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ORIGINAL RESEARCH

Combination of gambogic acid with cisplatin enhances the antitumor effects on cisplatin-resistant lung cancer cells by downregulating MRP2 and LRP expression

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Abstract: Cisplatin resistance is a main clinical problem of lung cancer therapy. Gambogic acid (GA) could prohibit the proliferation of a variety of human cancer cells. However, the effects of GA on cisplatin-resistant lung cancer are still unclear. The objective of the present study was to find out the antitumor effects of GA on cisplatin-resistant human lung cancer A549/DDP cells and further explore its underlying mechanisms. Cell Counting Kit-8 assay was used to observe the impacts of GA and/or cisplatin on the proliferation of lung cancer cells; flow cytometry was used to detect the effects of GA on cell cycle and apoptosis; Western blot was used to examine the effects of GA on the expression of lung resistance protein (LRP) and multidrug resistance-associated protein 2 (MRP2) protein in A549/DDP cells. Our results showed that GA dose- and time-dependently prohibited the proliferation and induced significant cell apoptosis in A549 and A549/DDP cells. GA also induced G0/G1 arrest in both A549/DDP and A549 cells. Moreover, GA upregulated protein expression level of cleaved caspase-3 and Bax and downregulated protein expression level of pro-caspase-9 and Bcl-2 in time- and dose-dependent way in A549/DDP cells. GA combined with cisplatin enhanced the cells apoptotic rate and reduced the cisplatin resistance index in A549/DDP cells. In addition, GA reduced the MRP2 and LRP protein expression level in A549/DDP cells. GA inhibits the proliferation, induces cell cycle arrest and apoptosis in A549/DDP cells. Combination of GA with cisplatin enhances the antitumor effects on cisplatin-resistant lung cancer cells by downregulating MRP2 and LRP expression.

Keywords: gambogic acid, cisplatin, drug resistance, lung cancer, lung resistance protein, multidrug resistance-associated protein 2, anti-tumor, cell cycle arrest, cell apoptosis

Introduction

Lung cancer is the commonest cause of cancer-related death around the world. Non-small-cell lung cancer (NSCLC) occupies ~75%–80% of lung cancer.[1] Platinum-based doublet chemotherapeutic regimens have become the primary therapeutic method for NSCLC. However, the 5-year survival rate is still very low.[2] Cisplatin resistance is a main clinical problem of lung cancer therapy.[3] Therefore, it is urgently needed to find novel agent that possess anticancer effects on the cisplatin-resistant lung cancer cells.

Gambogic acid (GA) is a main ingredient of gamboge, the resin secreted from *Garcinia hanburyi* tree in South-East Asia.[4] GA has been used as one of traditional medicine for centuries and it possesses many kinds of biological effects such as anti-infectious, anti-oxidant, anti-inflammatory, and antiviral.[5] In recent years, many researchers have found that GA could inhibit the proliferation of a variety of human cancer cells in vitro,

including lung cancer,[6] leukemia,[7] colorectal cancer,[8] prostate cancer,[9] hepatocarcinoma,[10] and breast cancer.[11] The possible antitumor mechanisms of GA are associated with the enhancement of reactive oxygen species accumulation,[8] inhibition of telomerase,[12] induction of apoptosis,[13] and interruption of nuclear factor-κB signal pathway.[14] Also, GA exhibits anticancer effects on NCI-H1993 xenografts by regulation of the MET signal pathway.[15] GA is a prospective antitumor drug with less toxic effects on the normal tissues,[16] which has been authorized for the treatment of variety of cancer in clinical trials by the Chinese Food and Drug Administration.[17] A Phase IIa clinical study suggests that GA administered at 45 mg/m² is safe.[18] Moreover, GA could enhance antitumor effects of cisplatin on both osteosarcoma[19] and lung cancer.[20] In addition, GA not only could sensitize esophageal cancer cells to radiation via blocking Akt/mammalian target of rapamycin pathway and nasopharyngeal carcinoma cells in hypoxic conditions,[21,22] but also reverse docetaxel resistance in gastric cancer,[23] doxorubicin resistance in breast cancer[24] and ovarian cancer,[25] 5-fluorouracil resistance in colorectal cancer,[13] and the multidrug resistance of human in epithelial cancer.[26]

However, the impact of GA on cisplatin-resistant lung cancer is still unknown. The purpose of this study was to find out effects of GA in aspect of cell growth, cell cycle, and apoptosis and observe the antitumor effects of GA combined with cisplatin in cisplatin-resistant lung cancer A549/DDP cells and explore its underlying mechanisms.

Materials and methods
Reagents
GA (>98% purity, Sigma-Aldrich, St Louis, MO, USA) was soluble in dimethyl sulfoxide and conserved at −20°C. The preservation solution was diluted to different concentrations in use, in which the dimethyl sulfoxide concentration was <0.1%. Cisplatin was purchased from Stockhausen Pharmaceutical Co., Ltd (Lianyungang, Jiangsu, People's Republic of China). Roswell Park Memorial Institute medium 1640 was obtained from HyClone (Logan, UT, USA). Cell Counting Kit-8 (CCK-8) was acquired from Dojindo (Rockville, MD, USA). Propidium iodide (PI), RNase A, and annexin V-fluorescein isothiocyanate was purchased from Keygen Biotechnology (Nanjing, Jiangsu, People's Republic of China) and dissolved in phosphate buffered saline (PBS). The primary antibodies against Bcl-2, Bax, anticaspase-9, anticaspase-3, multidrug resistance-associated protein 2 (MRP2), lung resistance protein (LRP), and β-tubulin were acquired from Cell Signaling Technology (Danvers, MA, USA). And goat antimouse or antirabbit IgG-HRP secondary

antibody was acquired from Beyotime Biotech (Nanjing, Jiangsu, People's Republic of China).

Cell culture
Human lung adenocarcinoma cisplatin-resistant cell lines (A549/DDP) and cisplatin-sensitive cell lines (A549) were acquired from BioLeaf Biotech (Shanghai, People's Republic of China) and American Type Culture Collection (Manassas, VA, USA) and cultured in Roswell Park Memorial Institute medium 1640 with 10% fetal bovine serum (FBS; Biological Industries, Cromwell, CT, USA), maintained in a suitable environment with 5% CO_2 at 37°C. This study was performed with the approval of the Institutional Ethic Committee of Guangdong Medical University.

Cell proliferation assay
A549 and A549/DDP cells were seeded in a 96-well plate at density of $5×10^3$ cells per well. Incubated ~24 hours later, the former medium was replaced by fresh medium with GA as following concentrations (0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 μM) for 24 and 48 hours in the presence of 1% FBS. Cell viability was tested by using the CCK-8 assay. The cells were incubated ~2 hours before absorbance read for the CCK-8 proliferation assay. Each well absorbance was tested at 450 nm by microplate reader. The proliferation rate was defined in terms of the percentage of each group surviving cells compared with the untreated group.

Cell cycle analysis
For flow cytometry analysis, $2×10^5$ cells were gathered by $1×10^3$ r/min centrifugation following GA treatment (0, 1.0, 2.0 μM) for 24 hours at 37°C. The cells were fixed with 70% ethanol at 4°C overnight after washing it twice with PBS. The fixed cells were washed one time with PBS and incubated with 50 μg/mL PI plus 100 μg/mL RNase A before the flow cytometry analysis.

Cell apoptosis assay
For flow cytometry analysis, $1×10^5$ cells were harvested after different treatment, collected by centrifugation (10 min, $1×10^3$ r/min) and washed with PBS three times. The cells were resuspended in binding buffer then incubated with 5 μL annexin V-fluorescein isothiocyanate and PI for 15 minutes in the dark before analyzing by flow cytometry.

Resistance index of cisplatin
The calculation way we learned from this article.[27] The A549/DDP cells cisplatin resistance index (RI) was also tested by

the CCK-8 assay. A549/DDP cells and A549 were seeded in 96-well plates at density of 5×10^3 cells per well. Incubated ~24 hours later, the former medium was replaced by fresh medium with cisplatin at the following concentrations (0, 2.5, 5.0, 10, 20, and 40 µg/mL) for 24 and 48 hours in the presence of 1% FBS. Cells were prepared for absorbance reading after 24 and 48 hours incubation. Cell inhibitory rate was calculated using the formula: inhibitory rate $= 100 \times (A_{con} - A_t)/A_{con}$, where A_{con} is the A value of the control group and A_t is the A value of the treatment group. The 50% inhibitory concentration (IC_{50}) was calculated from three independent experiments' dose–response data. The A549/DDP cells RI of cisplatin was calculated using the formula: $RI = IC_{50}A549/DDP/IC_{50}A549$. The experiment was repeated in the presence of 2 µM GA.

Western blot analysis

Cells were seeded in six-well plates at a density of 1×10^5 per well. After treatment with GA and cisplatin, the preparation of total protein samples from the culture cells for immunoblotting was carried out as previously described.[28] The membrane was incubated with the following indicated antibody: Bax, Bcl-2, caspase-3, caspase-9, MRP2, LRP, and β-tubulin. The interest protein was tested with goat antimouse or antirabbit IgG-HRP secondary antibody.

Statistical analysis

The data were expressed as mean ± standard deviation. Student's t-test method was used for two-group comparison. One-way ANOVA was used for more than two-group comparison. All statistical analyses were calculated by SPSS 13.0 software (SPSS, Chicago, IL, USA). $P < 0.05$ was considered to be statistically significant.

Results
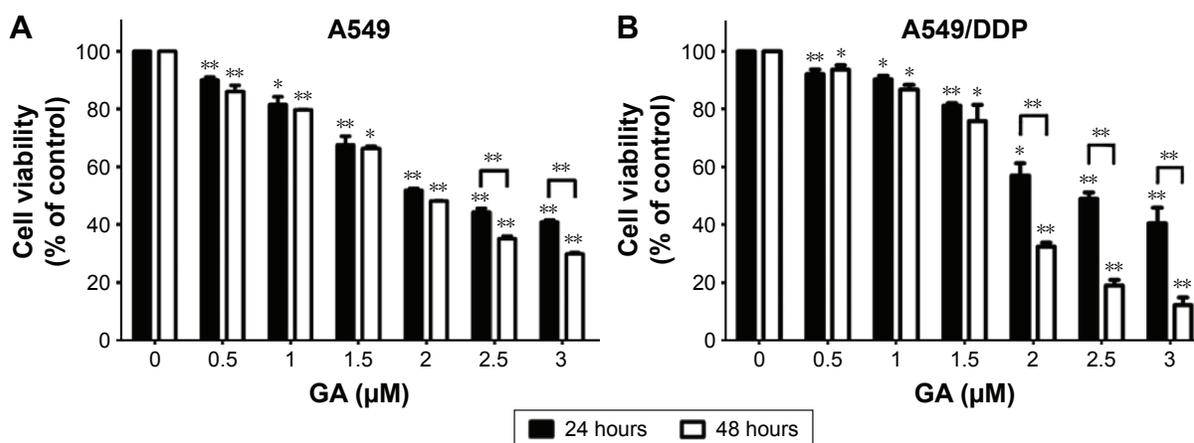
GA prohibits the growth of A549/DDP and A549 cells

Both A549/DDP and A549 cells were exposed to different concentrations of GA (0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 µM) for 24 and 48 hours. Cell viability was tested by CCK-8 assay. The results showed that GA prohibited A549/DDP and A549 cells proliferation in a time- and dose-dependent manner (Figure 1, $P < 0.05$). The IC_{50} after GA treatment for 24 hours in the A549/DDP and A549 cells were 2.591±0.782 and 2.261±0.218 µM, respectively.

GA induces cell cycle arrest at G0/G1 in A549 and A549/DDP cells

To figure out whether GA inhibits cell proliferation by promoting cell cycle progression changes, flow cytometry was used to detect the impact of GA on cell cycle. A549 and A549/DDP cells were exposed to 0, 1.0, and 2.0 µM GA for 24 hours. The results indicated that GA caused an increase in the G0/G1 stages in A549/DDP and A549 cells, suggesting that GA induced G0/G1 cell cycle arrest (Figure 2A and B, $P < 0.01$).

## GA induces A549/DDP cells apoptosis in time- and dose-dependent way

A549/DDP cells were treated with 0, 0.5, 1.0, and 2.0 µM GA for 24 hours and 2 µM GA for 24–72 hours. After GA

Figure 1 GA inhibits the growth of A549 (**A**) and A549/DDP (**B**) cells.
Notes: Human lung adenocarcinoma cells were treated with GA at the indicated concentrations (0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 µM) for 24 and 48 hours, and the proliferation rates were tested by CCK-8 assay. GA significantly reduced the viability of the two cell lines in a dose- and time-dependent manner. All data are presented as the mean values ± SD from three independent experiments. Cell proliferation in the untreated control cells was regarded as 100%. *$P < 0.05$; **$P < 0.01$ vs control cells; bars, SD.
Abbreviations: CCK-8, Cell Counting Kit-8; GA, gambogic acid; SD, standard deviation.
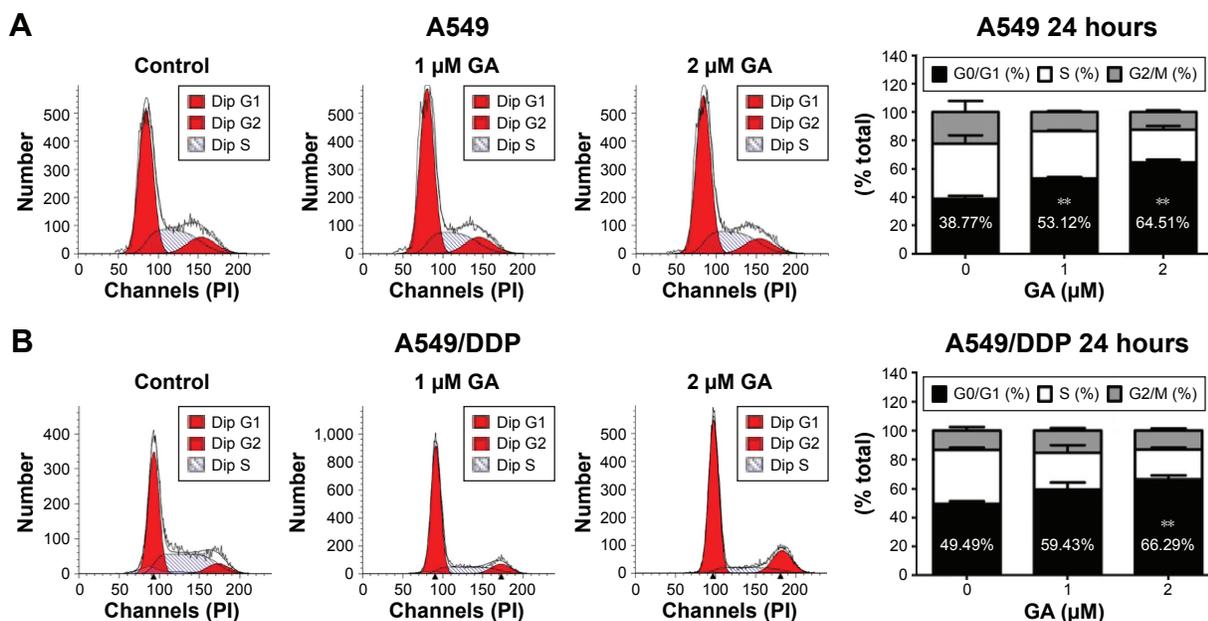
Figure 2 GA induces G0/G1 cell cycle arrest in A549 (**A**) and A549/DDP (**B**) cells.
Notes: A549 and A549/DDP cells were treated with 0, 1.0, and 2.0 µM GA for 24 hours. After staining with propidium iodide, cell cycle stage was analyzed by flow cytometry. The cell cycle distribution was quantified in GA-treated and -untreated control cells. All data are presented as the mean ± SD of at least three independent experiments. **P<0.01 vs control cells; bars, SD.
Abbreviations: GA, gambogic acid; SD, standard deviation; PI, propium iodide.

treatment at 0.5, 1.0, and 2.0 µM for 24 hours, the cells apoptosis rate was 14.15%±7.70%, 20.18%±7.91%, and 27.30%±7.69%, respectively, which were significantly different from the unexposed group (5.67%±2.54%) (Figure 3A, P<0.05). The GA treatment caused an increase in A549/ DDP cells apoptosis for 24–72 hours (Figure 3B, P<0.05). The results illustrated that GA could induce A549/DDP cells apoptosis in a time- and dose-dependent way. To further ensure that caspase activation was related to GA-induced A549/DDP cells apoptosis process, we found that GA upregulated the protein expression level of cleaved caspase-3 and Bax and downregulated protein expression level of pro-caspase-9 and Bcl-2 in A549/DDP cells (Figure 3C–F, P<0.01).

## GA reduces the resistance index of A549/DDP cells

Cell proliferation inhibited by cisplatin is apparently attenuated in A549/DDP cells compared with A549 cells. However, the sensitivity of A549/DDP cells with the presence of GA to cisplatin is more than absence of GA. In addition, cisplatin RI of the A549/DDP cells was significantly weakened by the GA and the reversal multiples was 1.81 (24 hours) and 2.05 (48 h) (Figure 4, P<0.01). Therefore, the data indicated that GA dramatically reduced the A549/DDP cells cisplatin resistance.

GA combined with cisplatin results in an enhancement of cell apoptosis effects in a time-dependent way

The A549/DDP cells were treated with a combination of cisplatin (10 µg/mL) and GA (2 µM). After treatment of GA and cisplatin for 24–72 hours, the apoptotic rate of A549/ DDP cells were 18.0%, 52.4%, and 74.8%, respectively. While the control and monotreatment groups were less than combined treatment group (Figure 5A, P<0.01). Meanwhile, GA and/or cisplatin induced A549/DDP cell apoptosis in a time-dependent way. Furthermore, after the same treatment Western blot was used to detect the expression of Bcl-2, Bax, caspase-3, and caspase-9 in the A549/DDP cells. The results showed that the combination of these two drugs caused a significantly greater increase in cleaved caspase-3 and Bax and decrease in pro-caspase-9 and Bcl-2 than either agent alone (Figure 5B–F, P<0.01). Taken together, these results showed that GA combined with cisplatin could be more efficient via apoptosis-inducing action on A549/DDP cells.

## GA downregulates the protein expression of MRP2 and LRP in a dose-dependent way

To further figure out the underlying mechanisms of GA reducing A549/DDP cells resistance index, we first examined the basic expression level of MRP2 and LRP protein in
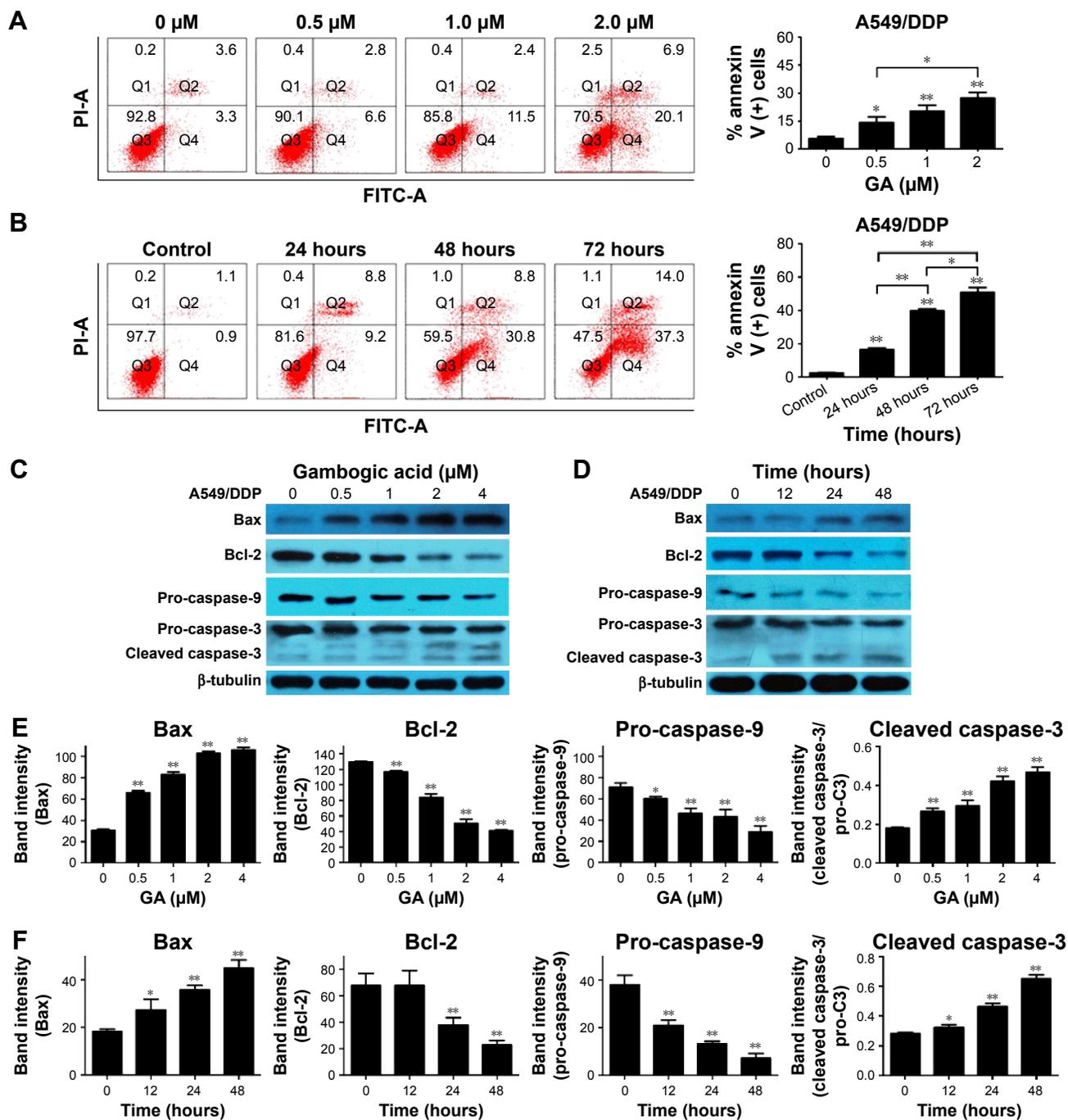
Figure 3 GA induces apoptosis in A549/DDP cells in a dose- and time-dependent way.
Notes: (**A**) A549/DDP cells were treated with 0, 0.5, 1.0, and 2.0 μM GA for 24 hours. (**B**) A549/DDP cells were treated with 2 μM GA for 0, 24, 48, and 72 hours. Cell apoptosis was analyzed by flow cytometry at various time points. The cell apoptotic rates were quantified. (**C**) A549/DDP cells were treated with GA at the indicated concentrations (0, 0.5, 1.0, 2.0, and 4.0 μM) for 24 hours. (**D**) A549/DDP cells were treated with GA for 0, 12, 24, and 48 hours. The levels of Bax, Bcl-2, caspase-9, and caspase-3 were determined by Western blot analysis. (**E** and **F**) GA upregulated the expression level of Bax and cleaved caspase-3 and downregulated Bcl-2 and pro-caspase-9 expression in A549/DDP cells. Data are shown as the mean values ± SD from three independent experiments. *$P<0.05$; **$P<0.01$; bars, SD.
Abbreviations: GA, gambogic acid; SD, standard deviation.

A549/DDP and A549 cells. The basic protein expression of MRP2 and LRP of A549/DDP cells was significantly more than A549 cells (Figure 6A). We further detected the changes of MRP2 and LRP protein after disposing with GA (0, 0.5, 1.0, 2.0, 4.0 μM) for 24 hours. The results showed that A549/DDP cells in response to GA had a distinct downregulation of protein expression level of MRP2 and LRP compared with untreated cells (Figure 6B, $P<0.01$).
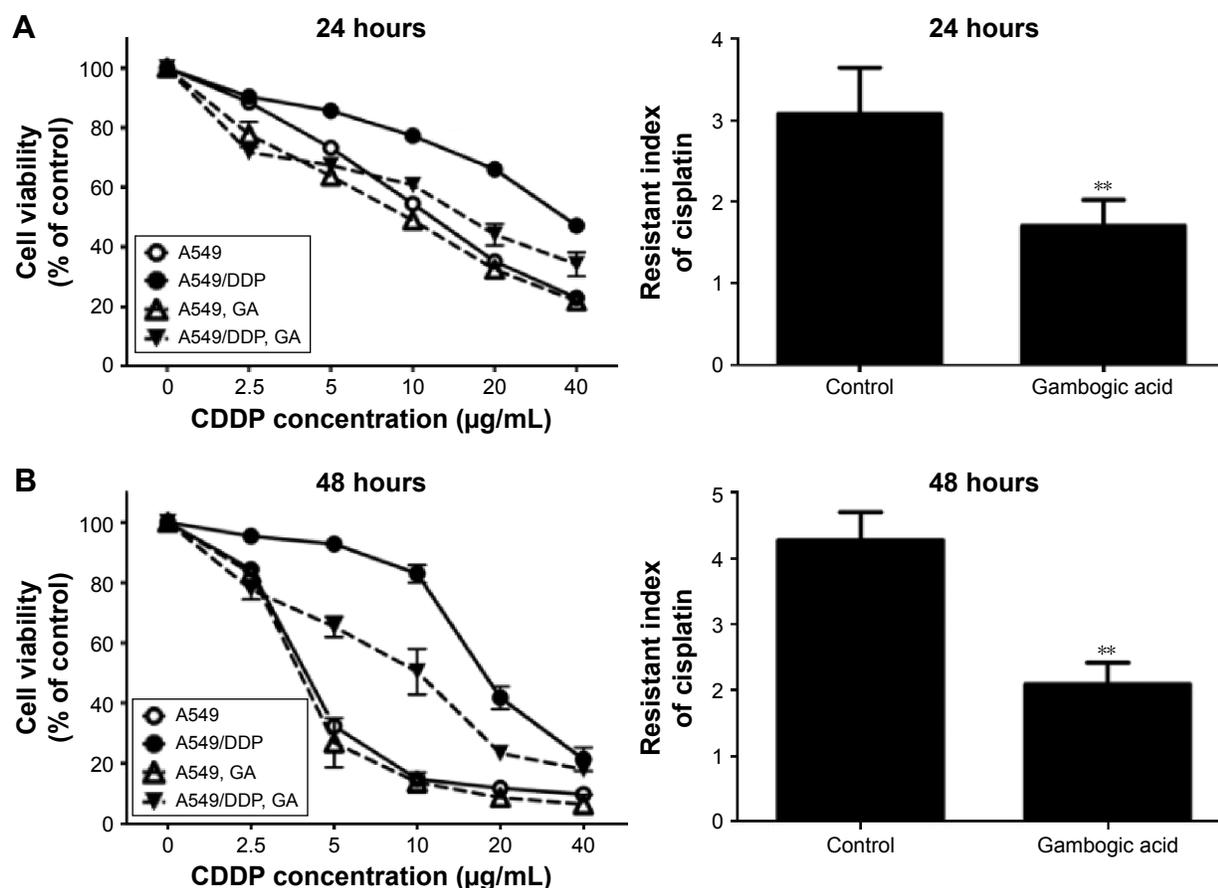
Figure 4 GA reduces the RI of A549/DDP cells. A549 and A549/DDP cells were exposed to increasing concentrations of cisplatin for 24 and 48 hours in the presence or absence of GA (2 μM). CCK-8 assay was used to detect cell growth. The rate of growth inhibition in A549 and A549/DDP cell line in the presence and absence of GA for 24 hours (**A**) and 48 hours (**B**). RI of cisplatin in A549/DDP cells in the presence and absence of GA was presented at the right side. Values are expressed as mean ± SD (n=3). Data were analyzed by Student's *t*-test. **P<0.01 vs control cells; bars, SD.
Abbreviations: CCK-8, Cell Counting Kit-8; GA, gambogic acid; RI, resistance index; SD, standard deviation; CDDP, cisplatin.

Discussion

Resistance to cisplatin-based chemotherapy is still one of the main obstacles in the therapy for lung cancer.[29] Hence, it is necessary to find novel drug to overcome cisplatin resistance. In this research, we reported for the first time that GA could effectively suppress the proliferation of A549/DDP cells in a time- and dose-dependent way (Figure 1A). Intriguingly, A549 cells showed a higher viability rate after GA treatment at 2, 2.5, and 3 μM for 48 hours when compared with A549/DDP cells (Figure 1B), although the conclusion was without too much influence, but which is an unexpected phenomenon worthy of further study. We suppose that cisplatin resistance A549/DDP cells may have some different expression levels of gene or protein compared with A549 cells, which make A549/DDP cells sensitive to GA. Maybe the counterbalance phenomenon of A549/DDP will provide another perspective to solve the problem of cisplatin resistance of lung cancer. Further investigation demonstrated that GA might exert its growth inhibition impact via inducing cell cycle arrest at G0/G1 stage (Figure 2B) and cell apoptosis

(Figure 3A and B) on A549/DDP cells. One of the limitations of this research was not to explain this phenomenon of cell cycle arrest by GA on A549/DDP and A549 cells.

To elucidate whether GA could reverse cisplatin resistance in lung cancer, we explored the effects of GA combined cisplatin on A549/DDP cells. Our results showed that cisplatin resistance index of the A549/DDP cells was significantly attenuated by GA and the reversal multiples were 1.81 (24 hours) and 2.05 (48 hours) (Figure 4), which implied that GA may enhance the antitumor effects of cisplatin on cisplatin-resistant lung cancer cells. Similar to this article, other studies also reported that GA could enhance K562/A02 cells' sensitivity to adriamycin with a reversal multiple of 1.53.[30] Furthermore, the apoptotic rate was higher when combining GA with cisplatin in comparison with single agent in A549/DDP cells (Figure 5). Consequently, we came to a conclusion that GA could enhance the cytotoxic effects of cisplatin in cisplatin-resistant lung cancer.

Previous studies have suggested that several mechanisms could lead to cisplatin resistance, including reduced
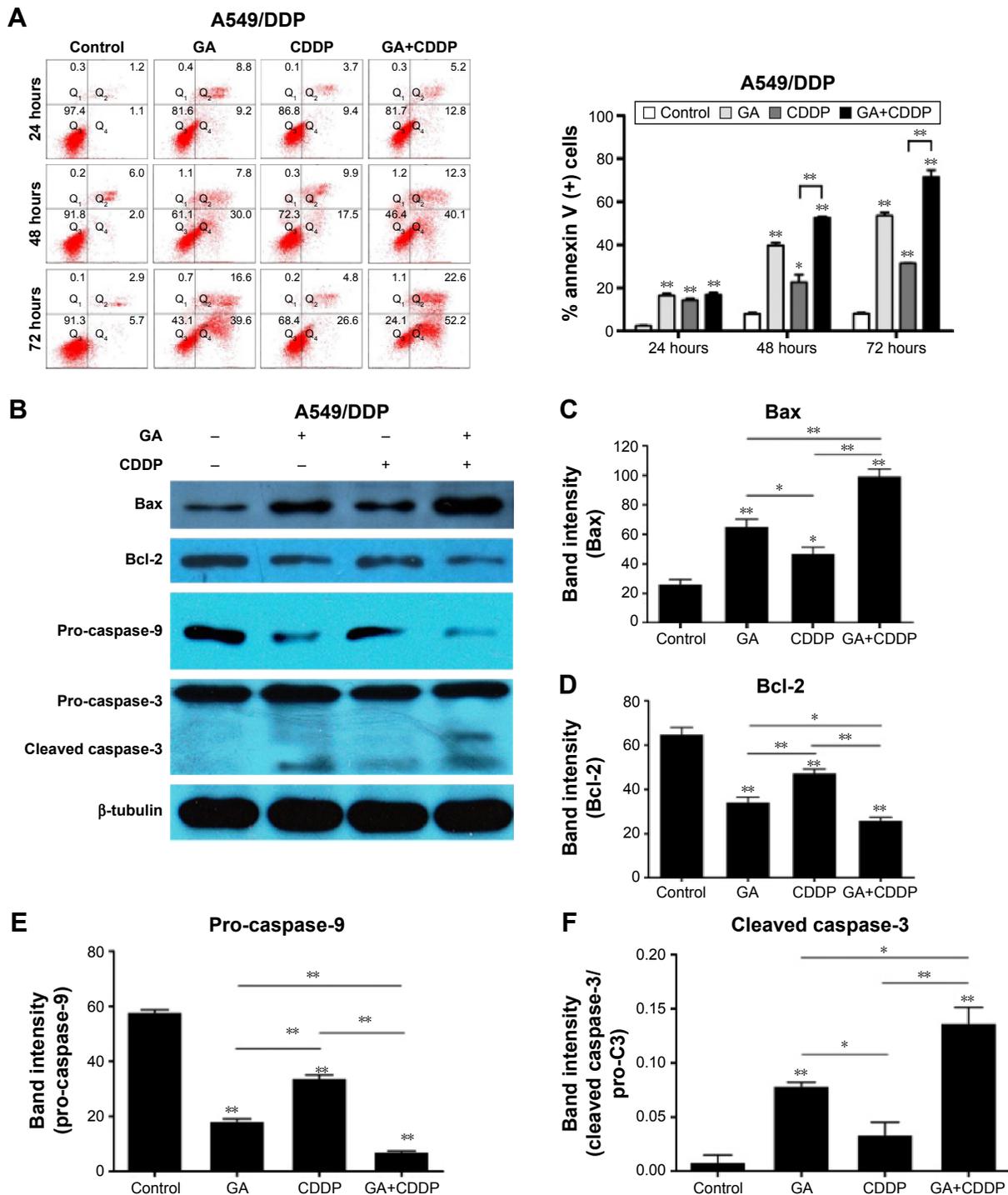
Figure 5 Combination of GA and cisplatin results in an enhancement of apoptosis effects in a time-dependent manner. (**A**) A549/DDP cells were treated with GA or cisplatin alone or combined with GA (2 μM) and cisplatin (10 μg/mL) for 24, 48, and 72 hours. (**B**) A549/DDP cells were treated with GA or cisplatin alone or combined with GA and cisplatin for 48 hours. The levels of Bax, Bcl-2, caspase-9, and caspase-3 were determined by Western blot analysis. (**C** and **F**) The Bax and cleaved caspase-3 protein in cells following the combined treatments was markedly increased compared with the cells exposed to GA or cisplatin alone. (**D** and **E**) The Bcl-2 and pro-caspase-9 proteins in cells following the combined treatments were markedly decreased compared with that cells exposed to GA or cisplatin alone. Data are shown as the mean values ± SD from three independent experiments. *$P < 0.05$; **$P < 0.01$; bars, SD.
Abbreviations: GA, gambogic acid; SD, standard deviation; CDDP, cisplatin.

drug accumulation,[31] enhanced drug inactivation,[32] restored DNA damage repair,[33] inactivated apoptotic signaling.[34] The reducing drug accumulation is on account of drug-resistant proteins.[35–38] Several studies have shown that reducing

cisplatin-resistant protein could reverse cisplatin resistance or enhance tumor sensitivity to cisplatin.[39–41] A host of cisplatin-resistant proteins have been reported, for instance, LRP, MRP2, and breast cancer resistance protein.[36,42,43] LRP is
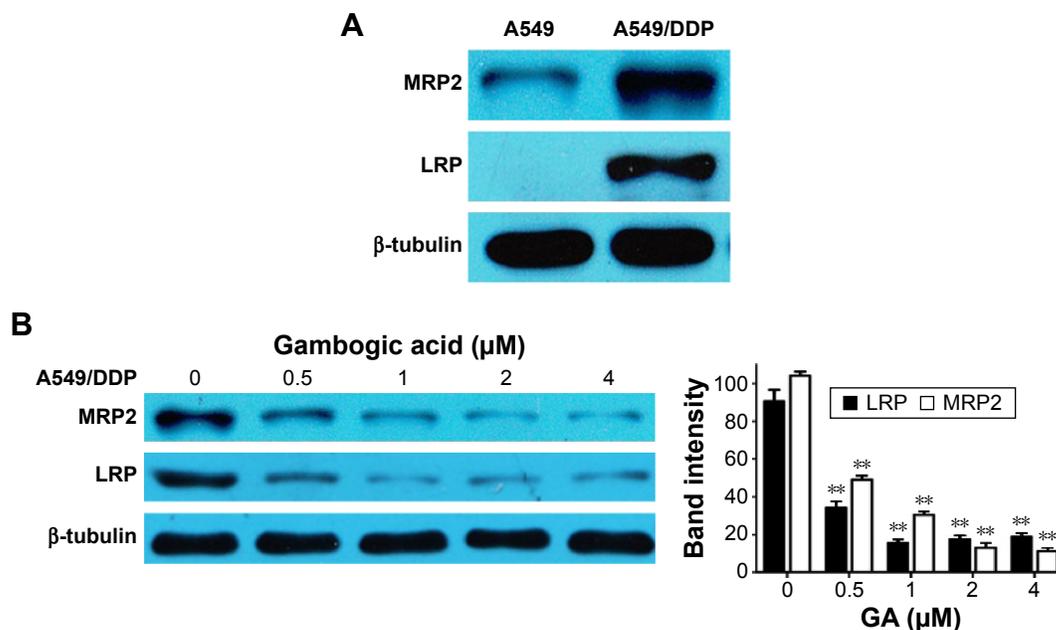
Figure 6 GA downregulates the protein expression of MRP2 and LRP in a dose-dependent way.
Notes: (**A**) The basic protein expression of MRP2 and LRP in untreated A549 and A549/DDP cells. (**B**) Cells were treated with GA at the indicated concentrations (0, 0.5, 1.0, 2.0, and 4.0 µM) for 24 hours. The levels of MRP2 and LRP were determined by Western blot analysis. Data are shown as the mean values ± SD from three independent experiments. **P<0.01; bars, SD.
Abbreviations: GA, gambogic acid; LRP, lung resistance protein; MRP2, multidrug resistance-associated protein 2; SD, standard deviation.

overexpressed in several non-P-glycoprotein cancer cell lines with performance of MDR.[44] LRP is a small subcellular structure located at cytoplasmatic vaults that may be in charge of subsequent exocytosis of agents from the cell.[45] In vitro, it has been found that LRP is related to resistance to melphalan, cisplatin, and doxorubicin.[44] MRP2 is overexpressed in a lot of cisplatin-resistant cell lines.[46,47] To figure out that whether the protein could lead to cisplatin resistance or not, we first detected the expression of LRP and MRP2 protein in A549/DDP and A549 cells. Western blot analysis demonstrated that the expression of LRP and MRP2 protein of A549/DDP cells is significantly more than A549 cells (Figure 6A), which is consistent with the previous researches.[48,49] Interestingly, GA could significantly downregulate the protein expression level of MRP2 and LRP of A549/DDP cells in a concentration-dependent manner (Figure 6B). Unexpected, there was not the same result on A549 cells. The MRP2 protein expression level of A549 cells was too little to be observed, letting alone LRP protein (Figure 6A). This was probably a consequence of the LRP and MRP2 protein of A549 cells, being too less than A549/DDP cells. For this reason, GA could not fully exert its same effects of downregulating resistance-associated protein on A549 cells. It seems that the phenomenon of the cell viability drop value of A549 cells was lower than A549/DDP cells after the same treatment sequence of cisplatin alone to GA plus cisplatin on 24 and 48 hours, respectively

(Figure 4A and B). Therefore, GA might enhance the cytotoxic effects of cisplatin by reducing cisplatin-resistant LRP and MRP2 protein on A549/DDP cells.

Excision repair cross-complementing 1 (ERCC1) has become a popular research subject in platinum resistance of NSCLC. ERCC1, as a DNA repair endonuclease, is charge of the 5′-incision in DNA excision repair and recognizing and removing platinum-induced nucleotide adducts in the nucleotide excision repair pathway.[50,51] ERCC1 overexpression participated in cisplatin resistance by inhibiting the EGFR pathway activation.[52] These discoveries are in accordance with the clinical phenomenon that ERCC1 in NSCLC prohibits cisplatin efficacy. High ERCC1 of NSCLC tumors will negative respond to cisplatin-based chemotherapy.[53,54] In this research, we applied the limited theory to explain this phenomenon of GA enhancing the cytotoxic effects of cisplatin on A549/DDP cells by downregulating LRP and MRP2 protein. GA could influence ERCC1 gene or protein expression, thus partially enhancing the cytotoxic effects of cisplatin on A549/DDP cells. Therefore, it is very necessary to confirm this hypothesis in next step.

In conclusion, our results demonstrated that GA has remarkable antigrowth effect by inducing cell cycle arrest and cell apoptosis. Downregulation of cisplatin-resistant protein LRP and MRP2 might represent a mechanism for the effects of GA on cisplatin-resistant human lung cancer A549/DDP cells.

Combination of GA with cisplatin enhanced the anti-tumor effects on cisplatin-resistant lung cancer cells by downregulating MRP2 and LRP expression. These findings implied that GA may be used as a promising adjuvant drug for the therapy of cisplatin-resistant human lung cancer.

Acknowledgments

Disclosure

The authors report no conflicts of interest in this work.

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