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*Original Article*

# Combination of icotinib and wogonin induces apoptosis and autophagy to overcome acquired resistance in lung cancer harbouring EGFR T790M mutation

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**Abstract:** Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) are widely used as targeted chemotherapeutic drugs in the treatment of advanced non-small cell lung cancer (NSCLC). However, acquired resistance against TKIs caused by T790M mutation of EGFR has become a major clinical problem. In this study, we assessed whether the combination of wogonin, a natural monoflavonoid, and icotinib could overcome T790M-mediated acquired resistance to icotinib, a novel EGFR-TKI with preclinical and clinical activity. Cell counting kit-8 assay showed that wogonin and icotinib caused time- and dose-dependent growth inhibition of NCI-H1975, which is a T790M-positive cell line. Wogonin plus icotinib combination produced a more pronounced growth inhibition and significantly increased the percentage of early apoptotic cells and cleavage of caspase 3 compared to cells treated with wogonin or icotinib alone. Furthermore, the number of intracellular autophagosomes, LC3B-I conversion to LC3B-II, and Beclin 1 expression after treatment with wogonin and icotinib combination were higher than those after treatment with wogonin or icotinib alone. The combined treatment with wogonin and icotinib upregulated the levels of phosphorylated mTOR. The suppression of mTOR phosphorylation by 3-methyladenine weakened the effects of wogonin and icotinib on cell autophagy and apoptosis. In conclusion, the combination of icotinib and wogonin exerted synergistic inhibitory effects on cell proliferation, and could induce apoptosis and autophagy in EGFR T790M-mutated lung cancer. The combination of icotinib and wogonin could become a potential strategy to overcome the acquired resistance to EGFR-TKIs in T790M mutant lung cancer.

**Keywords:** Non-small cell lung cancer, T790M, icotinib, wogonin

## Introduction

Lung cancer is the leading cause of cancer death and the most frequently diagnosed cancer in China [1]. It is mainly treated with surgery, chemotherapy, radiotherapy, and targeted therapies. Non-small cell lung cancer (NSCLC) accounts for approximately 80% of primary lung cancer. Because the initial symptoms are obscure, approximately two-third NSCLC cases are diagnosed at an advanced stage [2]. Therefore, chemotherapy, radiotherapy, and targeted therapies are the main treatment option for advanced stage NSCLC patients. Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR TKIs) have been widely used as targeted chemotherapeutic drugs in the treatment of advanced NSCLC. However, acquired resistance against them has become a major clinical problem. Several possible mechanisms for acquired resistance have been identified, such as aberrant downstream pathways, histologic transformation, ATP binding cassette transporter effusion, impairment of the EGFR-TKIs-mediated apoptosis pathway, and T790M-mediated acquired resistance, etc. [3]. Among these, T790M-mediated acquired resistance is the most prevalent mechanism and trials *in vitro* investigating the efficacy of new therapies targeting T790M for overcoming the acquired resistance to EGFR TKIs have been carried out [4-7]. However, only few drugs have reached clinical trials phase. Therefore, more studies are needed to overcome T790M-mediated acquired resistance.

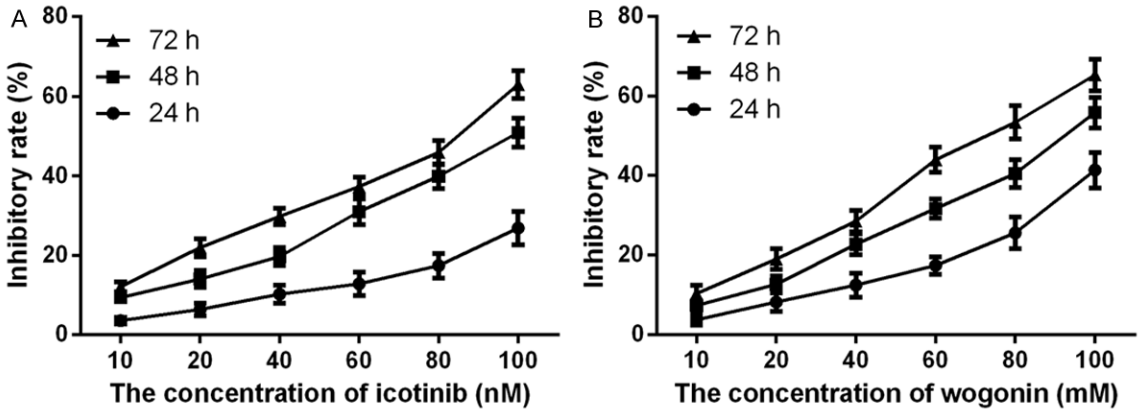


**Figure 1.** The rate of inhibition of NCI-H1975 cells after treatment with different concentrations of (A) icotinib (10 nM, 20 nM, 40 nM, 60 nM, 80 nM, and 100 nM) or (B) wogonin (10 mM, 20 mM, 40 mM, 60 mM, 80 mM, and 100 mM) for 24, 48, or 72 h.

Overcoming resistance of cancer cells is a main research area of Traditional Chinese medicine. Wogonin is a natural monoflavonoid isolated from *Scutellaria baicalensis* Georgi, *Scutellaria rivularis* wall, among others, which are important traditional Chinese medicine. Wogonin has been reported to have potent antitumor activities against many types of cancer cells, such as melanoma, malignant neuroblastoma, leukaemia, and cervical carcinoma [8-12]. For example, our previous studies showed that wogonin induced G1 phase arrest in human cervical carcinoma HeLa cells and human histiocytic lymphoma cell line U-937 via different regulatory mechanisms [11, 12]. Wogonin was also shown to increase doxorubicin sensitivity in breast cancer, and head and neck cancer [13, 14]. However, whether wogonin has a role in overcoming T790M-mediated acquired resistance is not clear, and the associated regulatory mechanism is not known.

In the present study, the role of wogonin in overcoming T790M-mediated acquired resistance to icotinib, a novel EGFR TKI with preclinical and clinical activity [15] in NSCLC, was investigated in NCI-H1975 cells, which have EGFR-T790M mutation. The effect of wogonin, icotinib, or combination of wogonin plus icotinib on cell proliferation, apoptosis, and autophagy was evaluated. The results showed that combination of wogonin and icotinib could overcome the acquired resistance to icotinib. In addition, we found that mTOR phosphorylation might be a regulatory mechanism through which wogonin overcomes the acquired resistance to icotinib.

## Materials and methods

### *Cell line, cell culture, and reagents*

Human NSCLC cell line NCI-H1975 was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). NCI-H1975 cells were cultured in RPMI-1640 medium with 10% foetal bovine serum (FBS) (GIBCO/BRL, MD, USA), supplemented with 100 U/ml penicillin G and 100 μg/mL streptomycin (Sigma-Aldrich Corp., St. Louis, MO). Cells were maintained at 37°C in a humidified 5% $CO_2$ incubator. Wogonin, icotinib, and 3-methyladenine (3-MA) were purchased from Abmole Bioscience Inc. (Houston, TX, USA). Wogonin was dissolved in dimethyl sulfoxide (DMSO) at 100 mM and stored at -20°C as a stock solution, and diluted to each designated concentration in the buffer solution before each experiment. The final concentration of DMSO did not exceed 0.1%.

### *Experimental design*

To detect the inhibitory concentration 50 ($IC_{50}$) of TKIs, icotinib and wogonin were diluted to different concentrations (10, 20, 40, 60, 80, 100, 200 μM) to treat NCI-H1975 cells for 24, 48, and 72 h. To choose the best concentration for icotinib and wogonin combination treatment, NCI-H1975 cells were treated for 48 and 72 h at following concentrations: 10 μM wogonin, 20 μM wogonin, 40 μM wogonin, 10 μM icotinib, 20 μM icotinib, 40 μM icotinib, 10 μM wogonin+10 μM icotinib, 20 μM wogonin+10 μM icotinib, 40 μM wogonin+10 μM icotinib, 10 μM wogonin+20 μM icotinib, 20 μM

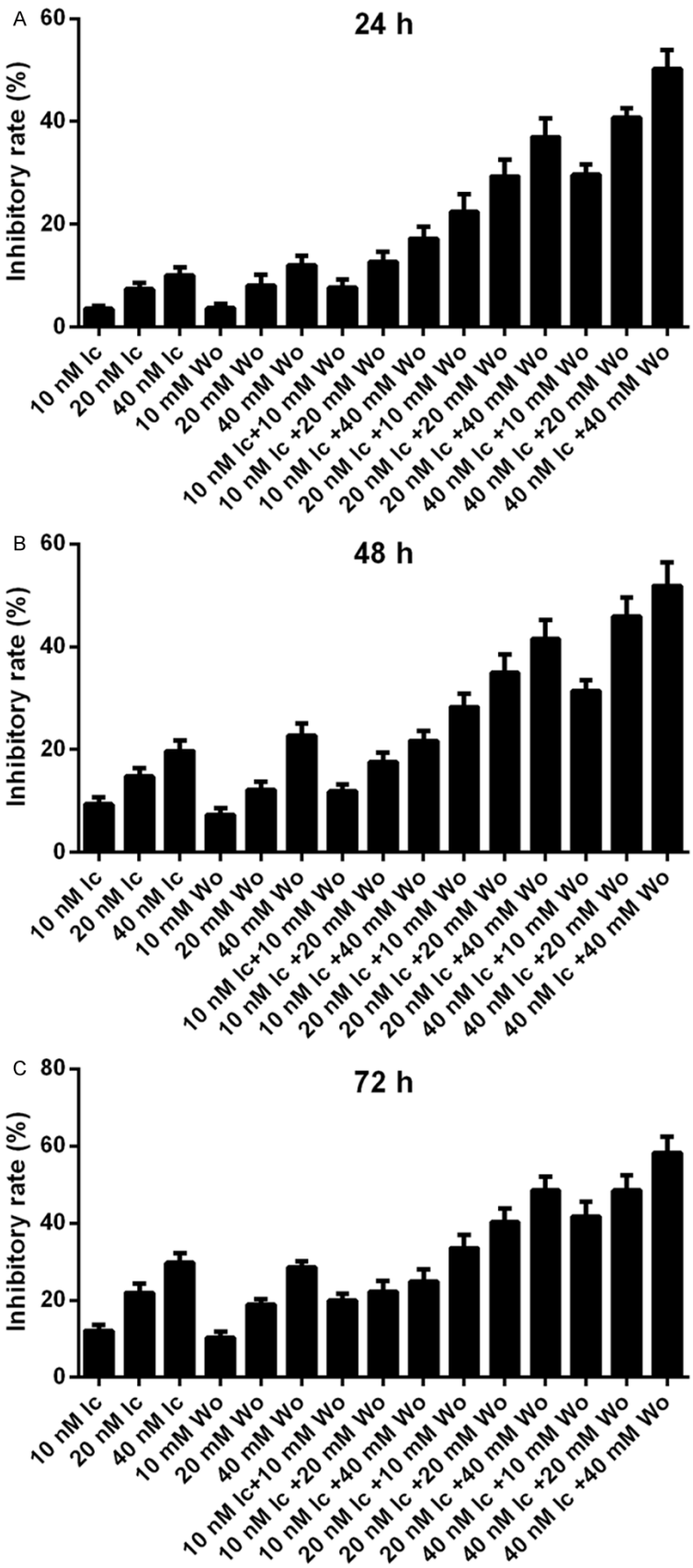


**Figure 2.** The inhibitory activity of low concentration icotinib (Ic) 10 nM, 20 nM, 40 nM plus low concentration wogonin (Wo) (10 mM, 20 mM, and 40 mM) treatment for 24 h (A), 48 h (B), and 72 h (C) in NCI-H1975 cells.

wogonin+20 μM icotinib, 40 μM wogonin+20 μM icotinib, 10 μM wogonin+40 μM icotinib, 20 μM wogonin+40 μM icotinib, 40 μM wogonin+40 μM icotinib. Based on the screening results, NCI-H1975 cells were divided into the following four groups for investigating the effects on apoptosis, the protein expression of caspase 3, LC3B, and Beclin 1 by western blot, and transmission electron microscope analysis: DMSO-treated group, wogonin-treated group, icotinib-treated group, and wogonin plus icotinib-treated group. To further investigate the relationship between apoptosis and autophagy, NCI-H1975 cells were divided into two groups: wogonin plus icotinib-treated group and wogonin plus icotinib plus 3-MA-treated group.

*Evaluation of the inhibitory activity*

Rate of cell inhibition was evaluated using the cell counting kit-8 (CCK-8) (Dojindo, Kumamoto, Japan) according to the manufacturer's protocol. Briefly, at the end of the treatment, 10 μL WST-8 reagent was added to each well, and after 4 h incubation at 37°C, OD value at 490 nm was measured using a microplate reader (Multiscan MK3; Thermo Fisher Scientific, Waltham, MA, USA). All experiments were performed in triplicate and repeated three times. A blank well that contained only

**Table 1.** The q value of each group

| Group | q value | | |
|---|---|---|---|
| | 24 h | 48 h | 72 h |
| 10 nM Ic+10 mM Wo | 1.08 | 0.74 | 0.94 |
| 10 nM Ic+20 mM Wo | 1.11 | 0.86 | 0.77 |
| 10 nM Ic+40 mM Wo | 1.13 | 0.72 | 0.67 |
| 20 nM Ic+10 mM Wo | 2.08 | 1.35 | 1.17 |
| 20 nM Ic+20 mM Wo | 1.97 | 1.39 | 1.09 |
| 20 nM Ic+40 mM Wo | 2.00 | 1.22 | 1.10 |
| 40 nM Ic+10 mM Wo | 2.21 | 1.23 | 1.13 |
| 40 nM Ic+20 mM Wo | 2.34 | 1.56 | 1.13 |
| 40 nM Ic+40 mM Wo | 2.41 | 1.37 | 1.17 |

q=EAB/(EA+EB-EA×EB); EAB: Inhibitory activity after icotinib (Ic) plus wogonin (Wo) treatment; EA: Inhibitory activity after treatment with icotinib alone; EB: Inhibitory activity after treatment with wogonin alone.

media and drug was used as a control for all the experiments. The inhibitory ratio was calculated using the following formula: inhibitory rate (%)=(1- the average absorbance of the treated group/the average absorbance of the control group)×100%. To examine the synergistic effect of wogonin and icotinib, q value was calculated using the following formula: q=EAB/(EA+EB-EA×EB), EAB: inhibition rate after icotinib plus wogonin treatment, EA: inhibition rate after icotinib treatment, EB: inhibition rate after wogonin treatment.

*Flow cytometry for investigating apoptosis*

Flow cytometric analysis for apoptosis was carried out using annexin V-FITC cell apoptosis analysis kit (KeyGEN BioTECH, Nanjing, China). Briefly, cells were collected by trypsin digestion, washed twice with PBS, centrifuged, and resuspended with 500 μL binding buffer. Subsequently, 5 μL annexin V-FITC was added to the cell suspension and mixed followed by addition of 5 μL propidium iodide (PI) and mixing. After incubation for 10 min at room temperature in the dark, cells were analysed using BD Accuri™ C6 flow cytometer (BD Biosciences, San Diego, CA, USA) to detect apoptosis.

*Western blotting analysis*

Cells were digested with trypsin and centrifuged at 2,000 rpm for 5 min. After collection, the cells were washed twice with PBS and centrifuged at 2,000 rpm for 5 min. The cell pellet ($1\times10^6$ cells) was lysed using RIPA lysis buffer. Total protein concentration was detected using the BCA Protein Assay kit (Thermo Scientific Pierce, Rockford, IL, USA). Total protein (30 μg) were loaded and separated on 10% sodium dodecyl sulphate polyacrylamide gels and transferred to PVDF membranes (PALL, New York, NY, USA). After blocking with 5% milk in TBS containing 0.05% Tween-20 (TBST) for 1 h at room temperature, all membranes were incubated for 1 h with primary antibody. Subsequently, the membranes were washed thrice with TBST and incubated with secondary antibody for 40 min. After washing three times with TBST, all membranes were visualized by enhanced chemiluminescence reagent obtained from Millipore Corporation (Billerica, MA, USA). GAPDH served as an internal loading control.

*Transmission electron microscopy*

At the end of the treatment, cells were digested with 0.25% trypsin and collected in centrifuge tubes, followed by centrifugation at 1,500 rpm/min for 10 min and at 800 rpm/min for another 10 min. The supernatant was discarded and cells were fixed by addition of 2.5% glutaraldehyde for 30 min. Subsequently, cells were dehydrated, embedded, and stained using standard methods. The autophagosomes were observed under a JEM-1010 electron microscope (Matsunaga Manufacturing, Japan).

*Statistical analysis*

The results shown in figures represent the means ± standard error of the means. Differences within or between normally distributed data were analysed by analysis of variance using SPSS (Version 13.0; SPSS, Inc., Chicago, IL, USA) followed by Tukey's post hoc test. A *p*-value of less than 0.05 was regarded as statistically significant.

## Results

*The effect of wogonin or icotinib on proliferation of NCI-H1975 cells*

To investigate the effect on proliferation of NCI-H1975 cells, the cells were treated with different concentrations of wogonin (10 mM, 20 mM, 40 mM, 60 mM, 80 mM, 100 mM) or icotinib (10 nM, 20 nM, 40 nM, 60 nM, 80 nM, 100 nM) for 24, 48, or 72 h. At the end of each time point, CCK-8 assay was used to detect the inhibitory activity. As shown in **Figure 1A** and **1B**, all concentrations of wogonin or icotinib could effectively inhibit NCI-H1975 cell proliferation

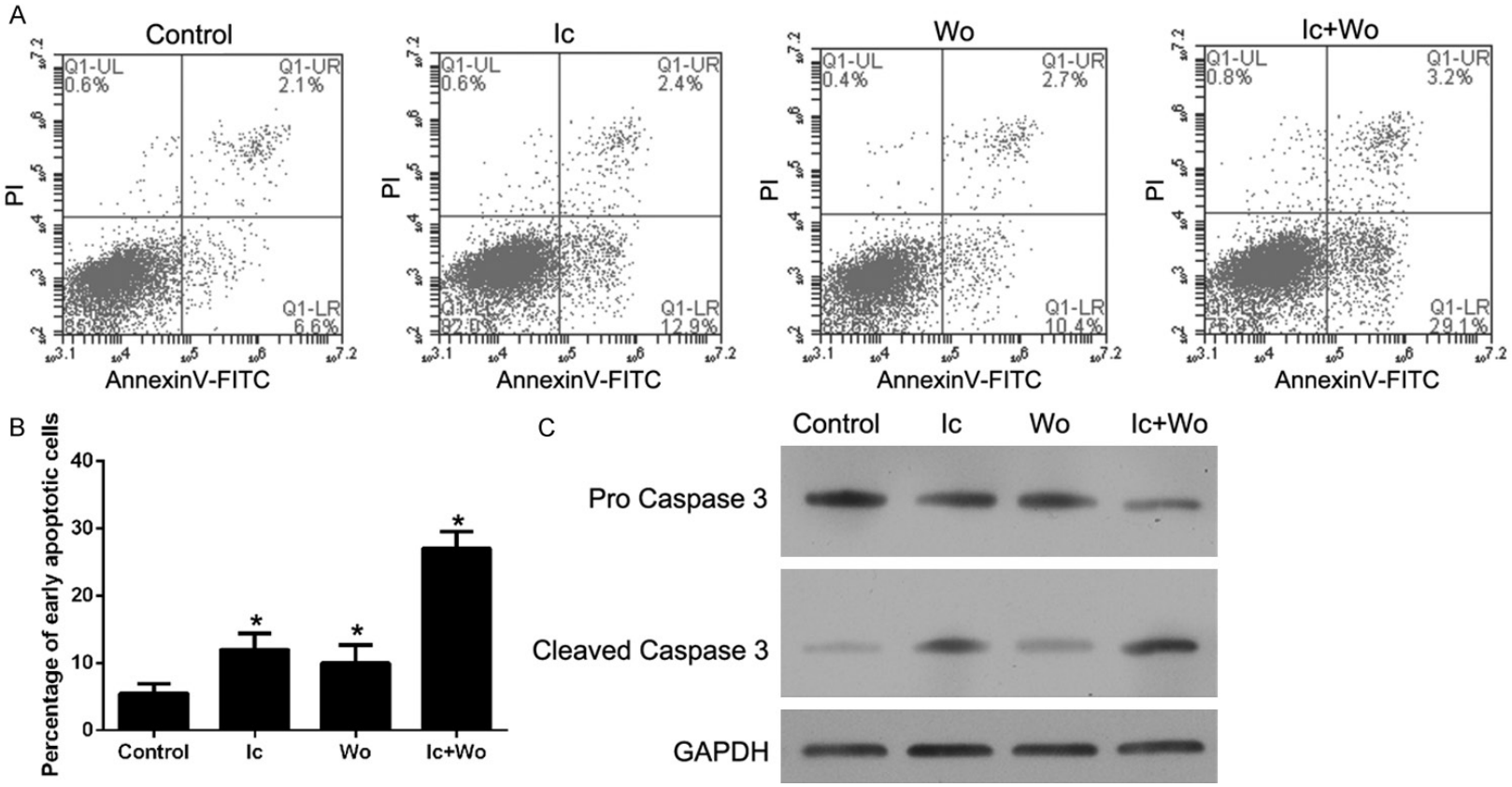


**Figure 3.** The effect of wogonin and icotinib, either alone or in combination, on apoptosis of NCI-H1975 cells. NCI-H1975 cells were divided into four groups: non-treated (Control), treated with 20 nM icotinib (Ic), treated with 10 mM wogonin (Wo), treated with 20 nM icotinib+10 mM wogonin (Ic+Wo). After treatment, cells were harvested for flow cytometry and western blot analysis. A: The representative graphs of flow cytometry analysis. B: The percentage of early apoptotic cells detected using flow cytometry. C: Procaspase 3 and cleaved caspase 3 protein expression levels detected by western blotting. GAPDH was used as an internal reference. *P<0.05, when compared to the control group.

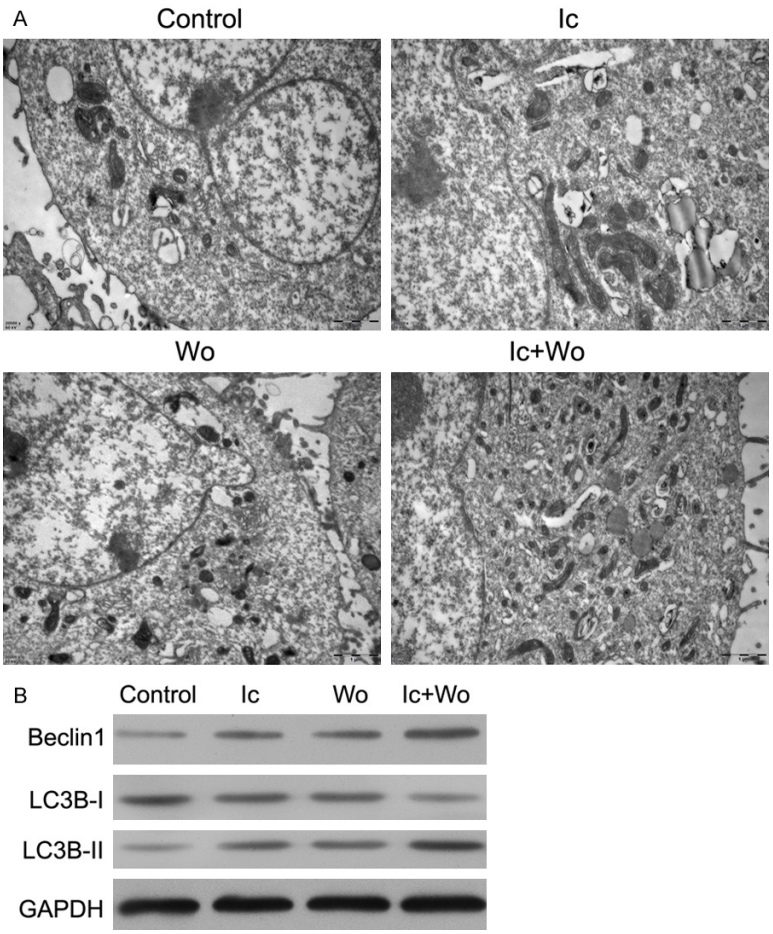


**Figure 4.** The effect of wogonin and icotinib, either alone or in combination, on autophagy of NCI-H1975 cells. NCI-H1975 cells were divided into four groups: non-treated (Control), treated with 20 nM icotinib (Ic), treated with 10 mM wogonin (Wo), treated with 20 nM icotinib+10 mM Wo (Ic+Wo). The cells were then harvested for transmission electron microscopy (A) and western blot detection of LC3B-I conversion to LC3B-II and Beclin 1 expression (B).

by different degrees. Furthermore, the inhibitory activity of wogonin or icotinib on NCI-H1975 was time-dependent and dose-dependent. Based on the effective inhibitory effect of wogonin or icotinib on proliferation of NCI-H1975 cells, three low concentrations of wogonin (10 mM, 20 mM, and 40 mM) or icotinib (10 nM, 20 nM, and 40 nM) were used in this study.

*The combined effect of wogonin and icotinib on proliferation of NCI-H1975 cells*

To investigate the combined effect of wogonin and icotinib, NCI-H1975 cells were treated with different concentrations of the combination for 24, 48, or 72 h. The results are shown in **Figure 2**. Treatment of NCI-H1975 cells with combination of wogonin and icotinib showed higher inhibitory activity than treatment with wogonin or icotinib alone at 24 h (**Figure 2A**), 48 h (**Figure 2B**), or 72 h (**Figure 2C**). To choose the best combination treatment concentration, the q value of each group was calculated, which showed whether wogonin and icotinib have synergistic effect on proliferation of NCI-H1975 cells. As shown in **Table 1**, the q values of 20 nM icotinib+10 mM wogonin and 40 nM icotinib+40 mM wogonin treatment groups were greater than 1.15 at 24, 48, and 72 h. These results indicated that 20 nM icotinib+10 mM wogonin and 40 nM icotinib+40 mM wogonin treatment have synergistic effect on proliferation of NCI-H1975 cells. Because the inhibitory activity of 40 nM icotinib+40 mM wogonin treatment was too high to be used, 20 nM icotinib+10 mM wogonin treatment was chosen for the following study.

*The effect of wogonin and/or icotinib on apoptosis of NCI-H1975 cells*

The effect of wogonin and icotinib, either alone or in combination, on apoptosis of NCI-H1975 cells was examined by flow cytometry and by western blot analysis for detecting cleaved caspase 3. As shown in **Figure 3A** and **3B**, percentage of early apoptotic cells after treatment with wogonin and icotinib, either alone or in combination, were markedly increased compared to the control group. Moreover, percentage of early apoptotic cells markedly increased in wogonin and icotinib combination treatment group compared to that reported for wogonin or icotinib individual treatment groups. Interestingly, the effect of wogonin and icotinib combination treatment on early apoptotic cells was greater than the sum of the effects of wogonin and icotinib individual treatment groups. Furthermore, the expression levels of cleaved caspase 3 after wogonin and icotinib treatment, either alone or in combination, were evaluated.

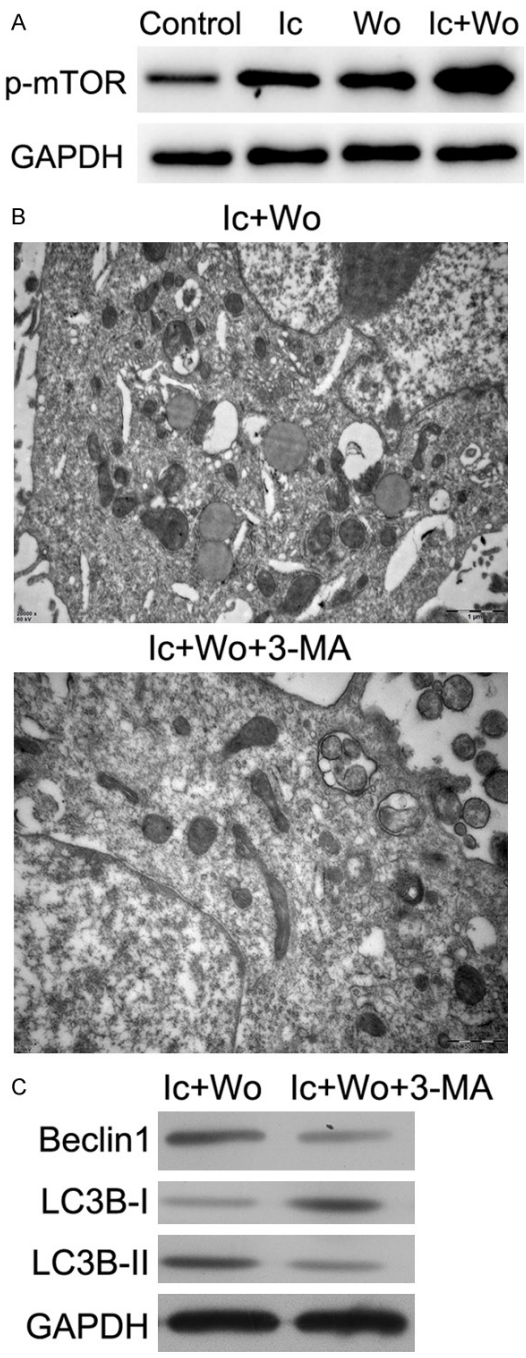


**Figure 5.** The suppression of mTOR phosphorylation weakened the effect of wogonin and icotinib on cell autophagy. NCI-H1975 cells were divided into four groups: non-treated (Control), treated with 20 nM icotinib (Ic), treated with 10 mM wogonin (Wo), treated with 20 nM icotinib+10 mM wogonin (Ic+Wo). The cells were then harvested for western blot analysis for detecting phosphorylated mTOR (p-mTOR) (A). After treatment with or without 3-MA, NCI-H1975 cells were treated with 20 nM icotinib (Ic)+10 mM wogonin (Wo). The cells were then harvested for transmission electron microscopy (B) and western blot analysis for detecting the conversion of LC3B-I to LC3B-II and Beclin 1 expression (C).

As shown in **Figure 3C**, expression level of cleaved caspase 3 significantly increased after treatment with wogonin, icotinib, and combination of wogonin plus icotinib. Similar to flow cytometry results, the effect of wogonin and icotinib combination treatment on caspase 3 cleavage was greater than the effect of treatment with wogonin or icotinib alone.

*The effect of wogonin and/or icotinib on autophagy of NCI-H1975 cells*

To detect the effect of wogonin and icotinib, either alone or in combination, on autophagy of NCI-H1975 cells, the formation of autophagosomes was observed using a transmission electron microscope. As shown in **Figure 4A**, the number of intracellular autophagosomes after treatment with wogonin and icotinib, either alone or in combination, were increased compared to the control group. The number of intracellular autophagosomes after wogonin plus icotinib combination treatment was more than that after treatment with wogonin or icotinib alone. The expression of autophagy-related genes LC3B and Beclin 1 was also examined using western blot analysis. As shown in **Figure 4B**, LC3B-I conversion to LC3B-II and Beclin 1 expression were increased by treatment with wogonin and icotinib, either alone or in combination, compared to control group. Moreover, the increase in LC3B-I conversion to LC3B-II and Beclin 1 expression after wogonin plus icotinib combination treatment was more than that after wogonin or icotinib individual treatment.

*The suppression of mTOR phosphorylation weakened the effect of wogonin and icotinib on cell autophagy and apoptosis*

To investigate the possible mechanism involved in regulating the effect of wogonin and/or icotinib on apoptosis and autophagy, the mTOR phosphorylation level was detected. As shown in **Figure 5A**, treatment with both wogonin and icotinib, either alone or in combination upregulated the mTOR phosphorylation level. The increased mTOR phosphorylation after wogonin plus icotinib combination treatment was more than that after wogonin or icotinib individual treatment. To further confirm the role of mTOR phosphorylation in regulating the effect of wogonin and icotinib on cell proliferation, apoptosis, and autophagy. As a control, 3-MA was used to prevent mTOR phosphorylation. The

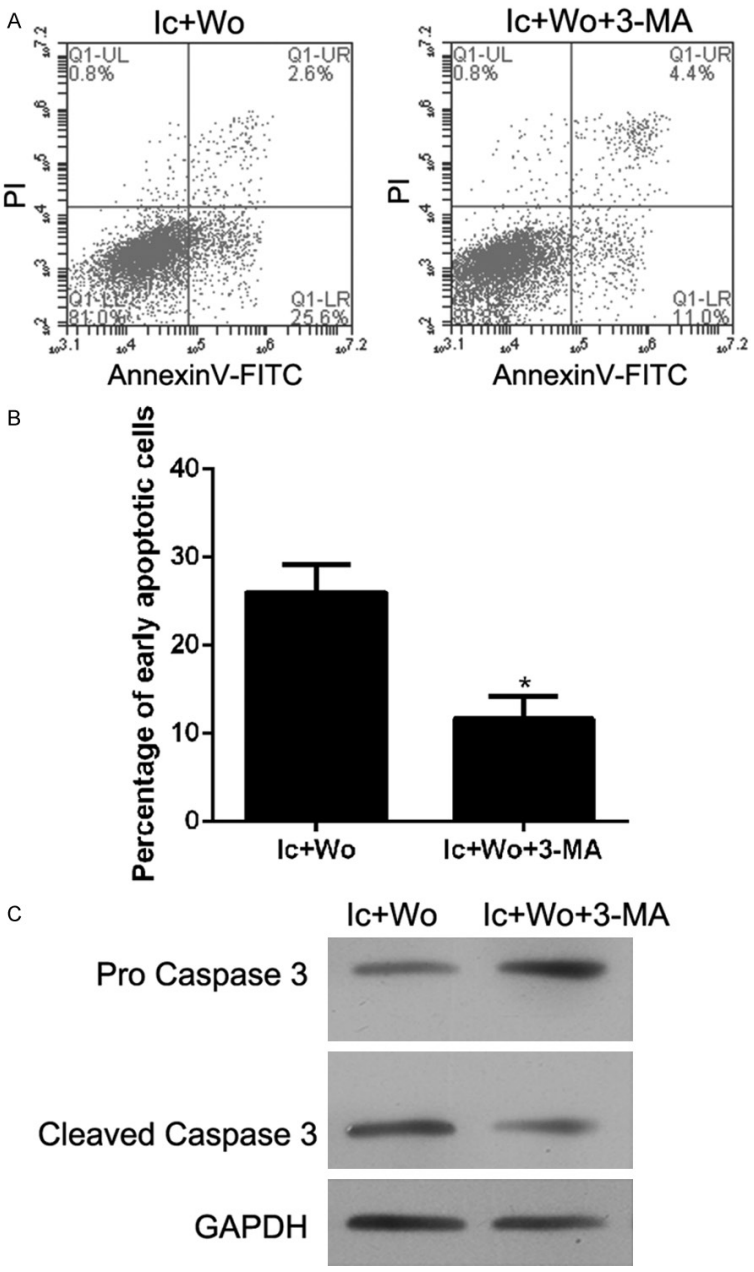


**Figure 6.** The suppression of mTOR phosphorylation weakened the effect of wogonin and icotinib on apoptosis. After treatment with or without 3-MA, NCI-H1975 cells were treated with 20 nM icotinib (Ic)+10 mM wogonin (Wo). The cells were then harvested for flow cytometry and western blot analysis. A: The representative graphs of flow cytometry analysis. B: The percentage of early apoptotic cells detected by flow cytometry. C: Procaspase 3 and cleaved caspase 3 protein expression levels detected by western blotting. GAPDH was used as an internal reference. *P<0.05, when compared to Ic+Wo group.

suppression of mTOR phosphorylation decreased the number of intracellular autophagosomes increased by treatment with wogonin plus icotinib combination (**Figure 5B**). The suppression of mTOR phosphorylation downregulated the wogonin plus icotinib combination treatment-mediated increased conversion of LC3B-I to LC3B-II and Beclin 1 expression (**Figure 5B**). In addition, the suppression of mTOR phosphorylation decreased the percentage of early apoptotic cells (**Figure 6A** and **6B**) and caspase 3 cleavage (**Figure 6C**). These results indicated that the suppression of mTOR phosphorylation weakened the effect of wogonin and icotinib on cell autophagy and apoptosis.

## Discussion

Advanced NSCLC is the main cause of cancer-related mortality. EGFR-TKIs are currently recommended as the standard first-line treatment for advanced NSCLC patients harbouring EGFR active mutation. However, about 60% of the patients unfortunately develop resistance to EGFR-TKIs after a favourable response for 9-13 months on average [16, 17]. To overcome acquired resistance to EGFR-TKIs, new strategies employing biologically synergistic combinations are needed. Until development of a successful treatment strategy to overcome the acquired resistance, only few treatment options are currently available. Some traditional Chinese medicines, such as gambogic acid, were shown to play a role in overcoming acquired resistance to EGFR-TKIs in a lung cancer cell line harbouring EGFR T790M mutations, NCI-H1975 [18]. Therefore, the present study firstly investigated the role of wogonin in overcoming acquired resistance to EGFR-TKIs in NCI-H1975 cell line. The EGFR-TKI used in this study was icotinib, which is the first self-developed small molecular drug for targeted therapy of lung cancer in China [18].

Wogonin was shown to potentiate the cisplatin-induced apoptosis and suppress migration and proliferation of lung cancer cells [19-21], which suggested that it has effective antitumor activities. The present study also demonstrated that treatment with wogonin alone could inhibit cell proliferation and induce apoptosis in NCI-H1975 cell line, indicating its antitumor activity. Furthermore, the combination of wogonin with icotinib had a synergistic effect on the inhibition of cell proliferation. For example, treatment with 20 nM icotinib alone produced less than 10% inhibition, but all combinations of 20 nM icotinib with different concentrations of wogonin produced more than 20% inhibition. In addition, the combination of wogonin with icotinib significantly enhanced the effect on apoptosis and autophagy. These results suggested that wogonin could enhance the inhibitory effects of icotinib in EGFR T790M-mutated lung cancer in vivo. Moreover, wogonin might play a role in overcoming acquired resistance to EGFR-TKIs in EGFR T790M-mutated lung cancers, which could be further add value to the antitumor role of wogonin in lung cancer cells.

Autophagy is the natural, regulated, destructive mechanism of the cell that disassembles unnecessary or dysfunctional components [22]. It plays double role in cancers by either contributing to cancer or acting as tumour suppressor [23]. Results of the present study showed that the combination of wogonin with icotinib activated autophagy and increased the rate of apoptosis in NCI-H1975 cells. Therefore, we suggest that combination of wogonin with icotinib increases apoptosis by activating autophagy. It was further based on the following results. The combination of wogonin with icotinib increased phosphorylation level of mTORC1 (mammalian or mechanistic target of rapamycin complex 1), a protein kinase mTOR, which is the classical regulator of autophagy [24, 25]. In contrast, inhibition of autophagy through autophagy inhibitor 3-MA could weaken the effect of wogonin plus icotinib combination treatment on apoptosis. These results indicated that autophagy acted as a tumour suppressor in overcoming acquired resistance to EGFR-TKI icotinib in NCI-H1975 cell line.

In conclusion, results of the present study showed that the combination of wogonin with icotinib could enhance the inhibitory activity of icotinib on cell proliferation, apoptosis, and autophagy. This shows that wogonin might play a role in overcoming acquired resistance to EGFR-TKIs in EGFR T790M-mutated lung cancer, in which autophagy acted as tumour suppressor.

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## Disclosure of conflict of interest

None.

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