

ORIGINAL ARTICLE

Gambogic acid and chloroquine synergistically induce cell death via increasing mitochondria damage in human NSCLC cells

Yanting Sun¹ | Xiaoliu Wu²  | Ming Zhu² | Yuanying Zhang² | Min Lv² | Liling Dai² | Jun Yu²

¹Hospital-Acquired Infection Control Department, Nanjing Medical University Affiliated Cancer Hospital, Cancer Institute of Jiangsu Province, Nanjing, Jiangsu Province, China

²Department of Science and Technology, Nanjing Medical University Affiliated Cancer Hospital, Cancer Institute of Jiangsu Province, Nanjing, Jiangsu Province, China

Correspondence

Liling Dai and Jun Yu, Department of Science and Technology, Nanjing Medical University Affiliated Cancer Hospital, Cancer Institute of Jiangsu Province, No. 42 Baiziting, Nanjing 210009, Jiangsu Province, China.
Email: 13815861801@126.com and fisheryj@hotmail.com

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Abstract

Gambogic acid is a natural bioactive compound from the brownish resin of the *Garcinia hanburyi* trees, which has been shown to induce cell death of cancer cells with a synergistic effect together with other compounds. In the present study, we aim to investigate whether the combination of gambogic and chloroquine, one of the inhibitor of autophagy, could increase cell death in human NSCLC cells. As a result, gambogic acid and chloroquine synergistically suppress the growth of NSCLC cells (CI <0.9). Moreover, the combination of gambogic acid and chloroquine significantly promotes apoptosis than that either alone does. Mechanistically, the results demonstrate that gambogic acid increases PINK1 expression at mRNA and protein level, accompanied with downregulation of Tom20, suggesting the mitophagy process is activated. Both silencing PINK1 and treatment of chloroquine could inhibit mitophagy, therefore disrupting the clearance of damaged mitochondria. Thus, co-treatment of gambogic acid and chloroquine results in an elevated level of ROS, which facilitates cell death. Taken together, our finding highlights that the synergistically inhibitory effect of gambogic acid and chloroquine on NSCLC cells is associated with the accumulation of mitochondrial damage.

KEYWORDS

apoptosis, chloroquine, gambogic acid, mitochondria, NSCLC

Abbreviations: BCA, bicinchoninic acid assay; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate isomer; HRP, horseradish peroxidase; MTT, (4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan; NAC, N-acetyl-L-cysteine; NSCLC, non-small-cell lung cancer; PBS, phosphate buffer saline; PCR, polymerase chain reaction; PI, propidium iodide; PINK1, PTEN-induced putative kinase 1; RIPA, radio immunoprecipitation assay; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Yanting Sun and Xiaoliu Wu contributed equally to this study.

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1 | INTRODUCTION

Gambogic acid (GA, structure as in Figure 1A) is one of the main components derived from the brownish resin of the *Garcinia hanburyi* trees which are mainly found in Southeast Asia, and have been widely used as traditional herb medicines. As the most studied member of natural product family that is structurally characterized by a xanthone backbone where the C ring has been converted into a caged tricyclic structure, GA has been reported to exert anticancer activity in a wide

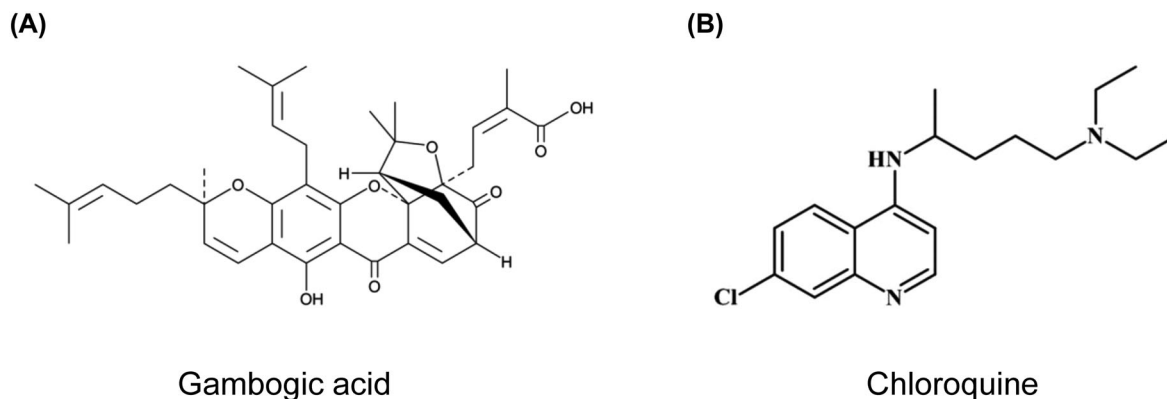


FIGURE 1 Structure of GA and CQ. (A) Chemical structure of GA; (B) Chemical structure of CQ.

spectrum of cancer cells including non-small-cell lung cancer (NSCLC) and multiple animal models.¹ Most importantly, GA has been proven to be safe and beneficial for cancer patients in phase I and phase IIA clinical trials in China.² Nevertheless, the underlying mechanisms remain to be investigated.

Mitochondria play essential roles in energy production, retention of redox balance, calcium buffering, and cell death.³ Evolutionally, cells have adopted multiple quality-control systems in response to mitochondrial damage, including biogenesis of healthy mitochondria, mitochondrial fusion and fission, as well as the selective removal of dysfunctional mitochondria by autophagy which is usually referred to as mitophagy. It has been believed that the mitophagy process is conserved in both mammalian and yeast cells, in which PINK1-Parkin-dependent mitophagy is the most characterized pathway. PINK1 senses damaged mitochondria, and subsequently recruits cytosolic Parkin to the outer mitochondrial membrane, which enables recognition of the marked mitochondria by receptor proteins such as SQSTM1/p62 for autophagic elimination.⁴ It has been recognized that mitophagy plays dual functions for cancer cell survival depending on cell content and stress condition. Mitophagy activation may reduce mitochondrial oxidative stress injury, and eliminate pro-apoptotic mitochondria, therefore playing cytoprotective roles in cancer cells.^{5,6} Indeed, inhibition of PINK1-Parkin mediated mitophagy increases the apoptotic cell death of multidrug-resistant cancer cells induced by betulinic acid analog B5G1 in liver cancer.⁷ Moreover, an E3 ubiquitin ligase ARIH1/HHARI which stimulates mitophagy protects cancer cells from chemotherapy-induced toxicity.⁸ These evidences suggest that mitophagy inhibition in cancer cells might improve the efficiency of chemotherapy. However, it remains necessary to explore the potential of targeting mitophagy as a therapeutic strategy for certain tumors.

Previously reports showed that GA could localize in mitochondria and then cause its damage.⁹ In the current study, our data demonstrate that GA induces the mitophagic process through increasing PINK1 expression in human NSCLC cells. Moreover, inhibition of mitophagy with chloroquine (CQ, structure as in Figure 1B) significantly enhances the cytotoxicity of GA against NSCLC cells, which may be attributed to excessive ROS production resulting from the accumulation of damaged mitochondria. Scavenging of ROS or

silencing of PINK1 dramatically decreases cell death induced by the combined GA and CQ in NSCLC cells.

2 | MATERIALS AND METHODS

2.1 | Reagents

GA and CQ were purchased from MedChem Express (Shanghai, China) and dissolved in DMSO as a stock solution at 2 and 80 mM, respectively. MTT and NAC solutions were obtained from Beyotime (Shanghai, China). Primary antibodies against PINK1, Tom20, and ACTB were from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2 | Cell culture

Human NSCLC cell lines (A549, H460) were purchased from Cell Bank of Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). Cells were grown in RPMI-1640 medium (Gibco, USA) supplemented with 10% heat-inactivated FBS (Gibco). All cells were kept in a humidified atmosphere of 5% CO₂ at 37°C.

2.3 | MTT assay and combination index

Briefly, cells (5×10^3) were seeded into 96-well culture plates for incubation overnight. After treatment, MTT solution was added into each well for further 4 h incubation at 37°C. Then MTT solution was aspirated, followed by the addition of 100 μ L DMSO solution into the wells for 15 min. After that, the absorbance of the sample was measured at 490 nm using a microplate reader (Bio-Tek, Winoosky, VT). For combined treatment with two drugs, cells were first exposed to CQ for 2 h, then treated with GA for another 24 h. The combination index (CI) was calculated according to the method of Chou and Talalay, using the CalcuSyn software (Biosoft, UK). If the CI < 0.90, this indicated synergism; a CI between 0.90 and 1.10 indicated an additive effect; and a CI > 1.10 indicated antagonism.

2.4 | Flow cytometry

Cells were cultured in six well plates overnight. After appropriate treatment, cells were washed with cold PBS, followed by staining with Annexin V-FITC/PI (KeyGEN BioTECH, Nanjing, China) and DCFH-DA (Beyotime) respectively, according to the manufacturer's instruction. The stained cells were subjected to a FACS Calibur flow cytometer (BD Biosciences, Bedford, MA) to analyze cell apoptosis and intracellular ROS production.

2.5 | RNA isolation and real-time PCR analysis

Total RNA was extracted from cells using the RNA-easy isolation reagent (Vazyme, Nanjing, China). RNA (1 µg) was reverse transcribed using a HiScript III cDNA synthesis kit (Vazyme) according to the manufacturer's instruction. Real-time PCR was carried out by SYBR Green Supermix (Roche, Pleasanton, CA) with the following primer sets: PINK1-sense (5'-GGACACGAGACGCTTGCA-3'); PINK1-antisense (5'-TTACCAATGGACTGCCCTATCA-3'); GAPDH-sense (5'-TCTGGTAAAGTGGATATTGTTG-3'); and GAPDH-antisense (5'-GATGGTGATGGGATTTCC-3'). Gene expression levels were analyzed by Applied Biosystems QuantStudio 5 Real-time PCR system (Life Technologies) using GAPDH as a house-keeping gene. The fold change in mRNA levels was calculated through the $2^{-\Delta\Delta C_t}$ method.

2.6 | Measurement of cytoplasmic mtDNA

Cells were seeded in six-well plates ($3-5 \times 10^5$ cells per well) and treated with GA for 24 h. After that, cytosolic mtDNA was isolated from the same number of untreated or GA-treated cells, and then quantified by real-time PCR analysis as described previously.¹⁰

2.7 | RNA interference

Cells were seeded on a 6-well plate (3×10^5 cells/well). After 24 h, siRNA for control and PINK1 (sense: 5'-UGGAUUUGUACCAUUCUU-CUGdTdT-3') were transfected at a final concentration of 30 nM using siRNA Lipofectamine RNAiMAX Transfection Reagent (Invitrogen, USA) according to the manufacturer's instruction. The efficiency of gene silencing was evaluated by real time PCR and western blot analysis.

2.8 | Western blot analysis

Briefly, cells were lysed using RIPA buffer with protease/phosphatase inhibitors. After centrifugation with highest speed, the supernatant protein concentration was determined by BCA protein assay (Pierce, Rockford, IL). The protein samples were mixed with

loading buffer and then boiled for 5 min. Equal amounts of denatured proteins (30 µg) was subjected to SDS-PAGE gel electrophoresis and then transferred onto a nitrocellulose membrane. After blocking by 5% non-fat milk in PBS, the membrane was incubated with specific primary antibodies overnight at 4°C. After three washing with PBS-T solution, the membrane was incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. The detection was performed by Amersham Imager 600 (GE Healthcare, Buckinghamshire, UK).

2.9 | Statistical analysis

The data represented as means $\pm$ SD from at least three independent experiments. Statistical analysis was performed by GraphPad Prism 8 using one-way ANOVA coupled with a post hoc test. Differences between the groups was considered as statistical significance at $P < .05^*$, $P < .01^{**}$, $P < .001^{***}$, and $P < .0001^{****}$.

3 | RESULTS

3.1 | GA and CQ acted synergistically against NSCLC cells

It has been documented that GA induces apoptosis in human NSCLC cells.¹¹ As Figure 2A shown, GA indeed suppressed cell growth in both A549 and H460 cells in dose- and time-dependent way, which was consistent with previous reports. To investigate whether CQ could promote cell death of NSCLC cells caused by GA, cell viability assay was performed when GA caused around 50% growth inhibition and CQ did not induce significant cell death. A549 and H460 cells were, respectively, pretreated with CQ for 2 h as indicated, followed by the incubation with GA at different concentrations for 24 h. As a result, the cytotoxicity of GA was enhanced in the presence of CQ (Figure 2B). The combination index of the synergistic effect between CQ and GA was calculated as shown in Figure 2C, suggesting GA had the synergism with CQ treatment in human NSCLC cells.

3.2 | CQ promoted apoptosis induced by GA

To further evaluate the synergistic effect of GA with CQ on cell death of NSCLC cells, A549 and H460 cell were treated with GA or CQ alone and the combination, and the apoptosis assay was performed. As shown in Figure 3A-D, either GA or CQ alone did not lead to significant apoptosis in A549 and H460, whereas the combination of GA and CQ dramatically result in about 50% cell apoptosis. Given that CQ is a well-known inhibitor for cellular autophagy, it is reasonable to speculate that GA caused certain cellular injury which could be removed by autophagy process, thus promoting cell survival.

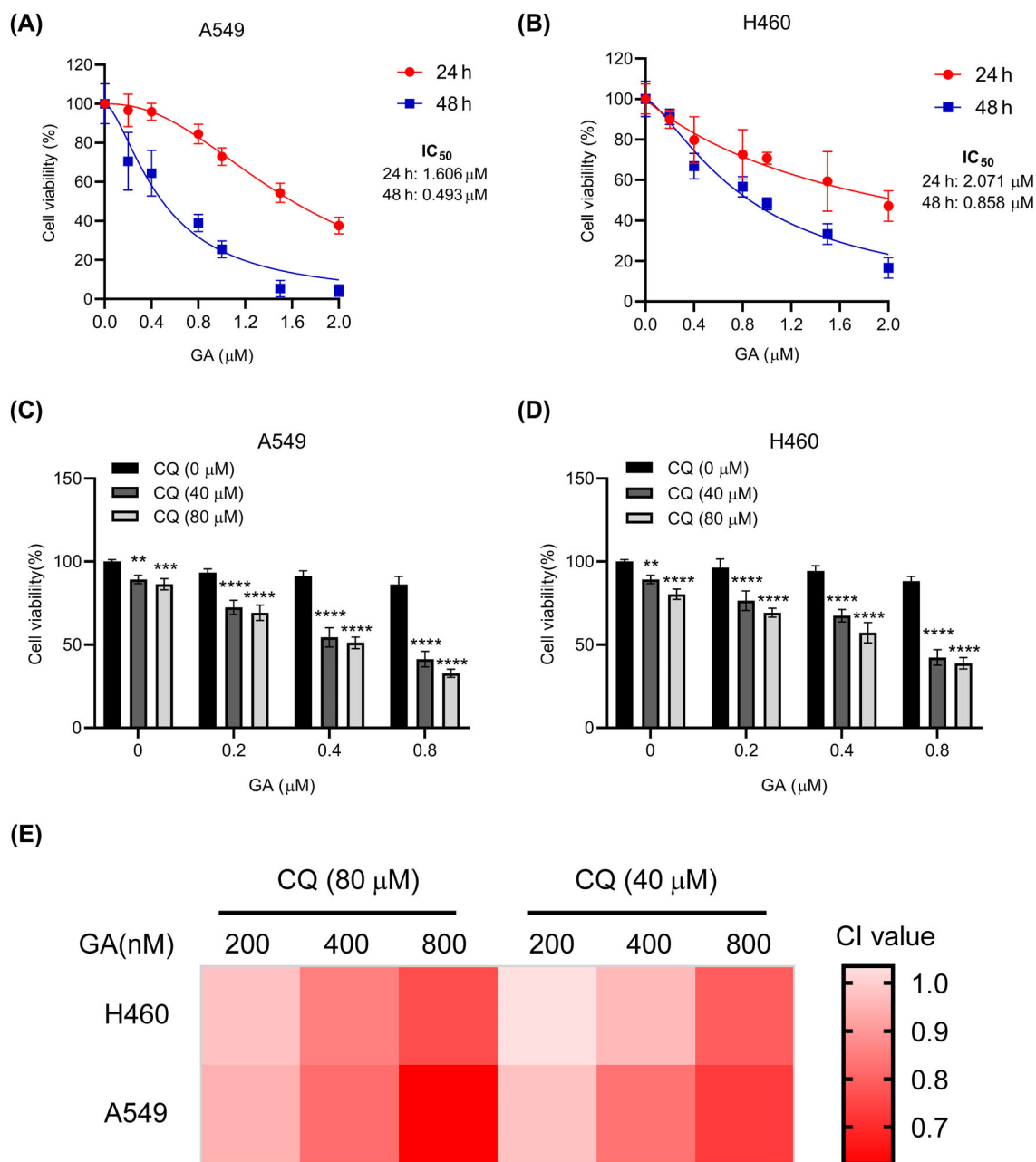


FIGURE 2 Effect of GA and CQ on NSCLC cell growth. (A) A549 and H460 cells were treated with GA as indicated for 24 h. Then MTT assay were performed for evaluating cell viability. (B) A549 and H460 cells were treated with either GA or CQ alone and the combination as indicated for 24 h. The MTT assay was carried out. (C) The combination index (CI) for GA and CQ was calculated using the Calcsyn software.

3.3 | GA promoted mitophagy in NSCLC cells

Since GA could target mitochondria, we next investigate that whether GA could induce cytoprotective mitophagy in NSCLC cells. Given that PINK1 plays vital roles in initiation of mitophagy, we examined the mRNA expression of PINK1 in NSCLC cells using qPCR. As showed in Figure 4A, compared with control group, GA treatment caused significant increase in PINK1 mRNA expression in both A549 and H460 cells. Moreover, GA also upregulated the level of PINK1 protein consistently as measured by immunoblotting, which was accompanied with decrease in mitochondrial protein Tom20, suggesting mitophagy

was activated (Figure 4B). These results suggested that GA could activate mitophagy processes in NSCLC cells, protecting cell from apoptosis.

3.4 | Inhibition of mitophagy increased the generation of damaged mitochondria by GA

Damaged mitochondria usually loss their membrane integrity which resulted in the release of its DNA into cytoplasm. To blocking the mitophagy induced by GA, A549 cell were treated with specific siRNA

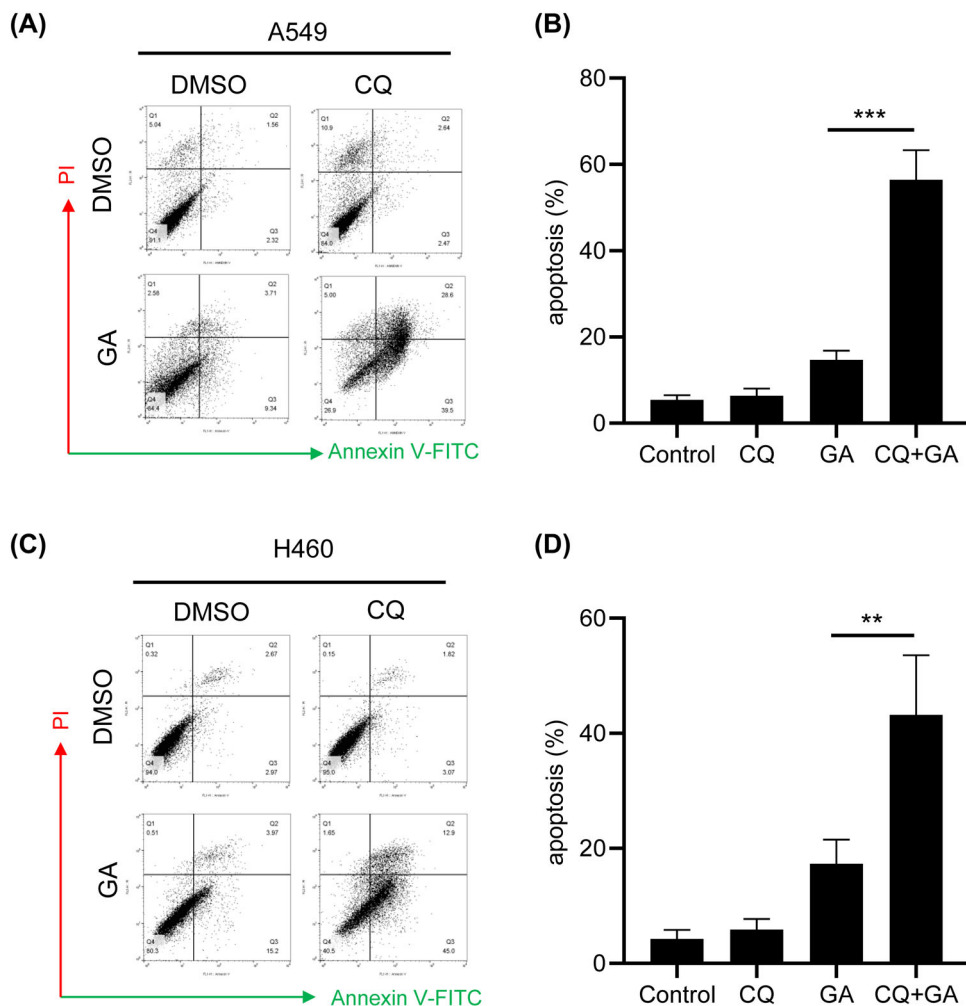


FIGURE 3 Effect of GA and CQ on apoptosis in NSCLC cells. (A–D) A549 and H460 cells were treated with GA (0.8 μ M) or CQ (40 μ M) alone and the combination for 24 h. Cells were harvested and stained using Annexin V-FITC and PI, and flow cytometry was conducted for apoptosis analysis. The representative data were shown on the left panel, and the quantitative data were present on the right panel, respectively.

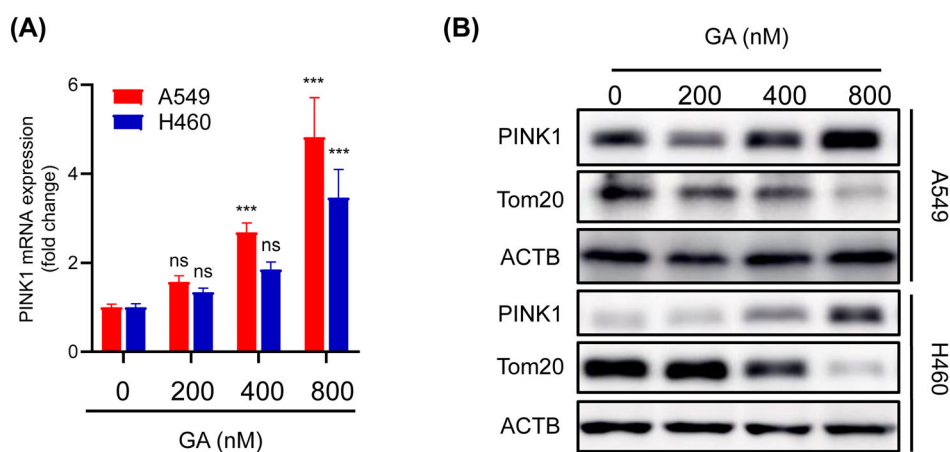


FIGURE 4 GA promoted mitophagy in NSCLC cells. (A) A549 and H460 cell were treated with GA as indicated for 24 h. The cells were harvested and total RNA was isolated. After synthesis of cDNA, it is subjected to qPCR analysis for PINK1 mRNA expression. (B) Cells were treated as (A), and the lysates were subjected to western blot analysis through specific antibodies using ACTB as loading control.

against PINK1 and chloroquine, and the level of cytosolic mitochondrial DNA (mtDNA) was determined to evaluate the mitochondrial damage under the inhibition of mitophagy. PINK1 was significantly downregulated by siRNA transfection at the mRNA and protein level with efficiency of about 70% (Figure 5A,B). As expected, silence of PINK1 promoted the level of cytosolic mtDNA compared

with GA treatment alone which caused slightly alteration (Figure 5C). Concomitantly, the combination of GA and CQ dramatically induced mtDNA translocation into the cytosol (Figure 5D) as well. These data demonstrated that genetic and pharmacological blockage of mitophagy increased the accumulation of damaged mitochondria in the presence of GA.

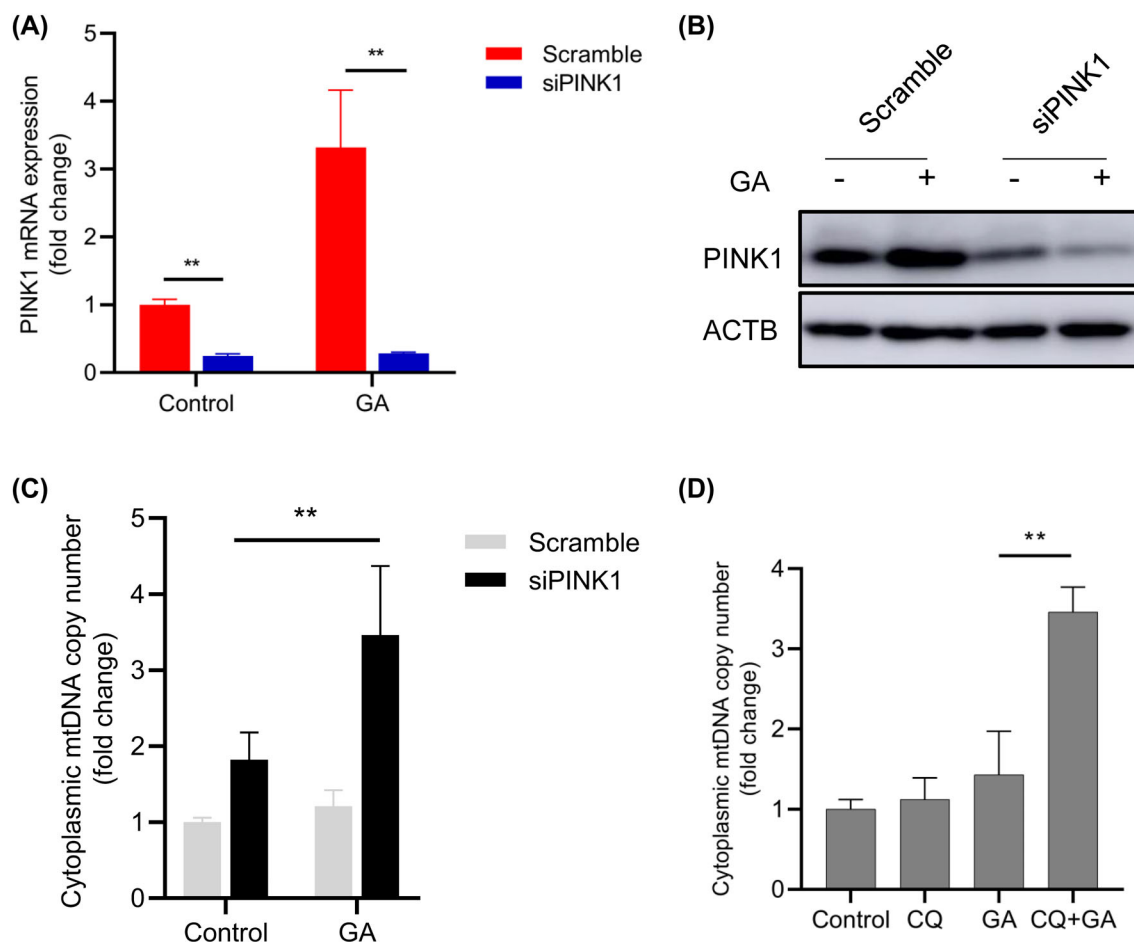


FIGURE 5 Effect of GA on mitochondria under the inhibition of mitophagy. (A–C) A549 cells were transiently transfected with Scramble or siRNA against PINK1 overnight, and then treated with GA (0.8 μ M) for 24 h. Total RNA was collected to synthesize cDNA, which was used for qPCR analysis. The relative mRNA expression of PINK1 was shown using GAPDH gene as internal reference (A). The lysates were subjected to western blot for detecting PINK1 expression (B). Cytoplasmic DNA was isolated and mtDNA was determined using specific primers by qPCR as described in Section 2. (D) A549 cells were treated with GA (0.8 μ M) or CQ (40 μ M) alone and the combination for 24 h. The mtDNA was measured as described above.

3.5 | GA and CQ induced cell death dependent on ROS

The dysfunction of mitochondria would produce excessive ROS which eventually lead to cell death. To further demonstrate whether the combination of GA and CQ could induce the accumulation of ROS in NSCLC cells, we detected the intracellular ROS level using flow cytometry analysis. As shown in Figure 6A–C, the level of ROS was elevated significantly due to treatment with GA and CQ combination in both A549 and H460 cells, whereas only slight change of ROS was observed for GA or CQ alone treatment, compared with control group. Furthermore, the addition of NAC, a ROS scavenger, almost restore the cell viability decreased by the combination of GA and CQ (Figure 6D,E). These results suggested that inhibition of mitophagy could impair the clearance of damaged mitochondria, therefore contributing to generation of excessive ROS, and eventually increasing cell death.

4 | DISCUSSION

Globally, lung cancer is still the leading cause of cancer-associated death.¹² Based on the clinical data, ~80%–85% lung cancer patients are classified as NSCLC.^{13,14} Despite recent progression made in NSCLC treatment, the outcomes remain poor due to drug resistance or toxicity.¹⁵ Furthermore, consistent clinical benefits of immune checkpoint inhibitor treatment (e.g., PD-L1/PD-1 inhibitor) are limited to a subset of patients. Therefore, additional therapeutic approaches with good efficiency must be developed to reduce the burden of lung cancer in clinic. For decades, substantial efforts have been focused on identifying compounds from natural organisms that could be able to suppress tumor growth with little or no side effects, or function synergistically with chemotherapeutic drugs for reducing side effects and overcoming drug resistance.¹⁶

As a well-documented natural compound of xanthenes family in terms of pharmaceutical study, GA has a high efficacy anticancer

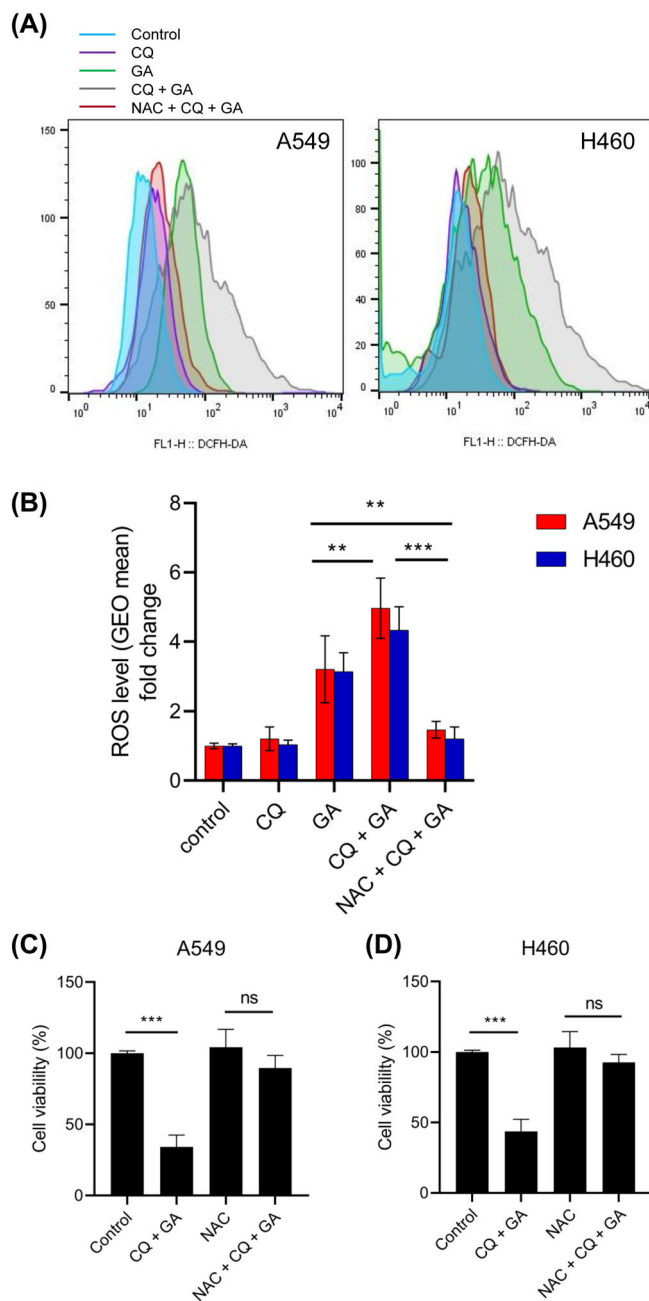


FIGURE 6 Effect of GA and CQ on ROS production in NSCLC cells. (A–C) A549 and H460 cells were treated with GA (0.8 μ M) or CQ (40 μ M) alone and the combination with or without NAC (5 mM) for 24 h. Cells were harvested and subjected to flow cytometry analysis. ROS level was represented as fold change compared with control group. The representative photos were shown at the upper panel and the quantitative data were plotted out at the middle panel. (D and E) A549 and H460 cells were treated with the combination of GA and CQ in the absence or presence of NAC for 24 h. MTT assay was performed for cell viability analysis.

effect in a large spectrum of cell lines or animal models.^{1,17} However, this compound may cause adverse effects on gastroenterology and blood system at high concentration in animal studies.^{18–20} Combining with other compounds or drugs could be better options to reduce

adverse reactions and enhance its anticancer activity. Indeed, several studies showed that GA exhibited synergistic antitumor effect with other compounds.^{21–25} In present study, we showed that the combination of GA with CQ could be potentially therapeutic strategy against NSCLC. CQ enhances cell death induced by GA in NSCLC cells, which might result from inhibition of mitophagy process, thereby increasing mitochondrial damage as well as intracellular ROS. However, the mitochondrial dysfunction has been associated with innate immune response and inflammation, which is also closely related with tumor progression.^{26,27} It is worthwhile to further investigate the effect of GA and CQ on NSCLC development in vivo in order to deeply understand the relationship of cancer cells and antitumor immune response.

Aberrant function of mitochondria largely contributes to tumor initiation and progression, such as aerobic glycolysis, imbalance of cellular redox status, excessive ROS production, and generation of apoptotic or proinflammatory signals in response to the tumor microenvironment.²⁸ As a classic mechanism for mitochondrial quality control, mitophagy as an evolutionarily conserved cellular process to remove damaged mitochondria could be involved in either promoting or limiting tumorigenesis. The potential of targeting mitophagy for tumor therapy remains under exploration. It has been documented that the failure of mitophagy is beneficial to the oncogenic stresses and tumorigenesis.²⁹ Moreover, several proteins responsible for mitophagy, including FUNDC1, Parkin, BNIP3, BNIP3L/NIX, and p62/SQSTM1, are deregulated in cancers.³⁰ Nevertheless, it has been documented that the inhibition of mitophagy is able to sensitize the esophageal squamous cell carcinoma (ESCC) patients for chemotherapy, suggesting the role of mitophagy in promoting chemoresistance and survival of the cancer cells and.³¹ Consistently, our data also showed that GA could activated mitophagy pathway through enhancing PINK1 expression in NSCLC cells, which then partially attenuated its anticancer effect. Interestingly, a very recent report showed garcisculenxanthone B (GeB), another natural xanthone compound, can stabilize PINK1 and induce mitophagy as well, suggesting that there could be some common mechanisms involved for compounds with similar structure.³²

Mitochondria are well-known central integrators for intrinsic and extrinsic apoptotic pathways, and the structure and/or function alterations has been long linked to apoptosis. In fact, most chemotherapeutic agents lead to mitochondrial damage directly or indirectly, therefore causing mitochondrial apoptosis in cancer cells. However, it is known mitochondria can function as a multifaceted regulator of cell death including apoptosis, necrosis, pyroptosis, and ferroptosis.³³ Whether certain type of cell death is dominant during treatment of GA and CQ combination remains unclear. On the other hand, as a highly conserved cellular process, autophagy is responsible for clearance of various damaged organelles, such as mitochondria, endoplasmic reticulum, lysosome, and so on. Suppression of autophagy by CQ cannot only block mitophagy, but it also reduces the removal of other intracellular organelle, which may collectively elevate cellular ROS production. Given the ambiguous roles of autophagy in tumorigenesis, it is still necessary to carefully evaluate the effect of GA combined with CQ on NSCLC.

In summary, we observed that GA caused mitochondrial damage which was accompanied with activated mitophagy through upregulating PINK1 expression. Inhibition of mitophagy by CQ leads to the cellular accumulation of damaged mitochondria and boosts ROS production, therefore promoting cell death. These finding demonstrated that GA-induced mitophagy is cytoprotective and CQ exhibited synergistical anticancer effect with GA together in NSCLC cells in vitro.

AUTHOR CONTRIBUTIONS

Xiaoliu Wu and Jun Yu designed the study; Shiye Jiang and Zhiling Dai performed experiments. Shiye Jiang, Zhiling Dai, Yuanying Zhang, Xiaoliu Wu and Ming Zhu analyzed the data; Xiaoliu Wu and Jun Yu drafted the article; All authors interpreted the data, revised the article.

CONFLICT OF INTEREST STATEMENT

The authors declared no potential conflicts of interest with respect to the research, authorship, and publication of this article.

PEER REVIEW STATEMENT

The authors have nothing to report.

ETHICS STATEMENT

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was exempt by Institutional Review Board (IRB) approval because the original data were from a public database.

ORCID

Xiaoliu Wu  <https://orcid.org/0009-0001-7287-4431>

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