# Baicalin attenuates DDP (cisplatin) resistance in lung cancer by downregulating MARK2 and p-Akt

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**Abstract.** DDP (cisplatin) resistance in lung cancer has been widely reported. Baicalin is a flavone glycoside found in genus *Scutellaria*. However, the effects of baicalin on DDP resistance in lung cancer are unclear. The aim of present study was to investigate effects of combination of baicalin and DDP on proliferation and invasion of human lung cancer cells, and explore possible mechanisms. MTT assay was utilized to evaluate effects of baicalin and DDP on the proliferation of A549 and A549/DPP (DPP-resistant) human lung cancer cells. The probability sum method was used to determine effects of the drug combination. Transwell invasion assay was utilized to detect tumor cell invasion. The mRNA expression of MARK2 in A549 and A549/DPP cells was detected by qPCR. Protein expression of MARK2, p-Akt and Akt was detected by western blot analysis. Baicalin and DPP when used alone inhibited the proliferation of A549 and A549/DDP cells in a dose-dependent manner at 24 and 48 h. For A549 cells, baicalin (8 $\mu$g/ml) antagonized DDP (1, 2, 4 and 8 $\mu$g/ml) at 24 h. For A549/DDP cells, baicalin and DDP were additive when the concentration of DDP was 4 $\mu$g/ml at 24 h. Effects of baicalin and DDP on proliferation inhibition were additive and synergistic when concentrations of DDP were 8 and 4 $\mu$g/ml, respectively, at 48 h for both A549 and A549/DDP cells. When baicalin (8 $\mu$g/ml) and DDP (4 $\mu$g/ml) were combined, the inhibitory rate of tumor cell invasion increased markedly compared to DPP or baicalin alone groups in both A549 and A549/DDP cells. A549/DDP cells had significantly higher MARK2 mRNA levels and protein expression of MARK2 and p-Akt. Baicalin decreased MARK2 mRNA and protein expression of MARK2 and p-Akt in A549/DDP cells dose-dependently. In conclusion, baicalin and DDP were synergistic at inhibiting proliferation and invasion of human lung cancer cells at appropriate dosages and incubation time in the presence or absence of DDP resistance. The attenuation of DDP resistance was associated with downregulation of MARK2 and p-Akt.

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## Introduction

Lung cancer is the most common cancer in men worldwide, and is the fourth most frequent cancer in women (1). The standard therapy of intermediate and advanced lung cancer is based on the combination of Cis-diamminedichloroplatinum (DDP, cisplatin) and other chemotherapy agents (2,3). DDP is a DNA cross-linking agent, which is used to treat cancers such as lung, ovarian and cervical cancers (4-6). Cisplatin, carboplatin and oxaliplatin induce cross-links between guanine bases. Cisplatin and carboplatin form an identical cross-link, whereas the cross-link of oxaliplatin is structurally different because of the bulky 1,2-diaminocyclohexane group. However, DDP resistance in lung cancer has been widely reported (7-9).

Baicalin is a flavone glycoside found in several species in the genus *Scutellaria*, such as *Scutellaria baicalensis* and *Scutellaria lateriflora*. Baicalin and its aglycone baicalein are positive allosteric modulator of benzodiazepine and non-benzodiazepine sites of GABAA receptor (10,11). Baicalin was shown to display anxiolytic effects without sedative effects in mice (12,13). Moreover, baicalin was revealed to inhibit prolyl endopeptidase (14), and induce apoptosis in pancreatic cancer cells (15). Baicalin also inhibited proliferation of other malignant tumors, such as hepatocellular carcinoma and glioma (16,17). However, the effects of baicalin on DDP resistance in lung cancer are unclear.

Microtubule affinity-regulating kinase 2 (MARK2) is serine/threonine-protein kinase that is involved in the control of cancer, microtubule stability, and cell polarity. MARK2 has been shown to interact with Akt (18). The phosphoinositide 3 kinase (PI3K)/Akt mammalian target of rapamycin (mTOR) regulates cell cycling, and is associated with cellular proliferation and the development of cancer. Once activated, PI3K phosphorylates and activates Akt, which has numerous downstream effects, including activating mTOR (19). Over-activation of the mTOR pathway leads to increased cell proliferation and reduced levels of cellular apoptosis involved in the pathogenesis of cancer. p-Akt is the activated form of Akt that has biological function. Therefore, we examined the

expressions of MARK2 and p-Akt as a means of exploring the effects of combination of baicalin and DDP on proliferation and invasion of human lung cancer cells.

## Materials and methods

*Cells and reagents. Cells.* A549 cells (human lung cancer cells) and A549/DDP cells (DDP-resistant human lung cancer cells) were purchased from MeiXuan Biological Science and Technology, Inc. (Shanghai, China), and cultured at 37˚C in F12K medium supplemented with 100 ml/l fetal bovine serum (FBS), 100 kU/l penicillin, and 100 mg/l chloramphenicol in a cell incubator with 5% $CO_2$. Drugs: the baicalin powder (Ronghe Inc., Shanghai, China) and cisplatin (DDP; Macklin Inc., Shanghai, China) were dissolved in dimethyl sulfoxide (DMSO). Both solutions were stored at -80˚C.

*Main reagents.* F12K culture medium; RPMI-1640 culture medium; fetal bovine serum (FBS); trypsin and antibodies (all from Gibco Inc., Grand Island, NY, USA); MTT cell proliferation and toxicity assay kits (Aladdine Inc., Shanghai, China); primary antibodies against GAPDH (Abcam Inc., Cambridge, MA, USA); primary antibodies against Akt and p-Akt (Cell Signaling Technology Inc., Danvers, MA, USA); primary antibodies against MARK2 (Proteintech Inc., Rosemont, IL, USA); goat anti-rabbit antibody (Invitrogen Inc., Grand Island, NY, USA); Martrigel (Becton-Dickinson Inc., Franklin Lakes, NJ, USA); Transwell (Corning Inc., Corning, NY, USA); TRIzol (Invitrogen Inc., Grand Island, NY, USA); Takara Reverse Transcriptase M-MLV; qPCR kit (Tiangen Biotech, Inc., Beijing, China); and specific primers for MARK2 and β-actin (Sangon Biotech Inc., Shanghai, China).

Main pieces of equipment: light microscope (Olympus Inc., Tokyo, Japan); microplate reader (Kehua Inc., Shanghai, China); table-type refrigerated centrifuge (USTC Zonkia Inc., Hefei, China); cell incubator (Thermo Scientific Inc., Waltham, MA, USA); vertical and horizontal electrophoresis system (Liuyi, Inc., Beijing, China); electric thermostatic drying oven (Huyue, Inc., Shangyu, China); PCR machine (Bio-Rad Inc., Irvine, CA, USA); and Step One Plus quantitative PCR machine (Applied Biosystems, Inc., Waltham, MA, USA).

*MTT assay.* A549/DDP cells were cultured for 24 h before baicalin was added. The final concentrations of baicalin were 1, 2, 4 and 8 $\mu$g/ml (n=3 wells/each concentration). The final concentrations of DDP were 1, 2, 4 and 8 $\mu$g/ml. No drug was added in control group. Meanwhile, other aliquots of cells were treated with baicalin (8 $\mu$g/ml), DDP (1, 2, 4 and 8 $\mu$g/ml), and baicalin (8 $\mu$g/ml) combined with DDP (1, 2, 4 and 8 $\mu$g/ml) respectively (n=3 wells/each concentration). Following addition of drugs, cells were cultured in a 37˚C incubator with 5% $CO_2$ for periods of 24 and 48 h, respectively. Following culture, 20 $\mu$l of MTT solution (5 mg/ml) was added to each well, and the cells were cultured at 37˚C for an additional 4 h. Following culture, the cell supernatants were removed and discarded, and 150 $\mu$l of DMSO was added to each well. The plates were then shaken for 15 min to dissolve crystals, and the absorbance of each sample was detected at 570 nm (A570) using an ELISA microplate reader. The degree of cell proliferation inhibition in each sample was calculated using the following formula: cell proliferation inhibition (%) = (1-absorbance of the experimental group/absorbance of the control group) x100%. The probability sum method was utilized to look for evidence of synergism achieved by combining baicalin with cisplatin (20). The formula used for this purpose was q=EAB/(EA+EB-EAxEB), where EAB is the effect achieved (e.g., inhibition rate) when drug A and B are combined, and EA and EB are the effects of drug A and B, respectively, when applied separately. A q-value between 0.85 and 1.15 indicates that the effects of drug A and B are additive. A q-value >1.15 indicates that the effects of drug A and B are synergistic, while a q-value <0.85 indicates that drug A and B have antagonistic effects.

*Transwell invasion assay.* A549 and A549/DDP cells were treated with baicalin (8 $\mu$g/ml), DDP (4 $\mu$g/ml), and baicalin (8 $\mu$g/ml) combined with DDP (4 $\mu$g/ml) for 48 h, respectively (n=3/group). No drugs were added in control groups. Cells were digested by trypsin-ethylene diamine tetraacetic acid (EDTA) solution (0.25% EDTA) and centrifuged. The cells were then diluted to make concentration of $5x10^5$/ml. The membrane of the upper compartment was coated with 50 $\mu$l of Matrigel (1 g/l), and incubated at 37˚C for 1 h in order to reconstruct its structure into basal membrane. Two hundred microliters of A549 and A549/DPP cell suspension were incubated in upper compartment of Transwell respectively, and 600 $\mu$l of 20% FBS were added into lower compartment. Cells were incubated at a humid incubator with 5% $CO_2$ for 48 h. Following culture, 4% paraformaldehyde was utilized to fix the microporous membrane. Cells were stained with 0.05% crystal violet for 10 min, and washed with phosphate-buffered saline (PBS) twice. Cells were then observed under microscope (x400), and the number of cells that penetrated the membrane were counted. The inhibition of tumor cell invasion was calculated utilizing the following formula: inhibition of cell invasion (%) = (1-the average number of cells that penetrated the membrane in the experimental group/the average number of cells that penetrated the membrane in the control group) x100%.

*Quantitative polymerase chain reaction (qPCR).* A549/DDP cells were treated with different concentrations of baicalin (0, 1, 2, 4, 8 and 10 $\mu$g/ml) for 48 h. Total RNA of A549/DDP cells was extracted and purified by TRIzol according to manufacturer's instructions. A universal cDNA synthesis kit (Tiangen Biotech, Inc.) was utilized for reverse transcription. Each reaction contained 1 $\mu$l of random hexamer primers (0.2 $\mu$g/$\mu$l) and 40 U M-MuLV reverse transcriptase (20 U/$\mu$l). The specific primer for detection of MARK2 gene was forward, ATGCTGC CCCAGAACTCTTC and reverse, GTGCCTCTCTTGCTGG GATT. The specific primer for detection of β-actin gene was forward, AGAAAATCTGGCACCACACC and reverse, AGA GGGTACAGGGATAGCA. miRcute miRNA qPCR detection kit was used for qPCR. PCR conditions were as follows: pre-denaturing at 95˚C for 15 min; denaturing at 95˚C for 10 sec; and annealing and polymerization at 60-66˚C for 20-32 sec. There were 40 PCR cycles. PCR was performed in an ABI Step One Plus qPCR system. The expression of MARK2 was determined as the ratio of relative optical density of target gene to β-actin.

*Western blot studies.* Expression levels of AKT, p-AKT and MARK2 proteins were detected by western blot analysis.

Table I. Effects of baicalin and DDP on the proliferation of A549 cells.

| A549 cells | Baicalin (8[a]) | DDP (1) | DDP (2) | DDP (4) | DDP (8) | Baicalin (8) + DDP (1) | Baicalin (8) + DDP (2) | Baicalin (8) + DDP (4) | Baicalin (8) + DDP (8) |
|---|---|---|---|---|---|---|---|---|---|
| A549 cells/24 h | | | | | | | | | |
| EA | 0.20 | | | | | | | | |
| EB | | 0.13 | 0.20 | 0.22 | 0.31 | | | | |
| EAB | | | | | | 0.10 | 0.18 | 0.28 | 0.36 |
| q=(EAB/ (EA+EB-EAxEB) | | | | | | 0.31 | 0.50 | 0.74 | 0.80 |
| A549 cells/48 h | | | | | | | | | |
| EA | 0.21 | | | | | | | | |
| EB | | 0.17 | 0.21 | 0.26 | 0.41 | | | | |
| EAB | | | | | | 0.26 | 0.29 | 0.49 | 0.50 |
| q=(EAB/ (EA+EB-EAxEB) | | | | | | 0.76 | 0.76 | 1.20[b] | 0.94[c] |

MTT assay was utilized to detect cell proliferation. Data for inhibition of cell proliferation (mean values). The probability sum method was used to determine effects of the drug combination. Formula: q=EAB/(EA+EB-EAxEB). EAB is the effect when drug A and B are combined. EA and EB are the effects of separate treatments with drug A and B alone. The effects of drug A and B are additive if q is between 0.85 and 1.15. Drug A and B are synergistic if q>1.15, and antagonistic if q<0.85. Baicalin (8 $\mu$g/ml) antagonized DDP when concentrations of DDP were 1, 2, 4 and 8 $\mu$g/ml at 24 h after A549 cells were treated. In addition, effects of baicalin and DDP were additive when the concentration of DDP was 8 $\mu$g/ml, and synergistic when the concentration of DDP was 4 $\mu$g/ml at 48 h. DDP, cis-diamminedichloroplatinum, cisplatin. [a]The unit in the table is $\mu$g/ml; [b]synergist effects; [c]additive effects.

A 549/DPP cells were treated with different concentrations of baicalin (1, 2, 4 and 8 $\mu$g/ml) for 48 h. No drug was added in control group. Cellular proteins were extracted and separated by electrophoresis (120 V) on a 10% SDS-polyacrylamide gel. The separated proteins were then electrophoretically (100 V for 120 min) transferred to polyvinylidene fluoride (PVDF) membranes. After being blocked with 5% non-fat milk powder for 1 h, the membranes were incubated with anti-Akt (1:1,000), anti-p-Akt (1:1,000), anti-MARK2 (1:2,000), and anti-GAPDH antibodies (1:5,000) respectively at 4˚C overnight. Following incubation, membranes was washed three times (10 min each) with a solution of Tris-buffered saline and Tween-20 (TBST). The membranes were then incubated for 1 h at room temperature with goat anti-rabbit secondary antibody labeled with horseradish peroxidase (HRP) (1:3,000); after which, they were washed and incubated for a short time period in electro-chemi-luminescence (ECL) solution. The film was exposed in a dark room.

*Statistical analysis*. The statistical data were analyzed and the figures were created using GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA). All statistical results are expressed as the mean ± SEM. Differences among 3 or more groups were compared by analysis of variance (ANOVA), followed by the Bonferroni post-hoc test for multiple comparisons. p-values ≤0.05 were considered statistically significant.

## Results

*Baicalin and DDP inhibit the proliferation of human lung cancer cells when used alone*. MTT assay was utilized to evaluate effects of baicalin and DDP on the proliferation of A549 and A549/DPP human lung cancer cells. Following addition of drugs, cells were cultured for periods of 24 and 48 h, respectively. The degree of cell proliferation inhibition was calculated as: inhibitory rate of cell proliferation (%) = (1-absorbance of the experimental group/absorbance of the control group) x100%. Our results showed that baicalin and DPP when used alone inhibited the proliferation of A549 cells in a dose-dependent manner at 24 and 48 h, respectively (Fig. 1). Similarly, baicalin and DPP inhibited the proliferation of A549/DDP cells in a dose-dependent manner at 24 and 48 h. The ability of baicalin to inhibit tumor cell proliferation was similar between A549 and A549/DDP cells, whereas the ability of DDP to inhibit cell proliferation was lower in A549/DDP cells (Fig. 1).

*Effects of combination of baicalin and DDP on the proliferation of human lung cancer cells*. MTT assay was utilized to evaluate effects of combination of baicalin and DDP on the proliferation of A549 and A549/DPP human lung cancer cells. Following addition of drugs, cells were cultured for periods of 24 and 48 h, respectively. The probability sum method was used to determine effects of the drug combination. Formula: q=EAB/(EA+EB-EAxEB). The effects of drug A and B are additive if q is between 0.85 and 1.15. Drug A and B are synergistic if q>1.15, and antagonistic if q<0.85. Baicalin (8 $\mu$g/ml) antagonized DDP when concentrations of DDP were 1, 2, 4 and 8 $\mu$g/ml at 24 h after A549 cells were treated (q<0.85). The effects of baicalin and DDP were additive when the concentration of DDP was 8 $\mu$g/ml (0.85<q<1.15), and synergistic when the concentration of DDP was 4 $\mu$g/ml at 48 h after A549 cells were treated (q>1.15) (Table I). In addition, at 24 h after

Table II. Effects of baicalin and DDP on the proliferation of A549/DDP cells.

| A549 cells | Baicalin (8[a]) | DDP (1) | DDP (2) | DDP (4) | DDP (8) | Baicalin (8) + DDP (1) | Baicalin (8) + DDP (2) | Baicalin (8) + DDP (4) | Baicalin (8) + DDP (8) |
|---|---|---|---|---|---|---|---|---|---|
| A549/DDP cells/24 h | | | | | | | | | |
| EA | 0.11 | | | | | | | | |
| EB | | 0.10 | 0.12 | 0.13 | 0.21 | | | | |
| EAB | | | | | | 0.07 | 0.09 | 0.21 | 0.21 |
| q=(EAB/ (EA+EB-EAxEB) | | | | | | 0.36 | 0.40 | 0.89[b] | 0.73 |
| A549/DDP cells/48 h | | | | | | | | | |
| EA | 0.19 | | | | | | | | |
| EB | | 0.11 | 0.12 | 0.19 | 0.29 | | | | |
| EAB | | | | | | 0.13 | 0.24 | 0.40 | 0.41 |
| q=(EAB/ (EA+EB-EAxEB) | | | | | | 0.48 | 0.84 | 1.17[c] | 0.95[b] |

The effects of drug A and B are additive if q is between 0.85 and 1.15. Drug A and B are synergistic if q>1.15, and antagonistic if q<0.85. Baicalin (8 $\mu$g/ml) antagonized DDP when concentrations of DDP were 1, 2 and 8 $\mu$g/ml, whereas effects of baicalin and DDP were additive when the concentration of DDP was 4 $\mu$g/ml at 24 h after A549/DPP cells were treated. In addition, effects of baicalin and DDP were additive when the concentration of DDP was 8 $\mu$g/ml, and synergistic when the concentration of DDP was 4 $\mu$g/ml at 48 h. DDP, cis-diamminedichloroplatinum, cisplatin. [a]The unit in the table is $\mu$g/ml; [b]additive effects; [c]synergist effects.

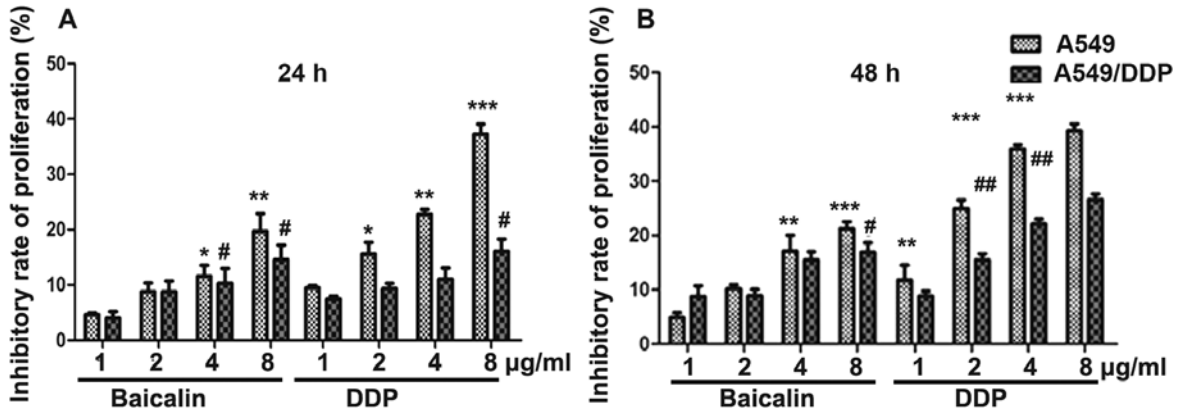


Figure 1. Baicalin and DDP inhibit the proliferation of human lung cancer cells when used alone. MTT assay was utilized to evaluate effects of baicalin and DDP on the proliferation of A549 and A549/DPP human lung cancer cells respectively. Following addition of drugs, cells were cultured for 24 and 48 h, respectively. The degree of cell proliferation inhibition was calculated as: inhibitory rate of cell proliferation (%) = (1-absorbance of the experimental group/absorbance of the control group) x100%. (A) Inhibitory rate of tumor cell proliferation at 24 h after drug treatment. (B) Inhibitory rate of tumor cell proliferation at 48 h after drug treatment. Baicalin and DPP when used alone inhibited the proliferation of A549 and A549/DDP cells in a dose-dependent manner at 24 and 48 h (mean ± SEM, n=3/group). $^{*}$p<0.05, $^{**}$p<0.01 and $^{***}$p<0.001, respectively when compared with the baicalin 1 $\mu$g/ml group; $^{\#}$p<0.05 and $^{\#\#}$p<0.01, respectively when compared with the DPP 1 $\mu$g/ml group. DDP, cis-diamminedichloroplatinum, cisplatin.

A549/DPP cells were treated, baicalin (8 $\mu$g/ml) antagonized DDP when concentrations of DDP were 1, 2 and 8 $\mu$g/ml (q<0.85), whereas effects of baicalin and DDP were additive when the concentration of DDP was 4 $\mu$g/ml (0.85<q<1.15). At 48 h after A549/DDP cells were treated, effects of baicalin and DDP were additive when the concentration of DDP was 8 $\mu$g/ml (0.85<q<1.15), and synergistic when the concentration of DDP was 4 $\mu$g/ml (q>1.15) (Table II). In conclusion, synergistic effects of baicalin and DDP on proliferation of both A549 and A549/DDP cells were observed when concentrations of baicalin and DPP were 8 and 4 $\mu$g/ml respectively. Therefore, we used the dosages to examine tumor invasion.

*Combination of baicalin and DDP enhances the invasion of human lung cancer cells*. A549 and A549/DDP cells were treated with baicalin (8 $\mu$g/ml), DDP (4 $\mu$g/ml), and baicalin (8 $\mu$g/ml) combined with DDP (4 $\mu$g/ml) for 48 h, respectively. Transwell invasion assay was used to detect the invasion of A549 and A549/DPP human lung cancer cells. When used alone, DDP and baicalin significantly inhibited the invasion of A549 (p<0.001) and A549/DDP cells (DDP, p<0.001; baicalin, p<0.01) (Fig. 2) as compared to the control group. When DDP and baicalin were combined, the inhibitory rate increased markedly as compared to DPP or baicalin single treatment groups (p<0.001) (Fig. 2).

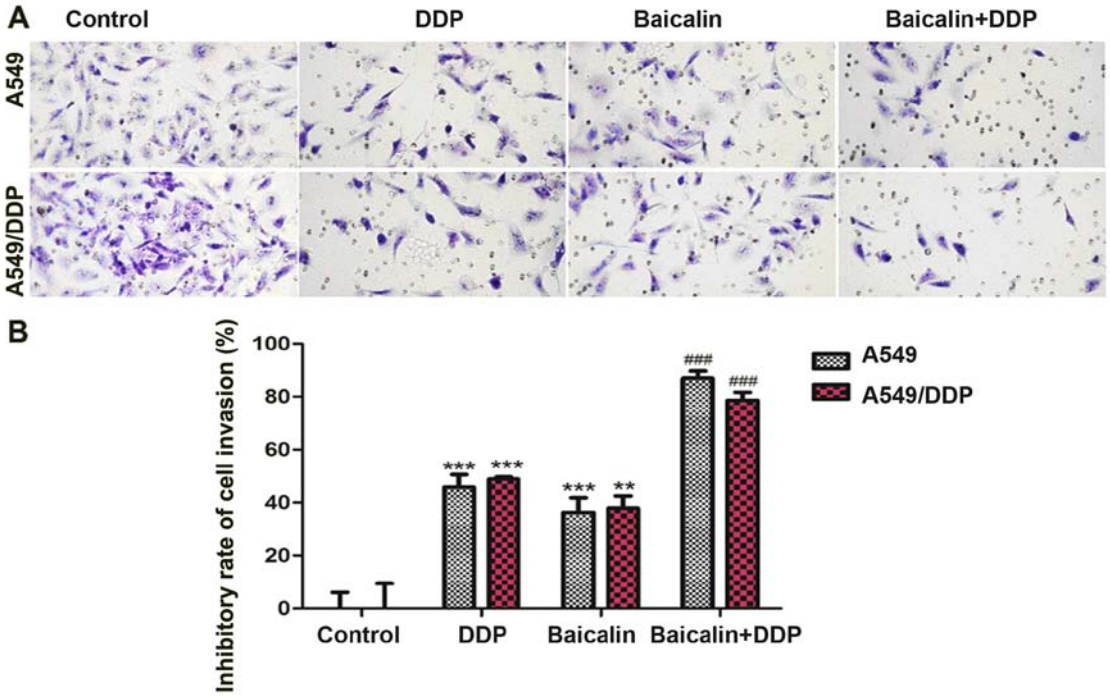


Figure 2. Effects of baicalin and DDP on the invasion of human lung cancer cells. A549 and A549/DDP cells were treated with baicalin (8 $\mu$g/ml), DDP (4 $\mu$g/ml), and baicalin (8 $\mu$g/ml) combined with DDP (4 $\mu$g/ml) for 48 h, respectively. Transwell invasion assay was used to detect the invasion of tumor cells. (A) Microscopic images of Transwell results (x400). (B) Statistical analysis of inhibitory rates of tumor cell invasion. When used alone, DDP and baicalin significantly inhibited the invasion of A549 and A549/DDP cells as compared to the control group. When DDP and baicalin were combined, the inhibitory rate increased markedly as compared to DPP or baicalin alone groups (mean ± SEM, n=3/group). $^{**}$, $^{***}$p<0.05 when compared with the control group; $^{###}$p<0.001 when compared with DDP or baicalin group. DDP, cis-diamminedichloroplatinum, cisplatin.

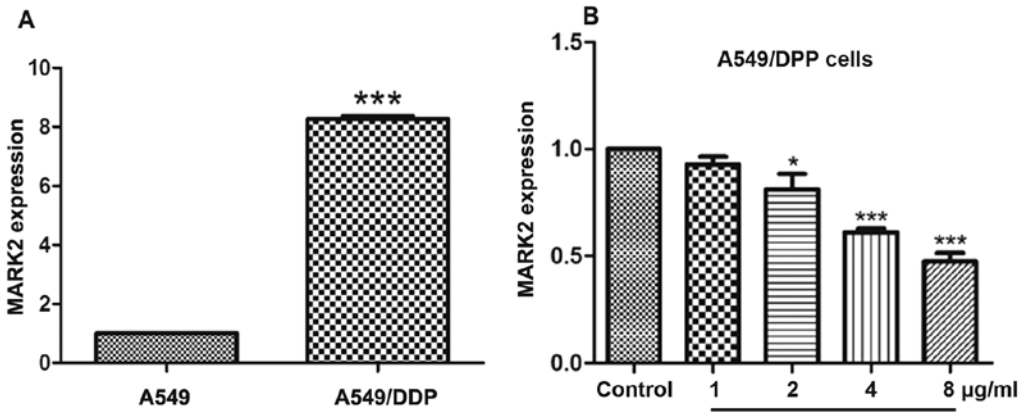


Figure 3. mRNA expression of MARK2 in human lung cancer cells. The mRNA expression of MARK2 in A549 and A549/DPP human lung cancer cells was detected by qPCR. (A) mRNA expression of MARK2 in A549 and A549/DDP cells. (B) Effects of different doses of baicalin on MARK2 mRNA expression in A549/DDP cells. A549/DDP cells had markedly higher MARK2 mRNA levels compared to A549 cells. Therefore, we chose A549/DDP cells to examine effects of different concentrations of baicalin on MARK2 mRNA expression. Baicalin decreased MARK2 mRNA levels in A549/DDP cells dose-dependently as compared to the control group (mean ± SEM, n=3/group). (A) $^{***}$p<0.001 as compared to A549 cells; (B) $^{*}$p<0.05 and $^{***}$p<0.001 when compared with the control group. Ctrl, control; DDP, cis-diamminedichloroplatinum, cisplatin.

*mRNA expression of MARK2 in human lung cancer cells.* The mRNA expression of MARK2 in A549 and A549/DPP human lung cancer cells was detected by qPCR. A549/DDP cells had markedly higher MARK2 mRNA levels compared to A549 cells (p<0.001) (Fig. 3A). Therefore, we chose A549/DDP cells to examine effects of different concentrations of baicalin on MARK2 mRNA expression. Baicalin decreased MARK2 mRNA levels in A549/DDP cells dose-dependently, and higher doses of baicalin (2, 4 and 8 $\mu$g/ml) markedly inhibited MARK2 mRNA expression (p<0.05, p<0.001 and p<0.001, respectively) (Fig. 3B) when compared to the control group.

*Protein expression of Akt, p-Akt and MARK2 in human lung cancer cells.* Protein expression of Akt, p-Akt and MARK2 was detected by western blot analysis. Relative protein expression of Akt, p-Akt and MARK2 to GAPDH was calculated. There were no differences in Akt expression between A549 and A549/DDP cells. The protein expression of p-Akt and MARK2 was markedly higher in A549/DDP cells as compared

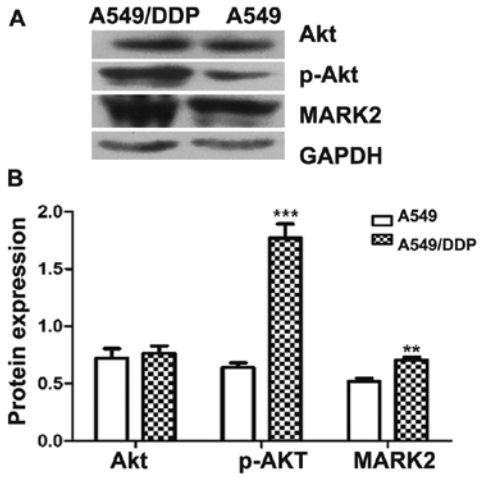


Figure 4. Protein expression of Akt, p-Akt and MARK2 in A549 and A549/DDP cells. Protein expression was detected by western blot analysis. Relative protein expression of Akt, p-Akt and MARK2 to GAPDH was calculated. (A) Western blot results show protein expression of Akt, p-Akt and MARK2 in A549 and A549/DDP cells. (B) Quantification of relative fold-change in blot density when compared with density of GADPH. The protein expression of p-Akt and MARK2 was markedly higher in A549/DDP cells as compared to A549 cells (mean ± SD, n=3/group). $^{**}$p<0.01 and $^{***}$p<0.001 when compared to A549 cells. DDP, cis-diamminedichloroplatinum, cisplatin.

to A549 cells (p-Akt: p<0.001; MARK2: p<0.01) (Fig. 4). Therefore, we chose A549/DDP cells to examine effects of different doses of baicalin on protein expression of MARK2, p-Akt, and Akt. Baicalin decreased protein expression of MARK2 and p-Akt in A549/DDP cells in a dose-dependent manner, whereas it did not alter protein expression of Akt. At higher doses (2, 4 and 8 $\mu$g/ml), baicalin significantly inhibited protein expression of MARK2 and p-Akt in A549/DDP cells as compared to the control group (p<0.01, p<0.001 and p<0.001, respectively) (Fig. 5).

## Discussion

We demonstrated that baicalin and DDP were synergistic at inhibiting proliferation and invasion of human lung cancer cells at appropriate dosages and incubation time in the presence or absence of DDP resistance. In addition, the attenuation of DDP resistance was associated with downregulation of MARK2 and p-Akt.

Lung cancer is the leading cause of cancer-related death worldwide (21). DDP was the first member of a class of platinum-containing anticancer drugs. These platinum complexes react *in vivo* and cause DNA cross-linking, which ultimately triggers cell apoptosis (22). Like other chemotherapeutic agents, resistance to DDP is inevitable and frequently occurs after several cycles of treatment. DDP resistance has been reported to be associated with mechanisms such as DNA damage/repair proteins, drug retention such as increased influx or decreased uptake, increased drug inactivation or prevention of drug to reach DNA target, growth signaling via different pathways or increase in anti-apoptotic proteins, and hypoxia-induced autophagy (23-28).

Studies have proposed measures to decrease DDP resistance. mTOR inhibitor (CCI-779) was revealed to be able to restore sensitivity to DDP in lung cancer (29). Inhibition of miR-196a reversed DDP resistance of A549/DDP cell lines, which may be linked to inhibition of drug efflux, downregulation of drug-resistant protein expression, cell apoptosis, and suppression of cell proliferation (30). Moreover, a fusion protein based on two tumstatin-derived sequences named recombinant VBMDMP (rVBMDMP) decreased cancer cell resistance to DDP in A549/DDP cell xenograft model of nude mice (31). Epigallocatechin-3-gallate (EGCG), the major polyphenol in green tea, was also shown to resensitize non-small cell lung cancer cells to DDP via demethylation of candidate genes.

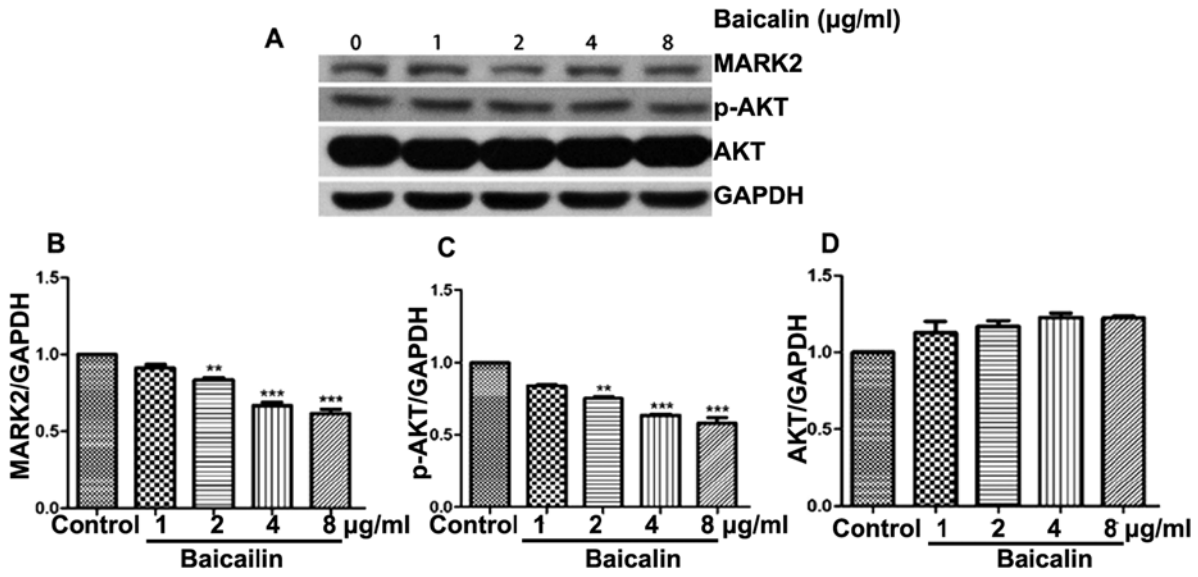


Figure 5. Effects of baicalin on protein expression of MARK2, p-Akt and Akt in A549/DDP cells. Protein expression was detected by western blot analysis. Relative protein expression of Akt, p-Akt and MARK2 to GAPDH was calculated. (A) Western blot results show protein expression of Akt, p-Akt and MARK2 in A549/DDP cells after baicalin treatment. (B-D) Quantification of relative fold-change in blot density when compared with density of GADPH. Baicalin decreased protein expression of MARK2 and p-Akt in A549/DDP cells in a dose-dependent manner, whereas it did not alter protein expression of Akt (mean ± SD, n=3/group). $^{**}$p<0.01 and $^{***}$p<0.001 when compared to the control group. DDP, cis-diamminedichloroplatinum, cisplatin; Ctrl, Control.

Some traditional Chinese medicines were revealed to protect cancer patients against treatment-related complications and reduce toxicity of conventional therapy (32-34). Baicalin, a flavone glycoside, was reported to inhibit proliferation of malignant tumors including hepatocellular carcinoma and glioma (16,17). However, effects of baicalin on DDP resistance in lung cancer were unclear. The main principle of lung cancer therapy is to induce cell death or inhibit cell survival (35). Therefore, we explored effects of combination of baicalin and DDP on proliferation and invasion of human lung cancer cells.

We demonstrated in the present study that effects of baicalin and DDP on proliferation inhibition of A549 and A549/DDP cells were synergistic when concentrations of baicalin and DDP were 8 and 4 $\mu$g/ml at 48 h after incubation. At these dosages, the inhibitory rate of tumor cell invasion increased significantly compared to DPP or baicalin alone groups in both A549 and A549/DDP cells. These findings indicate that baicalin increases the sensitivity and decreases resistance of DDP in lung cancer cells, no matter whether lung cancer cells already are resistant to DDP or not. These findings provide another novel approach to decrease DDP resistance in human lung cancer.

We then unveiled that baicalin dose-dependently decreased expression of MARK2 and p-Akt in A549/DDP cells. Interestingly, we showed that DDP-resistant A549 cells had significantly higher expression of MARK2 and p-Akt as compared to non-DDP-resistant A549 cells. Hence, the decreased expression of MARK2 and p-Akt after baicalin treatment may be associated with decreased DDP resistance in human lung cancer cells.

The role of MARK2 in lung cancer was recently identified. MARK2 was shown to activate cell cycle and DNA repair. High MARK2 expression levels correlated with resistance to DDP (36). In addition, Akt is an essential kinase enzyme component of the PI3K/Akt/mTOR pathway, and is a downstream effector of PI3K (37). The PI3K/Akt/mTOR pathway is an important intracellular signaling pathway related to cellular quiescence, proliferation and cancer. Over-activation of the PI3K/Akt/mTOR pathway reduces apoptosis and stimulates proliferation, and both of these processes are involved in the pathogenesis of cancer. Akt amplification was revealed to increase DDP resistance in human lung cancer cells through the mTOR/p70S6K1 pathway (38). IL-6 signaling contributed to cisplatin resistance in non-small cell lung cancer via upregulation of anti-apoptotic molecules including Akt (39). Meanwhile, DDP resistance due to loss of fragile histidine triad (FHIT) was reported to be conquered by Akt inhibitor perifosine in xenografts of non-small cell lung cancer (28). Furthermore, sorafenib reversed resistance of human gastric cancer cell line to DDP through downregulating expression MDR1 and Akt (40). As a result, baicalin is able to decrease DDP resistance, and inhibit proliferation and invasion of human lung cancer cells by downregulating MARK2 and p-Akt expression.

In conclusion, we demonstrated for the first time that baicalin and DDP were synergistic at inhibiting proliferation and invasion of human lung cancer cells at appropriate dosages and incubation time in the presence or absence of DDP resistance. The attenuation of DDP resistance was associated with downregulation of MARK2 and p-Akt. Although future research is needed to elucidate more underlying cellular and molecular mechanisms, baicalin appears to be a promising agent for reducing DDP resistance.

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