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Original Article

Gambogic acid improves non-small cell lung cancer progression by inhibition of mTOR signaling pathway

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Abstract Gambogic acid (GA) has been shown to inhibit cancer cell proliferation, induce apoptosis, and enhance reactive oxygen species accumulation. However, whether GA could improve multidrug resistance through modulating autophagy has never been explored. We demonstrated that the combination of GA and cisplatin (CDDP) resulted in a stronger growth inhibition effect on A549 and NCI-H460 cells using the MTT assay. Furthermore, treatment with GA significantly increased autophagy in these cells. More importantly, GA-induced cell death could be largely abolished by 3-methyladenine (3-MA) or chloroquine (CQ) treatment, suggesting that GA-induced cell death was dependent on autophagy. Western blot analysis showed that GA treatment suppressed the activation of Akt, mTOR, and S6. In addition, using a GA and rapamycin combination induced more cell death compared to either GA or rapamycin alone. In summary, GA may have utility as an adjunct therapy for non-small cell lung cancer (NSCLC) patients through autophagy-dependent cell death, even when cancer cells have developed resistance to apoptosis.

Conflicts of interest: All authors declare no conflicts of interest.

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Introduction

Lung cancer is one of the most common causes of cancer-related death worldwide [1]. It is estimated that more than 80% of lung cancer is attributed to non-small cell lung cancer (NSCLC), with chemotherapy being the standard of care for NSCLC patients [2,3]. However, the overall response rate of NSCLC patients is only 30—50%, even with the best regimens [4,5], and these poor response rates lead to failed treatment in lung cancer.

With respect to tumor progression, autophagy is regarded as a double-edged sword. During tumor initiation, autophagy functions as a tumor suppressor [6,7]. However, it allows malignant progression of tumors in harsh microenvironments once the tumor has seeded [8,9]. Clinical studies have shown that autophagy is enhanced after cancer therapy, thereby implicating this pathway in the development of chemotherapy resistance [10,11]. Therefore, understanding how to modulate the level of autophagy may shed light on the treatment of tumor cells.

Gambogic acid (GA), isolated from gamboge, is a brownish orange dry resin extracted from the Garcinia hanburyi tree in South-East Asia [12]. In previous years, GA has been used in Asian culture as a traditional medicine for human diseases. Reports have characterized GA as having anti-infectious, anti-oxidant, anti-inflammatory, and anti-viral properties [13]. Recently, researchers have been making an effort to evaluate GA as an anti-tumor reagent in vitro, including in lung cancer, leukemia, colorectal cancer, prostate cancer, and hepatocarcinoma [14—18]. It has been suggested that GA could inhibit cancer cell proliferation, induce cell apoptosis, and enhance reactive oxygen species accumulation [18,19]. However, whether GA could improve multidrug resistance through modulating autophagy has never been explored.

In the current study, we explored the effects of the combination of GA and cisplatin in NSCLC cancer cells in order to evaluate its potential clinical application.

Materials and methods

Cell lines and cultures

A549 and NCI-H460 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) F-12 culture medium (HyClone) supplemented with 10% heat-inactivated fetal calf serum (FBS), 100 U/ml penicillin, and streptomycin in a 25-cm^2 culture flask at 37 °C in a humidified atmosphere with 5% CO_2. Before treatment, the chemotherapeutic agent cisplatin (Sigma, St. Louis, MO, USA) was dissolved in RPMI 1640 medium to a stock concentration of 1 mM and added directly to media at indicated concentrations.

Cell proliferation assay

To investigate the influence of GA on cancer cell chemosensitivity, A549 and NCI-H460 cells were seeded in 96-well tissue culture plates at a density of 5×10^4 cells per well in medium containing cisplatin (CDDP) as indicated. Cell viability was measured using an MTT assay kit (Sigma). The blue formazan products in the A549 cells were dissolved in DMSO and spectrophotometrically measured at a wavelength of 550 nm. All experiments were carried out in triplicate.

Cell viability was calculated by measuring absorbance at 450 nm with a microplate reader (Bio-Rad). Growth inhibition was calculated as a percentage of the untreated controls. Experiments were done in triplicate, and the data are expressed as the means ± SD of 5 wells per treatment. For each cell line, IC_{50} was determined using the four-parameter logistic model.

GFP-LC3 transient transfection

A549 cells were seeded at a density of 5×10^5 cells/well in 6-well plates. After 24 h, a GFP-LC3 expressing plasmid was transfected into cells using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Twenty-four hours post-transfection, cells were treated with 10 μM vehicle or cisplatin and incubated for 16 h. Then, GFP-LC3-positive dots were counted under a confocal laser microscope, LSM700 (Carl Zeiss, Jena, Germany).

Electron microscopy

Cells were fixed with 2% glutaraldehyde/paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4 for 12 h at 4 °C and washed three times for 30 min in 0.1 M PB. Samples were postfixed with 1% OsO4 dissolved in 0.1 M PB for 2 h, dehydrated in an ascending gradual series (50—100%) of ethanol and infiltrated with propylene oxide. Specimens were embedded using a Poly/Bed 812 kit (Polysciences, Warrington, PA, USA). After pure fresh resin embedding and polymerization at 60 °C in an electron microscope oven (TD-700, DOSAKA, Kyoto, Japan) for 24 h, 350-nm sections were cut and stained with toluidine blue for light microscopy, and 70-nm thin sections were double stained with 7% uranyl acetate and lead citrate for contrast staining. Sections were cut with a LEICA Ultracut UCT Ultra-microtome (Leica Microsystems, Wetzlar, Germany). All thin sections were observed by TEM (JEM-1011, JEOL, Tokyo, Japan) at an acceleration voltage of 80 kV.

Western blot

Total protein was extracted from cells using RIPA buffer (Solarbio, Beijing, China) according to the manufacturer's instructions. Immunoblotting was carried out as described previously. Primary antibodies were purchased from Cell Signaling: anti-p62 (Abcam), anti-LC3 (Sigma), anti-beclin-1 (Santa Cruz), and anti-β-actin (Sigma). Goat anti-mouse or anti-rabbit IgG HRP-conjugated secondary antibodies were used.

Statistical analysis

Data are expressed as the means ± SE. Multiple comparisons were evaluated by ANOVA followed by Turkey's multiple-comparison test with $P < 0.05$ being considered significant.