Gambogenic Acid Inhibits Proliferation of A549 Cells through Apoptosis-Inducing and Cell Cycle Arresting

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Although anticancer effect of gambogic acid (GA) and its potential mechanisms were well documented in past decades, limited information is available on the anticancer effect of gambogenic acid (GNA), another major active component of Gamboge. Here we performed a study to determine whether GNA possesses anticancer effect and find its potential mechanisms. The results suggested that GNA significantly inhibited the proliferation of several tumor cell lines *in vitro* and *in vivo*. Treatment with GNA dose and time dependently induced A549 cells apoptosis, arrested the cells to G0/G1 phase *in vitro* and down-regulated the expression of cyclin D1 and cyclooxygenase (COX)-2 in mRNA level. In addition, anticancer effect was further demonstrated by applying xenografts in nude mice coupled with the characteristic of apoptosis in the GNA treated group. Taken together, these observations might suggest that GNA inhibits tumor cell proliferation *via* apoptosis-induction and cell cycle arrest.

Key words gambogenic acid; anticancer effect; apoptosis; cell cycle

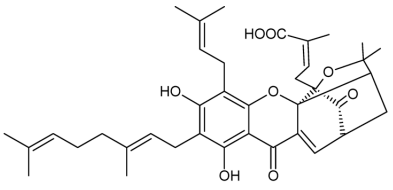
Gamboge is the dry resin of *Garcinia hanburyi* Hook. f. (Guttiferae) with various bioactivities, including detoxification, homeostasis, anti-inflammatory and parasiticide. Available evidence suggests that gamboge bears anticancer characteristics with gambogenic acid (GNA) and gamboge acid (GA) being its main components.[1)] Gamboge acid has been clinically applied to treat breast cancer, lymphatic sarcoma and carcinoma cutaneum, and its therapeutic effects have been well confirmed.[2,3)] Recently, accumulating evidence demonstrated equal antitumor effect of GNA, showing a wider spectrum of antitumor effect and lower toxicity than that of GA.[4—7)] However, the effect of GNA in anticancer and underlying mechanisms are yet to be elucidated.

A growing body of studies focused on apoptosis in screening cancer therapeutic drugs and apoptosis was affirmed to be one key pathway in the chemotherapeutic process of anticancer. For example, taxol is a typical drug with potential anticancer activity *via* apoptosis-induction.[8)] Oridonin, an active component extracted from Traditional Chinese Medicine, exerts similar anticancer activity not totally through apoptosis, while it plays a key role in the process.[9)] Thereafter, series of active components extracted from Traditional Chinese Medicine possess the characteristic of apoptosis-eliciting in cancer treatment. Previous studies suggested that GNA could be a potential candidate.[10)] Since rapid and uncontrollable proliferation is one hallmark of tumor cells. Arresting tumor cells to G0/G1, a sleeping phase, may offer therapeutic possibilities for treating malignant tumors.

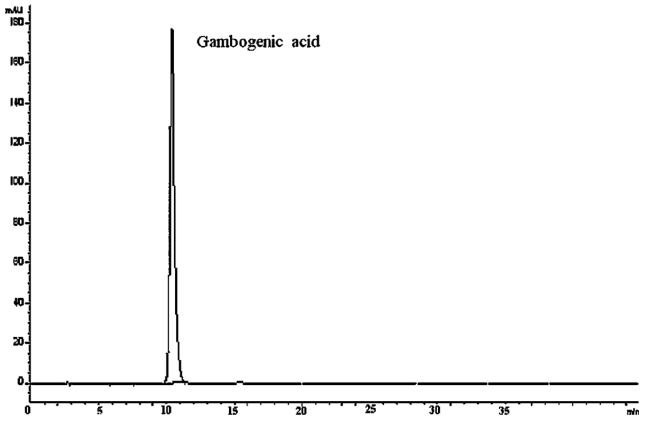
In this study, we aim to investigate whether GNA inhibit tumor cells growth *via* apoptosis-eliciting and cell cycle arrest. Based on *in vitro* and *in vivo* model, we determined that GNA inhibited series of tumor cells proliferation, induced A549 cell apoptosis, simultaneously arresting the cells to G0/G1 with regulating expression of cyclin D1 and cyclooxygenase (COX)-2.

MATERIALS AND METHODS

Materials Gambogenic acid (Fig. 1A) (norms: 20 mg/bottle; batch No.: 20070312; purity: 99%) was isolated from gamboge in Prof. Wang Xiaoshan's laboratory. The method of extraction and separation was as follows: dryed resin of gamboge was extracted with EtOAc, and then separated and purified by column chromatography RP-18 as packing materials. The structure was identified by comparing their ^{1}H-



A



B

Fig. 1. (A) Molecular Structure of GNA and (B) HPLC-UV Chromatograms of GNA

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NMR, ^{13}C-NMR and MS data with the literature data.[11] Their purities were 99% as determined by HPLC-diode array detector (DAD) (Fig. 1B). The chromatographic conditions were as follows: The Agilent HPLC system (Agilent, Waldbronn, Germany) was equipped with a quaternary pump (Agilent G1311A), an autosampler (Agilent G1313A), an online degasser (Agilent G1322A) and a column oven controller (Agilent G1316A), which was connected in a photo DAD (Agilent G1315A) for quantitative analysis and UV spectra acquisition. The column (ZORBAX Eclipse XDB-C8 column, 5 μm, ϕ4.6 mm×150 mm, Agilent, U.S.A.) was eluted isocratically with a binary mixture of acetonitrile and 0.20% acetic acid solution (volume ratio 85 : 15) at a flow rate of 1.0 ml/min. Elution was monitored at 360 nm on a diode array detector. The injection volume was 10 μl (5 $\mu g \cdot ml^{-1}$ gambogenic acid) and the column temperature was maintained at 25 °C.

Cell Culture Human lung adenocarcinoma cell line A549, human myelogenous leukaemia cell line K562, human colon cancer cell line HT-29, human breast adenocarcinoma cell line MDA-MB-231, human Hepatoma cell line BEL-7402, Mechanocyte cell line L929, human umbilical vein endothelial cell line HUVEC and human bronchial epithelium cell line HBE were obtained from Cell Bank of Shanghai Institute of Cell Biology, China. A549 cells, BEL-7402 cells and MDA-MB-231 cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100000 IU/l penicillin G and streptomycin 100 mg/l, L929 cell line, K562 cell line, HT-29 cell line were grown in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, benzylpenicillin 100000 IU/l, and streptomycin 100 mg/l, HUVEC were cultured in M199 medium supplemented with 20% heat-inactivated FBS with a humidified atmosphere of 95% air/5% CO_2 at 37 °C.

Animals Male and female BALB/cA nude mice, 4 weeks old and weighing 18—22 g, were supplied by Shanghai Institute of Materia Medica, Chinese Academy of Sciences (Shanghai, China). The mice were housed in air-conditioned rooms under controlled lighting (12 h lighting/d) and fed with standard laboratory food and water *ad libitum*. All 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 NIH, U.S.A.[12]

MTT Assay Approximately 2×10^4 cells/well were seeded in a 96-well plate. After cells were treated with indicated concentrations of GNA (dissolved in dimethyl sulfoxide (DMSO), terminal concentration not exceeding 1%) for 24 h, 48 h or 72 h, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5.0 mg/ml, Sigma, St. Louis, MO, U.S.A.) was added to culture medium and incubated for another 4 h at 37 °C. After the removal of culture medium, 150 μl DMSO was loaded to each well to dissolve precipitation. The absorbance (A) was measured at 570 nm using Automated Microplated Reader (Bio-Tek ELx 800uv, Bio-Tek Instrument Inc., Winooski, VT, U.S.A.). The inhibitory rate of cell growth was defined as the percentage of difference between control and treated group. Cell inhibitory ratio was calculated with the following formula: inhibitory rate (%)=(1−average absorbance of treated group/average absorbance of control group)×100%. IC_{50} was calculated according to the Log it method.

Inhibition of GNA on A549 Nude Mice Xenografts The mice were randomly divided into 5 groups: negative control, 5 mg/kg *cis*-diamminedichloroplatinum (cisplatin, C-DDP, Qilu Co., China, clinical formulation), 8, 16 and 32 mg/kg GNA, which was dissolved in DMSO (terminal concentration not exceeding 1%). All the mice were injected subcutaneously with 1×10^7 A549 cells in 0.1 ml into the right hind leg. The tumor volume reached 100—300 mm^3 five weeks after the injection. The tumor size was determined by using the formula[13,14]: $(a\times b)^2/2$ (a and b refer to the longer and shorter dimensions, respectively), which converted the subcutaneous tumor mass into tumor volume (TV). From the sixth week, the drugs were administered to the mice three times a week for another two weeks. C-DDP is typically dosed intraperitoneally (i.p.) in nude mice. C-DDP was diluted in 0.9% sodium chloride solution to an appropriate concentration and injected in 0.1 ml in nude mice. The body weights and tumor dimension were measured twice a week. In addition, the mortality of the mice was monitored daily. Relative tumor volume (RTV) was calculated using the equation: Vt/V0 (V0 was TV at day 0 when the mice were divided; Vt was TV obtained at each measurement). The evaluation index for inhibition was the relative tumor growth ratio T/C=TRTV/CRTV×100%, where TRTV and CRTV represented RTV of the treated and control groups, respectively.[15,16]

Cell Morphological Assessment A reported method was slightly modified and used in this study.[17] A549 cells treated with 2.5 μM GNA for 24 h were observed under phase contrast microscope (Olympus, Japan). A549 cells treated with GNA for 24 h were collected and fixed in 2.5% glutaraldehyde, washed with 0.01 mol phosphate buffered saline (PBS) buffer, dehydrated in alcohol, embedded in EPOR812, stained with acetic acid uranium and citromalic acid and observed by electron microscope (HITACHI-600, Japan). To further study the characteristic of apoptosis induced by GNA, fluorescence microscope was applied to detect morphological change. Cells were cultured in 6-well plate with different concentrations of GNA for 24 h. Afterwards they were stained with Hoechst 33258 at 1 mg/ml for 20 min. After washing with PBS, cell morphologic changes were observed under a fluorescence microscope (Olympus, Japan) through UV-filter.

PI Staining Assay for Evaluation of Cell Cycle Distribution A549 cells treated with different concentrations of GNA for 24 h were harvested by trypsinization, then washed twice with ice-cold PBS and fixed by 70% ethanol at −20 °C for at least 24 h. Cells were further washed twice with ice-cold PBS and stained with 50 mg/ml of propidum iodide (PI) in the presence of 100 mg/ml RNase A for 30 min. Cell cycle distribution was analyzed by using FACS Calibur (Becton & Dickinson, U.S.A.). At least 10000 cells per sample were collected and analyzed by using the Cell Fit Cell analysis program.[18]

Annexin V/PI Double-Staining Assay for Evaluation of Apoptosis A549 cells treated with GNA for 6, 12, 24 h were harvested by trypsinization, washed twice with ice-cold PBS, then double-stained by Annexin-V FITC and PI according to the instruction of the reagent (Nanjing Keygen Biotech. Co., Ltd.). The cells in apoptosis were determined

by Flow cytometry (FACScan, Becton Dickinson, U.S.A.).

Reverse Transcript Polymerase Chain Reaction (PCR) A549 cells were incubated with GNA for 6, 12, and 24 h. Total RNA was extracted using Trizol Isolation Reagent (Invitrogen, Carlsbad, CA, U.S.A.). Total RNA (5 μg) was used as template for cDNA synthesis, using the First Strand kit of Novagen (Madison, WI, U.S.A.) following manufacturer's instructions. The resulting cDNA products were used in each PCR reaction. Amplification within the linear range took place at subsequently 35 cycles of 94 °C for 45 s and 59 °C for 2 min followed by a final 72 °C extension for 5 min (cyclin D1) or 35 cycles of 94 °C for 30 s and 59 °C for 2 min (β-actin) or 35 cycles of 94 °C for 45 s and 54 °C for 90 s (COX-2) followed by a final 72 °C extension for 5 min, 4 °C paused. The PCR products were resolved on agarose gel, and densitometric analysis was conducted for quantification of individual band using Gel Imaging Analyser (Bio-Rad, San Diego, CA, U.S.A.). The primers (Sangon, Shanghai, China) specific for cyclin D1, COX-2 or β-actin were listed as below:

5′-GCGAGGAACAGAAGTGCG-3′ (cyclin D1 forward),
5′-TTCACGGGCTCCAGCGACA-3′ (cyclin D1 reverse);
5′-TGAAACCCACTCCAAACACAG-3′ (COX-2 forward),
5′-TCATCAGGCACAGGAGGAAG-3′ (COX-2 reverse);
5′-CTGGGACGACATGGAGAAAA-3′ (β-actin forward),
5′-AAGGAAGGCTGGAAGAGTGC-3′ (β-actin reverse).

TUNEL Assay For *in situ* detection of apoptotic cells, the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) assay (Nanjing Keygen Biotech. Co., Ltd.) was used to detect the DNA fragmentation. The solid tumor was dissected as described previously,[19] then fixed in 10% formalin, dehydrated, embedded in paraffin, sliced by microtome. The fixed slices were then incubated with digoxigenin-conjugated dUTP in a terminal deoxynucleotidyl transferase (TdT)-catalyzed reaction for 60 min at 37 °C in a humidified atmosphere and were then immersed in stop/wash buffer for 10 min at room temperature. The cells were then incubated with antidigoxigenin antibody conjugated with peroxidase for 30 min. DNA fragments were stained using 3,3-diaminobenzidine (DAB) as the substrate for the peroxidase.

Statistical Analysis All the values were represented as mean±S.E. of triple independent experiments. One way analysis variance (ANOVA) was used in multi comparisons between groups. Complete analysis was done by the Newman–Keuls test. Statistical significance is accepted at $p<0.05$.

RESULTS

Inhibition Effect of GNA on Tumor Cells To determine whether GNA exerts antitumor effects, we screened the effect of GNA on the proliferation of several types of malignant tumor cells, with L929 cell lines, a type of fibroblasts and human umbilical vein endothelial cell line by MTT assay. Figure 2 suggested that a strong inhibitory effect of GNA on the cell proliferation in various tumor cells was observed, especially in A549 cells. A549 cells exposure to GNA (0 to 10 μM) for 24 h resulted in a dose-dependent inhibition of cell proliferation, with the IC_{50} of 2.5 μM (data not shown). Prolonged exposure (48 or 72 h) led to a similar

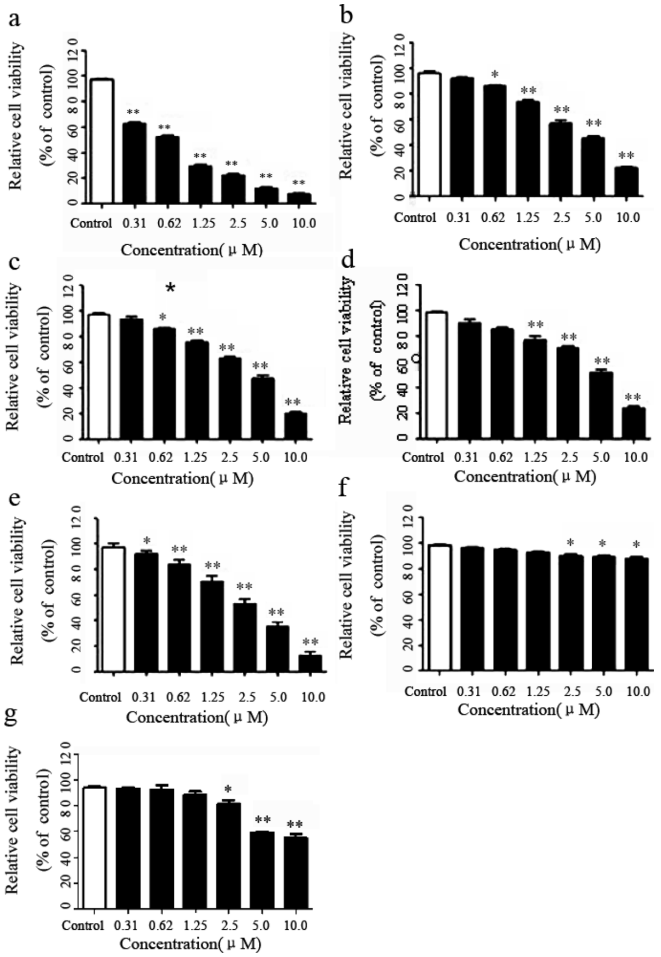

Fig. 2. Inhibitory Effect of GNA on Tumor Cell Line

After incubating with different concentrations of GNA for 72 h, cell viability of various cell lines were assayed by MTT analysis. Data are means±S.E. from three experiments respectively; compared to control, $*p<0.05$ and $**p<0.01$ (a. A549 cell line, b. HT-29, c. K562, d. MDA-MB-231, e. BEL-7402, f. L929, g. HUVEC).

dose-dependent reduction in the number of surviving cells. However, the same dose of GNA exhibited no apparent inhibitory effect on L929 cells for 72 h (Fig. 2f). Meanwhile, human umbilical vein endothelial cell line was also applied to assess the preferential inhibition and positive result was observed in our study (Fig. 2g).

Involvement of Apoptosis in Inhibiting Effect of GNA on A549 Nude Mice Xenografts To further assess the inhibiting effect of GNA on A549 cells *in vivo*, we measured the dimension of the xenografts. The results indicated that GNA inhibited tumor growth in A549 nude mice xenografts in the similar manner as Cisplatin did. Relative tumor volume (RTV) in GNA treated mice was less than that in negative control mice. RTV values in 16 and 32 mg/kg GNA treated groups were 12.16±7.39 and 7.65±2.84, respectively. Compared with vehicle treated negative control (26.36±14.10), the relative tumor growth ratio (T/C, %) values were 46.1% and 29.0%, respectively (Fig. 3), which indicated that GNA inhibited tumor growth in a dose-dependent manner. Meanwhile, no significant change was found in body weight, function of spleen, bone marrow and liver in the mice treated with GNA (data not shown), indicating the safety of GNA as a drug to treat the lung cancer. To further verify whether apoptosis was involved in the inhibiting effect, TUNEL assay

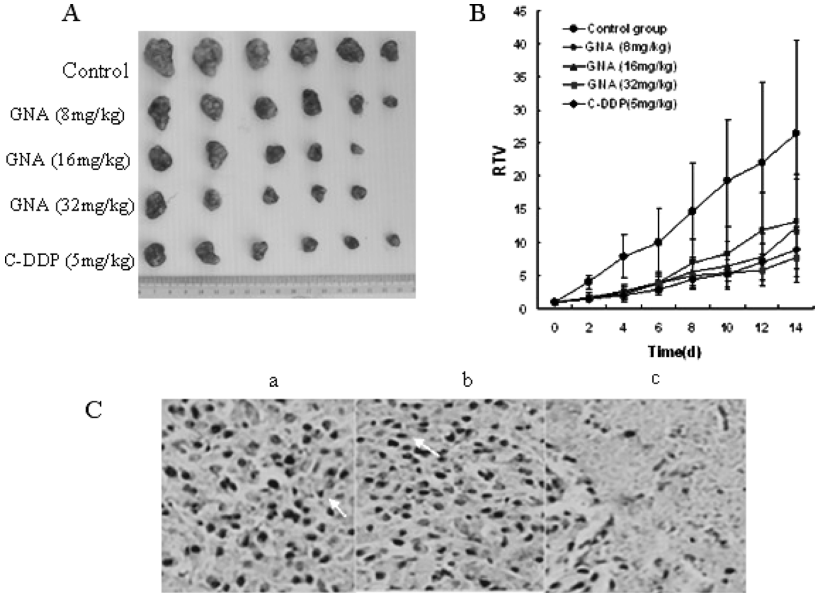

Fig. 3. Involvement of Apoptosis in Inhibiting Effect of GNA on A549 Nude Mice Xenografts

(A) Tumors isolated from different groups 7 weeks after subcutaneous injection of A549 cells. (B) Relative tumor volume (RTV) detected by sliding caliper. Data are means±S.E. from three experiments respectively; compared to control, $*p<0.05$ and $**p<0.01$. (C) Apoptosis elicited by GNA in mice xenografts, TUNEL assay was applied to detect apoptosis and the arrows indicated the apoptotic cells (a. control group, b. 16 mg/kg GNA, c. 5 mg/kg C-DDP).

was applied to detect the DNA fragmentation. The results suggested that the dose of 16 mg/kg GNA could significantly induced cell apoptosis in the tumor slice in comparison with control group. While only necrosis was observed in 5 mg/kg C-DDP treatment group.

GNA Induced Apoptosis in A549 Cells To examine the potential antitumor mechanisms, A549 cell, the optimal cell line in efficacy of GNA was chosen. Contrast phase microscope was applied to observe the morphological changes of the A549 cells treated by GNA for 24 h (Fig. 4Aa). The results obviously suggested that GNA inhibited A549 cell proliferation, even elicited cell death. To further evaluate the involvement of apoptosis in the GNA-induced A549 cell death, electron microscope (EM) and fluorescence microscope were used to detect the apoptotic morphological changes. After exposure to 2.5 μM GNA for 24 h, the aggregation appearance of chromatin was observed under the EM. The microvilli on the surface of creviced cells disappeared. There were various vacuoles and "apoptotic bodies" in the intra-cytoplasm or extra-cytoplasm (Fig. 4Ab). The results staining by Hoechst 33258 further verified that apoptosis was involved in the inhibiting effect (Fig. 4Ac). To quantitatively analysis apoptosis, Flow cytometer method double-stained by Annexin-V FITC and PI was applied to assess the apoptosis-eliciting effect of GNA. After treatment with 2.5 μM GNA for 6 h, 12 h and 24 h, apoptotic rate were 8%, 12% and 20%, respectively, in comparison with control group of 0.97% (Fig. 4B).

Cell Cycle of A549 Cells Was Arrested by GNA to G_0/G_1 Meanwhile, cell cycle distribution was assessed by flow cytometry stained by PI, and the results indicated that most GNA treated cells accumulated in the G1 phase compared to control group (Fig. 5). Meanwhile, we examined the potential role of two cell cycle related genes in GNA-triggered death of A549 cells by performing RT-PCR to assay cyclin D1 and COX-2 genes expression. As shown in Fig. 6,

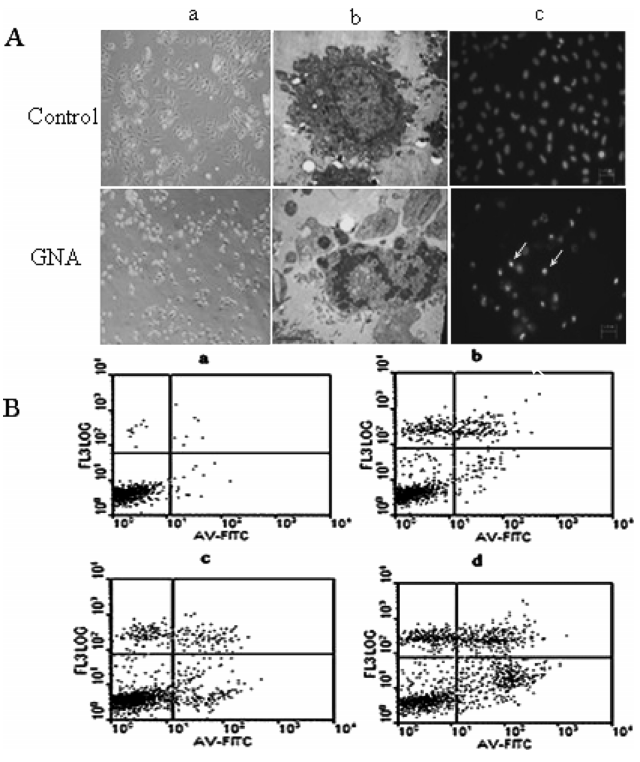

Fig. 4. GNA Elicited A549 Cells Apoptosis

(A) Cell morphological changes of A549 cells treated by 2.5 μM GNA about 24 h (a. phase contrast microscope, b. electron microscope, c. Hoechst 33258 fluorescent staining, apoptotic cells indicated by the arrows). (B) Flow cytometer method quantitatively analyzed A549 cells double stained by Annexin V-FITC and PI (a. control group, b. 2.5 μM GNA for 6 h, c. 2.5 μM for GNA 12 h, d. 2.5 μM for GNA 24 h).

treatment with GNA could significantly down-regulate the expression of cyclin D1 and COX-2 genes in mRNA level in a time dependent manner.

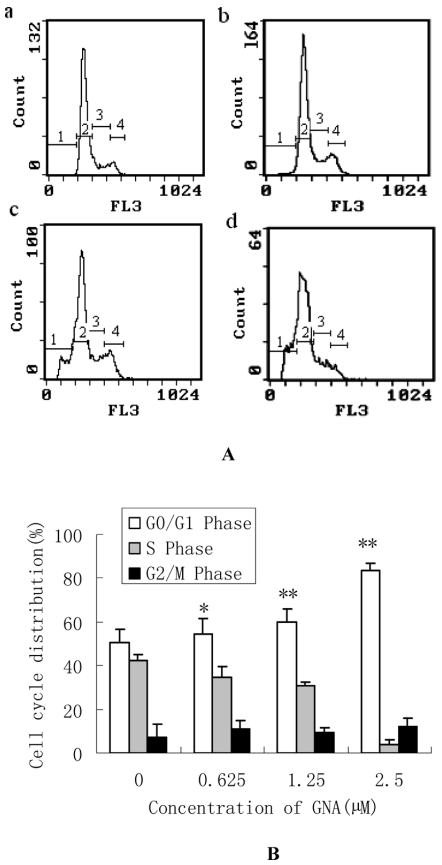

Fig. 5. Cell Cycle Distribution Detected by Flow Cytometric Analysis

(A) Fluorescence-activated cell sorter analysis for PI staining of A549 cells incubated with 0 μM (a), 0.625 μM (b), 1.25 μM (c), and 2.50 μM (d) of GNA for 24 h. Symbol 1, 2, 3, 4 represented apoptosis peak, G0/G1 phase, S phase and G2/M phase, respectively. (B) Graph of the cell cycle distribution of different groups. Data are means±S.E. from three experiments respectively; compared to control, $*p<0.05$ and $**p<0.01$.

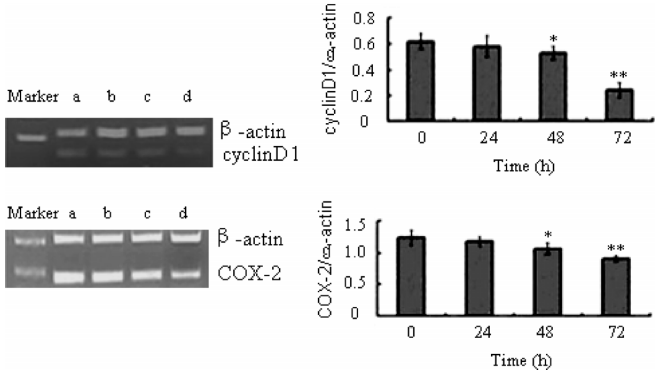

Fig. 6. RT-PCR Assay of Cyclin D1 (366 bp) Gene and COX-2 (232 bp) Gene Expression in A549 Cells

The cells were treated with 2.5 μM of GNA for 6, 12 and 24 h, respectively, and RNA was isolated and processed to PCR analysis. a. control; b. treated with 2.5 μM GNA for 6 h; c. treated with 2.5 μM GNA for 12 h; d. treated with 2.5 μM GNA for 24 h.

DISCUSSION

Chinese herbs applied to prevent or cure various diseases were marked with their high activity and low toxicity.[20] GNA, a type of water-insoluble substance isolated from Gamboge, is an active component in Gamboge with various pharmacological effects, especially its intriguing anti-cancer effect. Previously, we have already verified that GNA possesses selective anti-tumor activities in several types of cancer, including adenocarcinoma of the lung in a dose–response manner.[7] Here we further evaluate the anticancer effect of GNA on a variety of tumor cells and significant results were observed with the proliferation inhibited by GNA, especially apparent in A549 cells. The results indicated that GNA inhibited the proliferation of A549 cells in a dose-dependent and time-dependent manner with a highest inhibitory rate of 92.3%. Meanwhile, GNA had a relative low toxicity effect in fibroblasts and human umbilical vein endothelial cell line, which suggested that GNA might be an effective and safe anticancer agent. In addition, inhibiting effect of GNA on A549 nude mice xenografts, without significant changes in body weight, function of spleen, bone marrow and liver in the mice treated with GNA was observed in our study, which further suggested the safety of GNA as a cancer therapeutic drug.

Apoptosis is one type of cell death that plays a key role in the early development and growth of normal tissue. A growing body of evidence suggests that the derangement of apoptosis may lead to series of diseases including tumors. Meaningfully, various chemotherapeutic drugs have been applied in cancer therapy mainly through apoptosis-induction.[21—23] In this study, typical apoptosis in A549 cell induced by GNA was confirmed by morphological changes and flow cytometer method, which implied the involvement of apoptosis-elicited process in the GNA-triggered proliferation inhibition. Typical apoptosis was also affirmed in tumor slice obtained from GNA treated mice by using TUNEL assay. Based on experiments conducted both *in vivo* and *in vitro*, we could determine that apoptosis plays significance role in GNA elicited A549 cells death.

Cell cycle consists of four distinct phases: G1 phase, S phase, G2 phase (collectively known as interphase) and M phase. Activation of each phase is dependent on the proper progression and completion of the previous one. Accumulating evidence demonstrates that cell cycle redistribution would be a key stratagem in screening chemotherapeutic drugs.[24] Recent studies also suggest that cell cycle is always preferentially arrested in a special phase, such as G0/G1 or G2/M, in drugs-induced apoptosis cell death or autophagy mediated cell death. Hence, cell cycle distribution is of intriguing significance in finding potential chemotherapeutic drugs. In response to extracellular signals, cyclin D is of the priority expression and binds with CDK activating the downstream cascade reaction. Cyclin D binds to existing CDK4, forming the active cyclin D–CDK4 complex, which in turn, phosphorylates the retinoblastoma susceptibility protein (RB). The hyperphosphorylated RB dissociates from the E2F/DP1/RB complex, activates E2F and results in the transcription of various genes like cyclin E, cyclin A, DNA polymerase, thymidine kinase, *etc.* Cyclin E then binds to CDK2, forming the cyclin E-CDK2 complex, which pushes the cells from G1 to S phase (G1/S transition).[25,26] Gene amplification and abnormal expression of cyclin D1 have been described in several human cancers.[27] The results of our PI single-staining assay showed that the death of A549 cells treated with GNA was partially caused by regulating the G1/S phase cell cycle, which is in line with the previous studies. However, future study is needed to determine whether cyclin D1 cooperates in the development and progression of lung tumors and

whether GNA down-regulate the expression of cyclin D1.

COX, including COX-1, COX-2 and COX-3, is a type enzyme which is responsible for formation of important biological mediators called prostanoids (including prostaglandins, prostacyclin and thromboxane). It is well documented that chemoprevention based on COX-2 has already been achieved in a restricted group of patients. At the same time, COX-2 has been characterized as one of the proteins induced during the transformation of cells by the viral oncogene, v-src, and as a mitogen inducible protein. Meanwhile, COX-2 is also mediated in series of tumors or cancers curing involving angiogenesis. Increased levels of COX-2 are found in both premalignant and malignant tissues, and genetic engineered animals that are COX-2 deficient or animals treated with a selective COX-2 inhibitor show reduced tumor formation and growth. However, the mechanism correlating lung cancer with COX-2 intervening by GNA is not well established. COX-2 is preferentially expressed in the A549 cell line which previously demonstrated inflammatory cytokine interleukin-1β (IL-1β).[28] Here reverse transcription PCR showed that both expressions of cyclin D1 and COX-2 genes took on a down-regulating trend treated by GNA, which implied the potential role of COX-2 and cyclin D1 involved in GNA-triggered cell death.

In conclusion, although other potential mechanisms would be involved in the GNA-elicited cell death, our study could affirm that GNA inhibited the proliferation of the A549 cells by inducing cell apoptosis and arresting cell cycle to G0/G1, simultaneously mediating the expression of cyclin D1 and COX-2 genes. However, clear mechanism underlying the relationship among cyclin D1, COX-2 and apoptosis need further study.

Acknowledgements We thank Ms. Jenny Lin (Iowa State University, U.S.A.) for her valuable suggestions and comments to the manuscript.

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