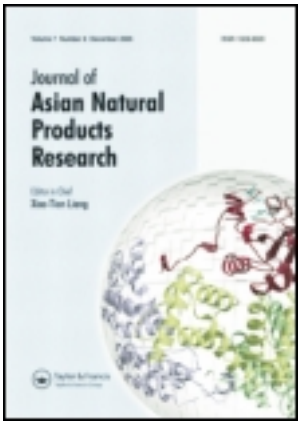

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Gambogenic acid inhibits proliferation of A549 cells through apoptosis inducing through up-regulation of the p38 MAPK cascade

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Gamboge is a dry resin secreted from *Garcinia hanburryi*, and gambogenic acid (GNA) is one of the main active compounds of gamboge. We have previously demonstrated the anticancer activity of GNA in A549 cells and pointed out its potential effects in anticancer therapies. Previous studies reported that GNA induced apoptosis in many cancer cell lines and inhibited A549 tumor growth in xenograft of nude mice *in vivo*. However, the anticancer mechanism of GNA has still not been well studied. In this paper, we have investigated whether GNA-induced apoptosis is critically mediated by the p38 mitogen-activated protein kinase (MAPK) pathway. Our findings revealed that GNA could induce apoptosis, inhibit proliferation, down-regulate the expression of p38 and MAPK, increase the activations of caspase-9, caspase-3, and cytochrome c release. Furthermore, using SB203580, an adenosine triphosphate-competitive inhibitor of p38 MAPK, inhibit the expression of p-p38 and the experimental results show that it may promote the occurrence of apoptosis induced by GNA. Taken together, these results suggested that up-regulation of the p38 MAPK cascade may account for the activation of GNA-induced apoptosis.

Keywords: gambogenic acid; A549; apoptosis; p38 MAPK

1. Introduction

Cells sense changes in their environment by activating signal transduction pathways that direct biochemical programs to mediate proliferation, differentiation, and survival [1]. Mitogen-activated protein kinases (MAPKs), a family of serine/threonine kinases, are essential components of intracellular signal transduction and are usually activated in response to various extracellular stimuli, including growth factors, cytokines, and environmental stress. The three major MAPK pathways in mammalian cells include the extracellular signal-regulated kinases, the c-Jun NH2-terminal protein kinases (JNKs), and the p38 MAPKs. The JNKs and p38 MAPKs are activated in response to a variety of stress signals including UV irradiation, chemotherapeutics, osmotic shock, hypoxia/anoxia, hyperthermia, and so on [2–4]. It mediates various cellular processes including apoptosis, senescence, inflammation, and tumorigenesis through decreased p38 MAPK activity, which has been associated with tumor progression [5].

Lung cancer is one of the most common malignancies and nowadays becomes the leading cause of cancer

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death worldwide. Moreover, its incidence has still increased in China [6]. Chemotherapy is a standard treatment option for patients with cancers. Thus, the development of new therapeutic strategies of the molecular mechanisms is needed. Natural products are the most important sources of promising leads for the development of novel cancer therapeutic drugs [7]. As one of the main active compounds from *Garcinia hanburryi*, gambogenic acid (GNA) ($C_{38}H_{46}O_8$, MW 631.32) (Figure 1), possesses various bioactivities including detoxification, detumescence, hemostasis, and even as a parasiticide [8–12]. Previous studies reported that GNA has broad-spectrum antitumor effect and we have reported that GNA had potent anticancer activity because of its activation on the apoptotic pathways in tumorous cells both *in vitro* and *in vivo*. More recent studies have revealed that GNA inhibits proliferation of cultured cancer cells by causing G_1-S phase cell-cycle arrest or apoptosis induction [13,14]. In this study, we investigated the effects of GNA-induced apoptosis in A549 cells and its underlying molecular mechanisms.

2. Results and discussion

Apoptosis is an important cellular process that eliminates unwanted cells during normal development or damaged cells after removal of trophic factors or exposure to toxic chemicals [15]. Most of the key players in cellular apoptosis regulation have been identified and can be targeted by therapeutic strategies. We have previously demonstrated that GNA significantly inhibited the growth of A549 cells both *in vitro* and *in vivo*. Flow cytometric detection of normal cells, early apoptotic or necrotic cells using fluorescein labelled Annexin-V/propidium iodide (PI) at different times after GNA treated with A549 cells and the result indicated that GNA could induce A549 cells apoptosis in a time-dependent manner.

The p38 MAPK cascade has been shown to participate in the induction of apoptosis in various cell types, including normal cardiomyocytes [16]. The mechanism that is not well studied may involve the phosphorylation of members of the Bcl-2 family and activation of the mitochondrial apoptotic pathway [17]. The p38 MAPK cascade is an inhibitor of apoptosis and is also overexpressed in many treatment-resistant cancers [18]. Both SB203580 and a dominant-negative p38 MAPK blocked phytosphingosine-induced Bax translocation, mitochondrial cytochrome c release, and cleavage of caspase-9. We have shown that GNA down-regulates the expression of the antiapoptotic protein Bcl-2 (result is not shown). To further investigate whether GNA-induced apoptosis is also mediated through the p38-MAPK signal pathway, we pretreated the A549 cells with the p38-MAPK-specific inhibitor SB203580.

The morphologic changes of A549 cells and GNA-treated A549 cells are depicted in Figure 2(A). GNA-treated cells display condensed nuclei, cell detach-

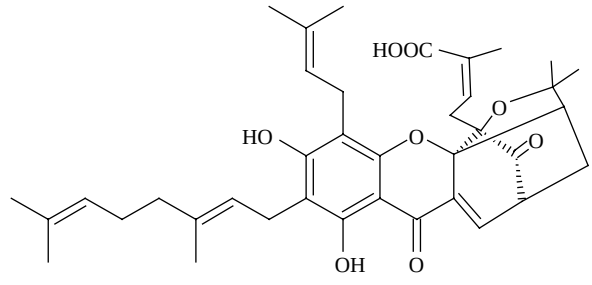

Figure 1. Molecular structure of GNA.

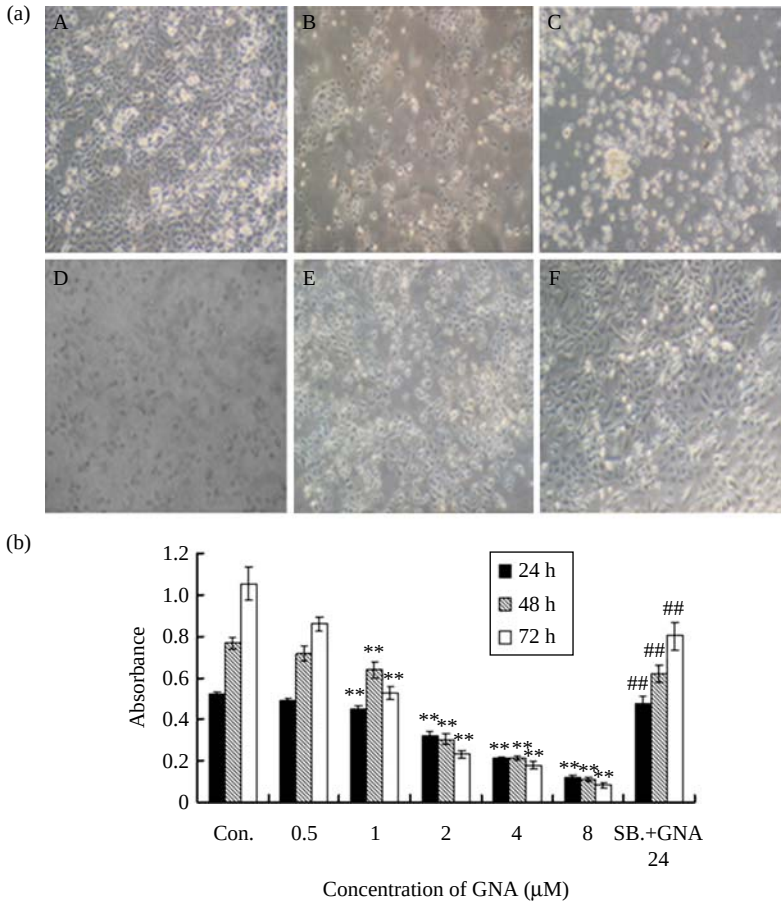

Figure 2. Morphological observation of GNA inhibits A549 cells growth and the effects on cell viability. (A) Cell morphological changes of A549 cells treated by 2 μM GNA for 12, 24, and 36 h in the presence or absence of SB203580 under a phase contrast microscope. (A, Con. 36 h; B, GNA 12 h; C, GNA 24 h; D, GNA 36 h; E, GNA 24 h + SB203580; F, GNA 36 h + SB203580). (B) Cells in 96-well plates were treated with various concentrations of GNA in the presence or absence of SB203580 for 24, 48, and 72 h. Cell viability was determined by MTT assay. Values are mean ± SE of at least three independent experiments. (Statistical analysis: vs. control $^{**}P < 0.01$; vs. GNA (2 μM) $^{\#\#}P < 0.01$).

ment, and irregular shape compared with the untreated A549 cells. The inhibitory effect of GNA on cell growth was measured by 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Figure 2(B)). Our result showed that GNA could significantly inhibit the proliferation of the cells, compared with the negative control group at the same exposure time and dose. In this study, we chose 2 μM GNA based on the 50% inhibiting concentration (IC_{50}) value for GNA-treated A549 cells at 72 h [19]. In contrast, SB203580 could decrease GNA-induced cell death. These results suggest that GNA-mediated cell death in A549 cells is predominantly mediated through p38 MAPK.

To assess GNA's interactions in A549 cells, cells were exposed to 2 μM GNA for 12, 24, and 36 h in the absence or presence of SB203580. Then, apoptosis was assessed by Annexin-V/PI analysis. The results revealed that control cells did not show any significant apoptosis, but the percentage

of apoptotic cells increased remarkably in GNA-treated cells in a time-dependent manner. Compared with GNA-treated cells, pretreatment with SB203580-protected cells almost completely offset GNA-induced apoptosis (Figure 3).

Apoptosis involves two main pathways: death receptor and mitochondria death pathway [20,21]. The intrinsic or mitochondrial apoptotic pathway is a major cell death pathway, and these include death receptors triggering apoptosis from the cell surface, Bcl-2 proteins as the gatekeepers of the mitochondrial pathway, caspases as the executioner enzymes, endogenous caspase inhibitors, and various transcriptional regulators. The identification of the major regulators of apoptosis has boosted an intense research in developing therapeutic approaches to intervene with cell death, either in a pro- or antiapoptotic direction. The mitochondrial membrane potential, which is necessary in the process of the formation and maintenance of oxidative phosphorylation, regulates the selectivity and permeability of the mitochondrial membrane to all kinds of material. By this means, it maintains the normal structure and function of the mitochondrial membrane [22]. To examine the relative effect of GNA to the mitochondrial membrane potential, we investigated the use of rhodamine 123 (Rho-123; MolecularProbes, Eugene, OR, USA), as fluorescent probes to monitor the membrane potential of mitochondria. Our result revealed that mitochondrial membrane potential of GNA-treated A549 cells was remarkably significantly decreased as indicated by a 40–50% decrease in fluorescence intensity ratio in a time-dependent manner compared with that of untreated A549 cells. There was a significant increase in fluorescence inten-

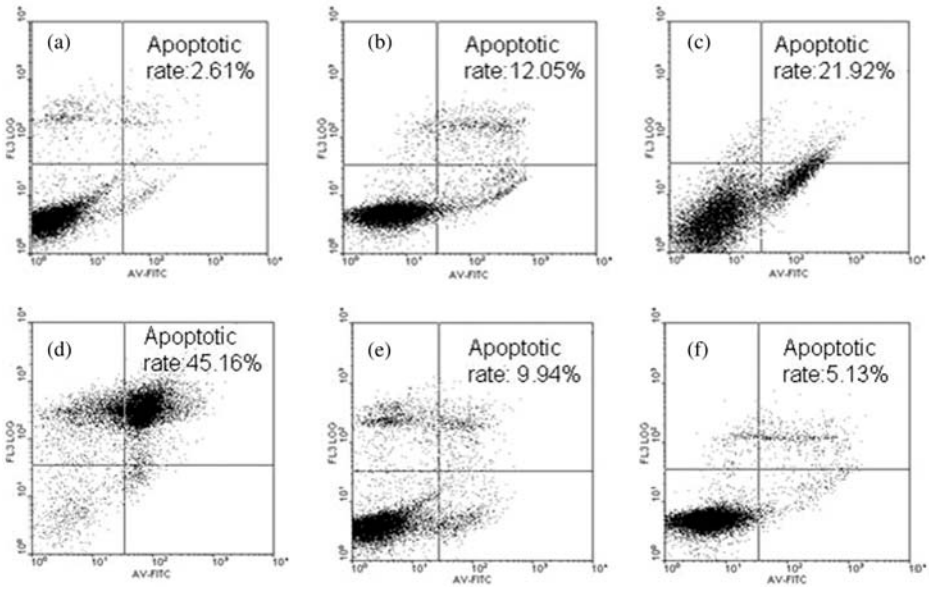

Figure 3. Fluorescence-activated cell sorter analysis for Annexin-V and PI staining of A549 cells incubated with GNA (2 μM). (A, control; B, GNA 12 h; C, GNA 24 h; D, GNA 36 h; E, GNA + SB203580 36 h; F, GNA + SB203580 24 h). Up right (UR): Necrotic cells and late apoptotic cells labeled with PI and Annexin V-FITC. Lower left (LL): Fully viable cells. Lower right (LR): Early apoptotic cells labeled with Annexin V-FITC but not with PI. After exposure to GNA, the cells of LR had increased from 2.61% (Con.) to 45.16% (GNA, 36 h), but the cells of LR which were treated with both SB203580 and GNA for 24 or 36 h were 5.13 and 9.94% respectively.

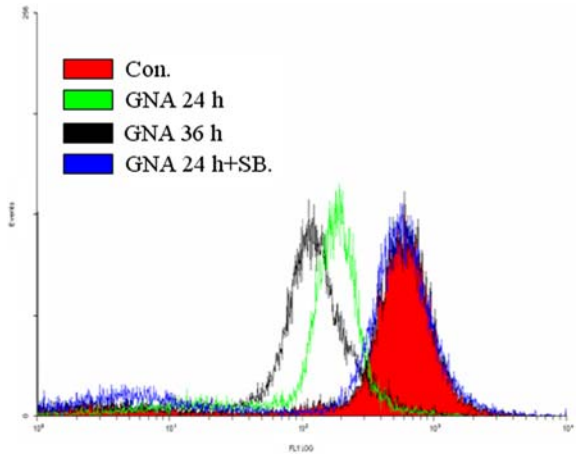

Figure 4. Cells were treated with 2 μM GNA for 24 and 36 h in the presence or absence of SB203580. Mitochondrial membrane potential is detected using Rho-123 staining for 24 and 36 h.

sity of mitochondrial membrane potential in A549 cells treated with 2 μM GNA plus SB203580 at 24 h (Figure 4). The data suggested that mitochondrial dysfunction was involved in the cell death induced by GNA. The mitochondria damage caused GNA treatment partially offset by SB203580.

To examine the potential role of mitochondria in GNA-induced cell death, we measured cytochrome c release, a hallmark of mitochondrial integrity disruption. The cytochrome c content in each fraction was determined by ELISA. As shown in Figure 5, compared with untreated cells, cells treated with 2 μM GNA caused a substantial increase in the cytochrome c content in the cytosol and culture media in a time-dependent manner. It has suggested a significant increase ($P < 0.01$) after GNA treatment in A549 cells. In addition, SB203580 almost completely abolished the increase of cytochrome c in cytosol. It implied that GNA-induced apoptosis might be dependent on the p38-MAPK pathway.

Mitochondrial homeostasis plays a pivotal role in regulating apoptosis. Pro-apoptotic signals can trigger the mitochondria to release caspase-activating proteins into cytosol, such as cytochrome c [23]. We then questioned whether the increased apoptotic response induced by GNA was sustained by caspase activation in A549 cells. Western blotting was used to detect apoptotic-related protein changes induced by GNA treated with A549 cells for 12 h, 24 h and 36 h. The results showed a dramatic increase in cleavage caspase-9 and caspase-3 levels in a time-dependent manner (Figure 6). These results revealed

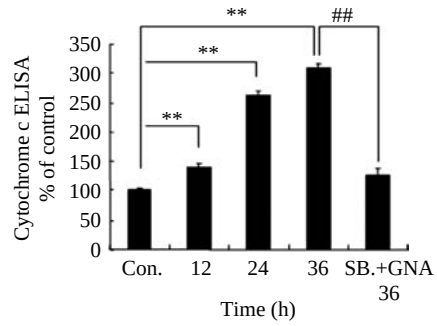

Figure 5. Cells were treated with 2 μM for 12, 24, and 36 h in the presence or absence of SB203580. GNA-induced release of cytochrome c from mitochondria and SB203580 prevented apoptosis mediated through p38 MAPK. The expression of cytochrome c was analyzed by ELISA. The summarized data are presented as mean ± SE of three independent experiments. (Statistical analysis: vs. control $^{**}P < 0.01$; vs. GNA (2 μM) $^{\#\#}P < 0.01$).

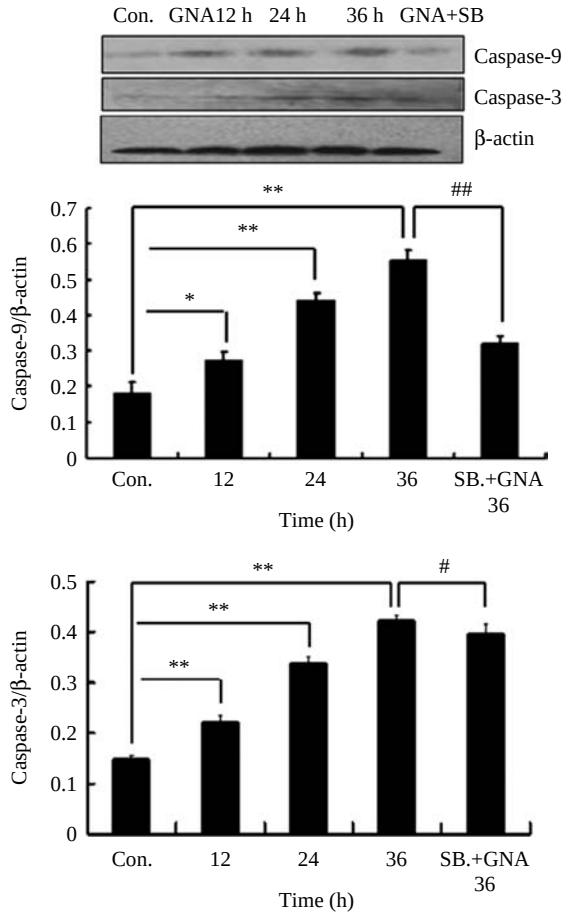

Figure 6. Expression of caspase-3 and caspase-9 in the absence or presence of the p38-MAPK inhibitor SB203580 in A549 cells treated with 2 μM GNA for 12, 24, and 36 h. The amount of caspase-3 and caspase-9 was quantitated by densitometric analysis normalized on the level of β-actin. Data are represented as the means ± SE of triplicate experiments. (Statistical analysis: vs. control $*P < 0.05$, $**P < 0.01$; vs. GNA (2 μM) $^{\#}P < 0.05$, $^{\#\#}P < 0.01$).

that mitochondrial apoptosis pathway was involved in the apoptosis induced by GNA in A549 cells, and apoptosis induced by GNA in A549 cells could be reduced when combined treatment of GNA and SB203580.

To further understand the mechanism of GNA, we next examined whether GNA induced phosphorylation of p38. As shown in Figure 7(A), after treatment with GNA in A549 cells, the expression of phosphorylation of p38 protein increased in a time-dependent manner. Meanwhile, total p38 protein levels showed a decrease in a time-dependent manner. Pretreatment with SB203580, a specific inhibitor of p38 MAPK, markedly inhibited the accumulation of phosphorylation p38 at 24 h. Furthermore, GNA also induced a decrease in MAPK protein levels in a time-dependent manner (Figure 7(B)).

In conclusion, our data have demonstrated that GNA can induce A549 cell death, primarily by apoptosis. The decline of p38 MAPK activities may account for the resistance to apoptosis, and thereby leading to unrestricted cell growth of A549 cells. Furthermore, inhibition of p38 kinase

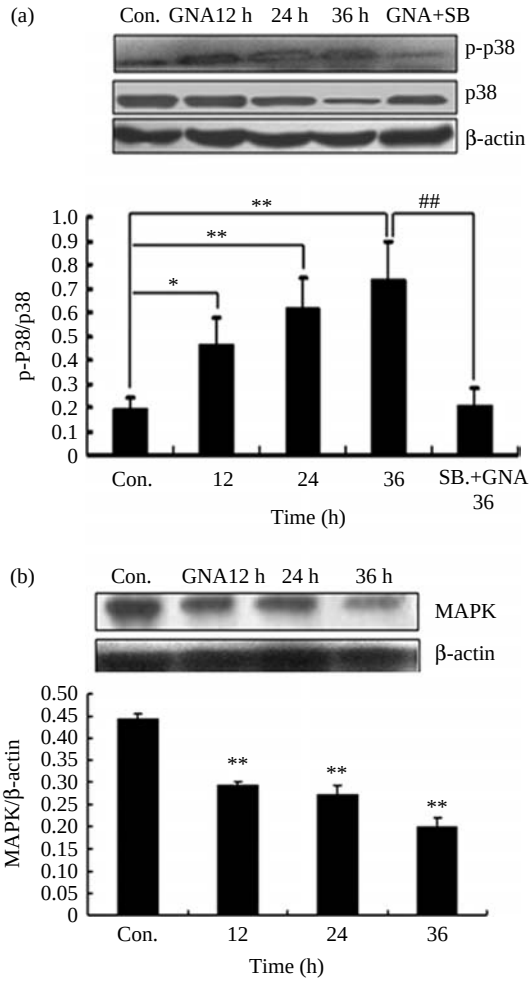

Figure 7. A549 cells were treated with 2 μM GNA for 12, 24, and 36 h in the presence or absence of SB203580. (A) Expression of p-p38 and p38 in A549 cells was analyzed by Western blotting and (B) Expression of MAPK in A549 cells was analyzed by Western blotting. Quantitative data are presented as mean ± SE of three independent experiments. (Statistical analysis: vs. control $*P < 0.05$, $**P < 0.01$; vs. GNA (2 μM) $^{\#\#}P < 0.01$).

activity in A549 cells reduced GNA-induced apoptosis. These findings are important in providing a better understanding of the antitumor mechanisms of GNA.

3. Materials and methods

3.1 Materials

GNA powder was supplied with the purity above 99.0% as determined by high-performance liquid chromatography by Prof. Xiao-Shan Wang in Anhui University of Traditional Chinese Medicine. GNA was dissolved in DMSO, which was kept at −20°C, then diluted as needed in complete culture medium immediately before use. The primary antibodies against p38, p-p38, MAPK, and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), caspase-3 and caspase-9 were from Bioworld Technology (Louis Park, MN, USA). All other common chemicals were from Abcam (Cambridge,

MA, USA) or Sigma-Aldrich (St Louis, MO, USA).

3.2 Cell lines and cell culture

Human lung adenocarcinoma cell line A549 cells supplied by the Cell Bank of Shanghai Institute of Cell Biology (Shanghai, China) were maintained in RPMI-1640 medium (Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10% heated inactivated fatal bovine serum (Hyclone, South Logan, Utah, USA), benzylpenicillin 100 kU/l, streptomycin 100 mg/l, pH 7.4 in an incubator (Sanyo, Tokyo, Japan) with a humidified 95% air and 5% CO_2 at 37°C.

3.3 Cell growth inhibition (MTT assay)

The A549 cells were digested with 0.25% trypsin/0.02% ethylene diamine tetraacetic acid to prepare a 6×10^4/ml cell suspension, then partitioned into wells of 96-well plates at 180 μl/well for overnight in a 5% CO_2 incubator at 37°C. A549 cells were treated with different concentrations of GNA in the presence or absence of SB203580 for 24, 48, and 72 h. Then, 5 mg/ml MTT (Sigma, St Louis, MO, USA) solution (20 μl/well) was added to the wells and the plates were further incubated for 4 h at 37°C, the supernatant was discarded and 150 μl DMSO was added to each well to solubilize the water-insoluble purple formazan crystals. The absorbance (*A*) was measured at 570 nm by Automated Microplated Reader ELx800 (Bio-Tek Instrument Inc., Winooski, VT, USA).

3.4 Assessment of apoptosis on A549 cells by Annexin-V/PI double-staining assay and mitochondrial membrane potential

Cells (4×10^5) were seeded in 6-well plates and incubated for 24 h at 37°C. Then, GNA (2 μM) was added to 6-well plates and incubated for an additional 12, 24, and 36 h, respectively. Apoptotic cell death was identified by double supravital staining with recombinant fluorescein isothiocyanate (FITC)-conjugated Annexin-V and PI, using the Annexin V-FITC apoptosis detection kit (Becton Dickinson, Canaan, CT, USA) according to manufacturer's instructions. Flow cytometric analysis was carried out immediately after supravital staining. Cells were excited at 488 nm and the emissions of Annexin-V at 525 nm. PI was collected through 610 nm band-pass filters. Data acquisition and analysis were carried out in a Becton Dickinson FACS Calibur flow cytometer using the CellQuest software (Becton Dickinson, San Jose, CA, USA).

After treatment with GNA for different times, are harvested cells by trypsinization and stained with Rho-123 at a final concentration of 10 μg/ml for 10 min at 37°C, then cells were washed twice with serum-free RMPI-1640. Cell suspension was incubated at 37°C, 5% CO_2 incubator for 60 min. At least 10,000 cells were analyzed for each sample. The changes were analyzed by flow cytometry using Rho-123 as the indicator, with the single beam at 488 nm excitation wavelength and 530 nm emission wavelength. Data acquisition and analysis were carried out in a Becton Dickinson FACS Calibur flow cytometer using CellQuest software.

3.5 Cytochrome c release assay

A549 cells were washed three times with cold phosphate-buffered solution, then resuspended in cell lysis buffer to a concentration of 1.5×10^6 cells/ml, and incubated for 1 h at room temperature with gentle mixing. After that, cells were centrifuged at 1000 *g* for 15 min. For quantitative comparisons, cytochrome c release was also determined using an ELISA kit (R&D Systems, Minneapolis,

MN, USA) according to the instructions of the manufacturer.

3.6 Western blot analysis

Protein of A549 cells was isolated by lysis buffer (100 mmol/l Tris–Cl, pH 6.8, 4% (m/v) sodium dodecyl sulfonate (SDS), 20% (v/v) glycerol, 200 mmol/l β-mercaptoethanol, phenylmethanesulfonyl fluoride 1 mmol/l, aprotinin 1 μg/ml) and measured using the Bradford assay with BioPhotometer (Eppendorf, Hamburg, Germany) at 595 nm. Equal amount of lysate protein was separated on 8%–10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The nonspecific binding sites were blocked with Tris-buffered saline tween-20 (TBST) containing 5% nonfat milk for 2 h. The membranes were incubated overnight at 4°C with specific primary antibodies. Then, the membranes were washed three times with TBST buffer and incubated at room temperature for 2 h with horseradish peroxidase-conjugated secondary antibody. After three washes with TBST buffer, the immunoreactive bands were detected with enhanced chemiluminescence reagents (Thermo, Scientific, Rock ford, IL USA).

3.7 Statistical analysis

Each experimental point was carried out in triplicate per experiment unless stated otherwise; the data shown represent means ± SE. Statistical significance of differences between control and sample groups was determined by Student's *t*-test. The minimal level of significance was $P < 0.05$.

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Note

1 These authors contributed equally to this work.

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