of the supernatant was added to a mixture (final volume of 50 μl) containing dNTP, biotin-labeled TS primer, Taq DNA polymerase, and CX primer. After

30 min primer elongation at 25 °C and 5 min telomerase inactivation at 94 °C, PCR amplification was cycled 30 times: 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 90 s. Telomerase activity was quantified by ELISA assay for the PCR products following the manufacturer's instruction. Briefly, 5 μ l of amplification product which had been denatured at room temperature for 10 min with 20 μ l denaturation reagent was hybridized with a digoxigenin-labeled probe specific for human telomeric repeats. The probe bound to the strand with the labeled biotin at the 5' end. The hybrid was immobilized to a streptavidin-coated microtiter plate *via* the biotin-labeled primer at 37 °C on a shaker for 2 h and washed 3 times. The reaction product was detected with 100 μ l of anti-digoxigenin-peroxidase and 100 μ l of peroxidase substrate 3,3',5,5'-tetramethylbenzidine (TMB). Color intensities were measured with a model 450 microplate reader (Bio-Rad, U.S.A.) at 450 nm.

Semi-quantitative Detection for hTERT mRNA Expression by RT-PCR Total RNA was extracted from 1.25 μ g/ml GA or media-treated cells using Tripure Isolation Reagent and RT-PCR was performed for 1 μ g of the total RNA in each group (quantitated by ultraviolet spectrophotometer) with the RobusT One Tube RT-PCR kit for detecting hTERT mRNA expression. The primers were 5'-CG-GAAGAGTGTCTGGAGCAA-3' and 5'-GGATGAAGCG-GAGTCTGGA-3' for hTERT which generated a PCR fragment of 126 bp,¹⁸ and 5'-CCAAGGCCAACCAGCGAGA-AGATGAC-3' and 5'-AGGGTACATGGTGGTGCCGC-CAGAC-3' for β -actin (internal control, 540 bp). PCR consisted of one cycle at 50 °C for 30 min, 94 °C for 2 min, and subsequently 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s by using a thermal cycler (PTC-100, MJ Research). Amplification products were subjected to electrophoresis through 2% agarose gels, stained with ethidium bromide, and quantified using Gel Base/Gel Blot/Gel Excel/Gel Sequence analysis software (UVP).

Statistical Analysis Data are expressed as the mean \pm S.D. and were statistically compared by one-way ANOVA with Dunnett's test or unpaired Student's *t*-test in different experiments. A $p < 0.05$ was taken as statistically significant.

RESULTS

Inhibitory Effect on Growth of Transplantable Tumors

Tumor xenografts transplanted by SPC-A1 cells were used to evaluate the antitumor effect of GA *in vivo*. RTV in GA- or OPT-treated mice were less than that in negative control mice at the same measurement day (*e.g.* at day 3). In particular, RTV in the 8 mg/kg GA group at days 3, 7, 10, and 17 were inhibited significantly, relative to that of the negative control at the same measurement day ($p < 0.01$, unpaired Student's *t*-test). Values of T/C in the 8 mg/kg (4 mg/kg) GA group were of 57.1% (64.3%), 45.0% (65.0%), 46.2% (61.5%), 72.7% (78.8%), 51.1% (55.6%), and 57.4% (67.1%) on days 3, 7, 10, 14, 17, and 21, respectively, indicating that GA inhibited tumor growth in a dose-dependent manner during the 21-d treatment. Meanwhile, body weight in the mice in the 2 GA groups remained essentially unchanged (8 mg/kg: from 20.2 \pm 1.1 g to 21.2 \pm 1.9 g, 4 mg/kg: from 21.5 \pm 1.3 g to 23.8 \pm 2.5 g) and none of the mice died during the treatment (see Fig. 2).

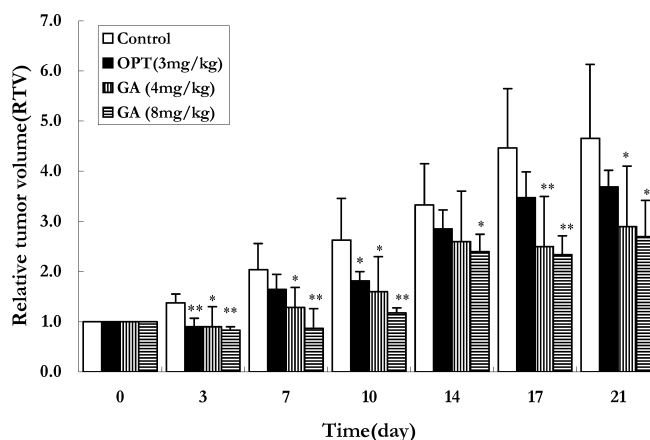


Fig. 2. Inhibitory Effect of GA on SPC-A1 Transplantation Tumors in BALB/cA Nude Mice

In the experiment, mice in the negative and positive control groups were given normal saline (NS) and OPT, respectively. Each data point is the average from six mice (*n*) each in the negative control group, GA groups (8, 4 mg/kg), and OPT group (3 mg/kg). * $p < 0.05$, ** $p < 0.01$, relative to the RTV in the negative control group at the same day point (unpaired Student's *t*-test). The experiments were repeated twice, and the results were similar to those above (data not shown).

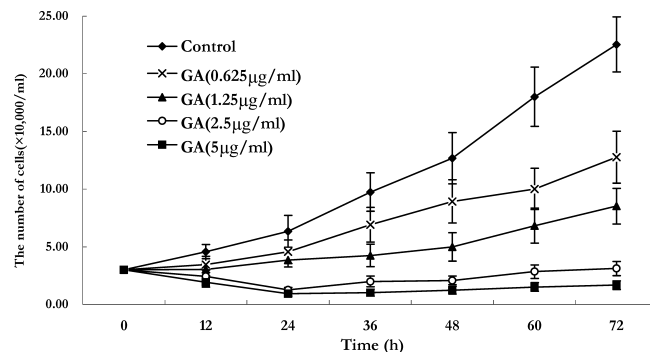


Fig. 3. Effect of GA on Growth of SPC-A1 Cells

Live cells that could exclude the trypan blue were counted manually under an optical microscope at $\times 100$. The data were obtained from triplicate wells and the experiments were repeated twice. The results were similar to those of the above experiment (data not shown).

Inhibition of Cell Proliferation To investigate the inhibitory effect of GA on cell growth, cells were cultured in the presence of various concentrations of GA and living cells were counted by trypan blue exclusion. At the same time of exposure, when the concentration of GA increased from 0.625 μ g/ml to 5 μ g/ml, the numbers of living cells decreased gradually, suggesting that GA exhibited dose-dependent inhibition of cell growth (see Fig. 3). MTT assay was used to evaluate the *in vitro* cytotoxic activity of GA on SPC-A1 cells. Generally, both GA and OPT could significantly inhibit the proliferation of the cells, compared with the negative control group at the same exposure time ($p < 0.01$, unpaired *t*-test). Figures 4A and B show that at the same exposure time, the higher the concentration of GA administered, the higher was the growth inhibitory ratio, and at the same dose, the longer the cells were exposed to GA, the higher was the inhibitory ratio. These results indicate that GA could inhibit SPC-A1 cells in time-dependent and dose-dependent manners. Meanwhile, the IC_{50} values at the same exposure time for GA were less than that for OPT (*e.g.*, IC_{50} value of GA at 24, 48 and 72 h was 1.74 \pm 0.08, 1.01 \pm 0.04, and 0.81 \pm 0.06, respectively, and the value of OPT was

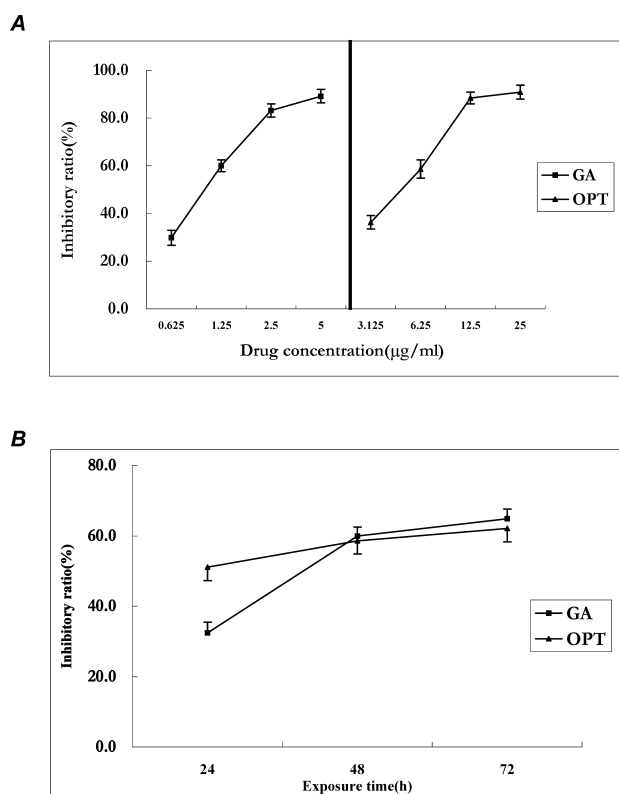


Fig. 4. Dose-Dependent and Time-Dependent Inhibition by GA and OPT of the Growth of SPC-A1 Cells

The results are expressed as a percentage of growth inhibition obtained from 3 separate experiments (3×triplicate wells). (A) Dose-dependent curves of GA (left panel) and OPT (right panel), exposure time was 48 h. (B) Time-dependent curves, dose of GA was 1.25 µg/ml and 6.25 µg/ml for OPT.

5.69±0.30, 4.44±0.35, and 3.54±0.30, respectively), showing that the *in vitro* cytotoxic activity of GA was more potent than that of the reference drug OPT.

Effect on Morphology of Cells We used transmission electron microscopy to determine the subcellular changes evoked by GA in SPC-A1 cells. It was observed that the nuclei of cells exposed to 1.25 µg/ml GA for 48 h were pyknotic and some nuclei were smashed. The surface microvillus of creviced cells had disappeared and there were various vacuoles and “apoptotic bodies” in the intra-cytoplasm or extra-cytoplasm (see Figs. 5A, B).

Repression of Telomerase Activity To evaluate the role of GA in the regulation of telomerase activity in SPC-A1 cells, the effect of GA treatment on the telomerase activity was measured by telomerase PCR-ELISA assay. After treatment with 1.25 µg/ml GA for 24, 48 and 72 h, telomerase activity was significantly repressed relative to the untreated control (at 24 h $p<0.05$, and at 48 and 72 h $p<0.01$, one-way ANOVA with Dunnett’s test). A reduction of 80.7% in telomerase activity was detectable after treatment for 48 h, and when treatment time rose to 72 h, 84.9% inhibition was achieved. However, when the cells were treated for 24 h, the inhibition was only 25.9% (see Fig. 6).

Effect of Down-Regulation on Expression of hTERT mRNA To determine whether GA affected telomerase activity by regulating hTERT expression, hTERT mRNA was measured by RT-PCR after treatment of SPC-A1 cells for 24, 48 and 72 h. As shown in Figs. 7A and B, after treatment for

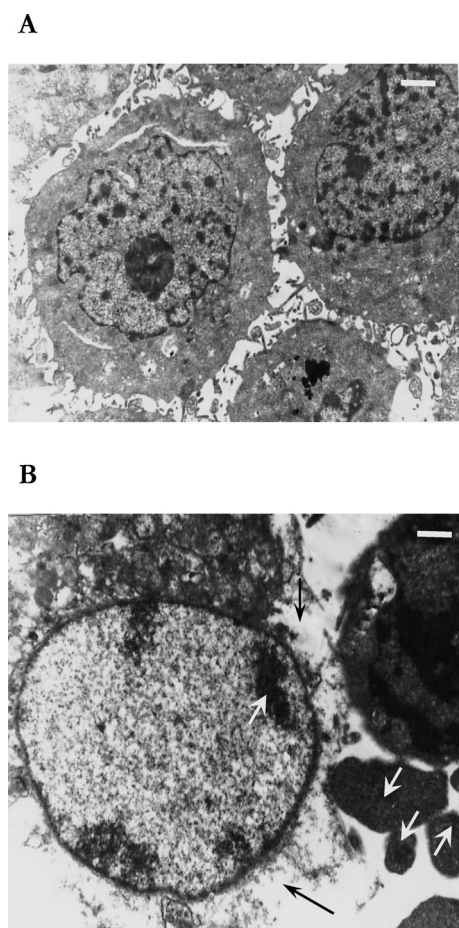


Fig. 5. Morphological Changes in SPC-A1 Cells Observed by Electron Microscope

(A) Morphology of SPC-A1 cells (untreated by GA); (B) morphology of SPC-A1 cells treated with 1.25 µg/ml GA for 48 h. White and black arrows indicate apoptotic bodies and vacuoles, respectively. Bar, 2 µm.

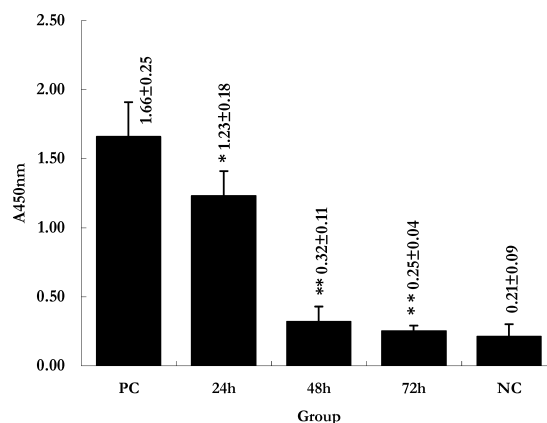


Fig. 6. Repressive Effect of GA on Telomerase Activity in SPC-A1 Cells

Results were obtained from 3 separate experiments and are expressed as absorbance at 450 nm. NC: negative control (treated with RNase); PC: positive control (untreated with GA); 24, 48 and 72 h represent the group treated with 1.25 µg/ml GA for 24, 48 and 72 h, respectively. * $p<0.05$, ** $p<0.01$, compared with PC.

48 and 72 h, the relative expression of hTERT mRNA was 0.12±0.04 and 0.09±0.02, respectively, compared to untreated cells ($p<0.01$, one-way ANOVA with Dunnett’s test). The relative expression only decreased from 1.87±0.28 to 1.31±0.23 when the cells were treated for 24 h ($p<0.05$).

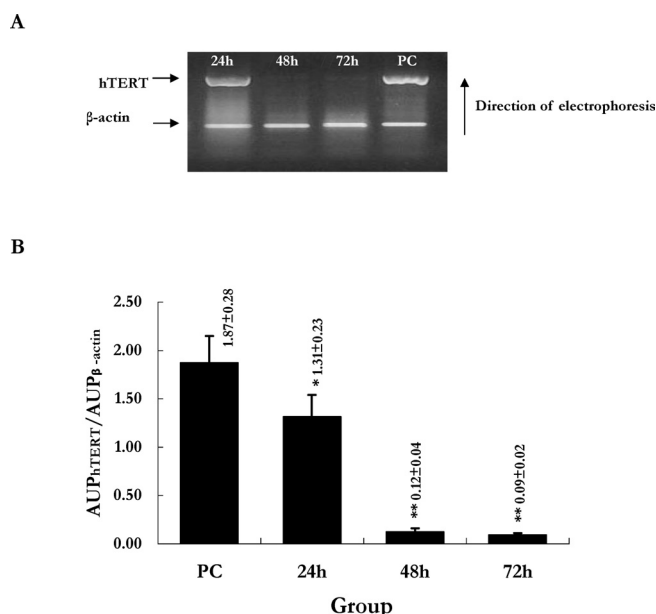


Fig. 7. Inhibition of GA on hTERT mRNA Expression in SPC-A1 Cells

hTERT mRNA was co-amplified with β -actin by RT-PCR. Amplified DNA was electrophoresed through 2% agarose gels and visualized by staining with ethidium bromide (A), and quantified by Gel Base/Gel Blot/Gel Excel/Gel Sequence analysis software (B). Data were obtained from 3 independent experiments and expressed as a ratio of AUP_{hTERT}/AUP_{β-actin}. AUP: area under peak. 24, 48 and 72 h represent the group treated with 1.25 μ g/ml GA for 24, 48 and 72 h, respectively; PC: Positive control (untreated with GA). * p <0.05, ** p <0.01 relative to PC.

DISCUSSION

GA, an active compound extracted from the gamboge resin of *Garcinia hanburyi*, was selected for further study due to its potent antitumor activities. As described in our previous study, GA has potent antitumor activities against Lewis lung carcinoma, Heps and S180 sarcoma, cultured human hepatocellular carcinoma SMMC-7721 and BEL-7402 cells, and human gastric adenocarcinoma MGC-803 and SGC-7901 cells (unpublished observations and ref. 11, 12). In the present study, to determine whether or not GA could inhibit proliferation of human lung adenocarcinoma cells, we chose SPC-A1 cells for *in vivo* and *in vitro* study. Data obtained from MTT and trypan blue exclusion assays showed that GA exerted significant inhibition of the proliferation of SPC-A1 cells in dose-dependent and time-dependent manners. The activity of GA was more potent than the reference drug OPT and on average, was over 3-fold more cytotoxic than that of OPT. Interestingly, when the IC₅₀ values at 48 h of exposure time served as the standard for evaluating the *in vitro* antitumor activity of GA, the inhibition of SPC-A1 cells by GA is similar to that of MGC-803 and SGC-7901 cells, but slightly lower than that on SMMC-7721 and BEL-7402 cells. Results from the *in vivo* study showed that GA could obviously inhibit the growth of transplantable tumors in a dose-dependent manner. In this experiment, none of the mice died, experienced substantial weight loss, or a gastrointestinal reaction during the entire treatment. The pathology slices showed there were no obvious pathological changes in the vital organs of the nude mice treated with 4 and 8 mg/kg (*i.v.*) (data not shown). The experiment undertaken on nude mice suggested that GA had no obvious toxicity on the nude mice. We also found in previous toxicity experiments in healthy rats

that GA at doses of 30 and 60 mg/kg (intragastrically, *i.g.*) had no significant influence on body weight, number of white blood cells in peripheral blood, or karyocytes in bone marrow (p >0.05, unpaired Student's *t*-test).¹⁹⁾ In addition, a long-term follow-up study was undertaken to determine the systemic adverse effects against normal tissues and organs in rats. The results showed that GA at doses of 30 and 60 mg/kg (*i.g.*) had no significant toxicity on vital organs such as the liver, heart, spleen, kidneys, lungs, testicles, uterus, and prostate, and when treated with 120 mg/kg GA (*i.g.*), the main toxic target organs were the liver and kidneys (unpublished observations). Therefore, it is partly safe to draw a conclusion from the results above that GA exerts potent antitumor activities without obvious side effects or toxicity in preclinical experiments, except for the toxicity on liver and kidneys at a dose of 120 mg/kg in the long-term follow-up study.

Telomeres are structures located on the ends of eukaryotic chromosomes.²⁰⁾ The progressive shortening of telomeres after each cell division²¹⁾ is believed to be the basis for the limited life-span of normal human cell lines.²²⁾ Human telomerase is a specialized ribonucleoprotein (RNP) in which a catalytic transcriptase subunit (hTERT) uses human telomerase RNA(hTR) as the template to add telomeric repeats to the end of chromosomes.^{23,24)} Telomerase activity is inactivated or repressed in the majority of normal somatic tissues but is activated in germ cells and in most malignant tumors. Telomerase reactivation may thus be a major step in human carcinogenesis.^{25–27)} Several studies have demonstrated that hTERT expression is a rate-limiting determinant of the enzymatic activity of human telomerase and that up-regulation of hTERT expression may play a critical role in human carcinogenesis.²⁸⁾ Moreover, inhibition of hTERT results in telomere loss and limits the growth of human cancer cell lines *in vitro* and their tumorigenic capacity *in vivo*.²⁹⁾ The data presented in the present study show that GA dramatically represses telomerase activity in SPC-A1 cells. To identify whether GA affected telomerase activity by regulating hTERT expression, we measured the hTERT mRNA level in SPC-A1 cells. We found that in the presence of GA, the hTERT mRNA level decreased dramatically when the cells were treated for 24, 48 and 72 h, a finding which coincided with the results of the determination of telomerase activity. These observations suggest that one possible mechanism for the growth inhibitory effect of GA on SPC-A1 cells may be that the drug regulates telomerase activity by modifying, at least in part, the expression of hTERT in tumor cells.

However, the inhibition of telomerase activity normally causes retardation of the cultured cells after several days or weeks. In the present study, when treated with GA for 24, 48 and 72 h, tumor cell proliferation was inhibited significantly, especially for 48 and 72 h. This is because, we assume, there may be at least two mechanisms (including inhibition of telomerase activity and induction of apoptosis) underlying the inhibitory effect of GA at 24, 48 and 72 h of exposure time. In fact, when treated with GA for 24, 48 and 72 h, the apoptosis ratio of SPC-A1 cells reached 33.82%, 73.61% and 82.77%, respectively (manuscript in preparation).

In addition, considering inactivated or repressed telomerase activity in the majority of normal somatic tissues, it is quite conceivable that inhibition of telomerase activity may

be one of the possible causes for the low toxicity and relative high selectivity of GA. However, it is also known that several normal tissues show weak but significant levels of telomerase activity. Therefore, in order to conclude affirmatively that the telomerase activity inhibition by GA was one of the possible causes of the low toxicity and high selectivity of the drug, further studies examining telomerase activity in various mouse tissues (including normal and tumorous tissues) before and after GA treatment should be conducted in the future.

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