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Baicalin induces apoptosis and autophagy in resistant human hepatocellular carcinoma cell line Bel-7402/5-FU cells via PI3K/AKT pathway

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ABSTRACT

Objective: Multidrug resistance (MDR) is one main cause of chemotherapy failure. Baicalin is an important active ingredient with anticancer potential in many Chinese herbal medicines. In order to understand the function of baicalin reversing MDR in hepatocellular carcinoma (HCC) and the molecular mechanisms that underlie it, the current study was designed.

Methods: Bel-7402 and Bel-7402/5-FU cells were cultured, and MTT assay was applied to detect cell viability and the cross-resistance of Bel-7402/5-FU cells. The pump function, apoptosis, and autophagy were detected by flow cytometry. The related proteins were detected by Western blot assay. The PI3K agonist (740Y-P) was used to verify whether baicalin overcomes the drug resistance of HCC cells by blocking the PI3K/AKT pathway.

Results: The findings showed that Bel-7402/5-FU cells were cross-resistant to different chemotherapeutic drugs. Baicalin inhibited cell viability in both Bel-7402/5-FU and Bel-7402 cells, and baicalin increased sensitivity of Bel-7402/5-FU cells to 5-FU in time- and dose-dependent manners. Baicalin increased the accumulation of doxorubicin and rhodamine-123 in Bel-7402/5-FU cells and inhibited the protein expression of ABCG2, ABCB1, and ABCC1, associated with pump function. In addition, baicalin induced apoptosis of Bel-7402/5-FU cells via up-regulating Bax expression. Furthermore, baicalin increased autophagy through regulating LC3-II, p62, and Beclin-1. Baicalin reversed drug resistance in Bel-7402/5-FU cells by inhibiting the PI3K/AKT pathway, which promoted autophagy and apoptosis to restore chemosensitivity.

Conclusion: Baicalin increased accumulation of chemotherapy drugs and induced apoptosis and autophagy in Bel-7402/5-FU cells by inhibiting the PI3K/AKT signaling pathway, that may be the important mechanism by which baicalin reverses the MDR of HCC.

1. Introduction

As the third most prevalent cause of cancer-related deaths globally in 2022, liver cancer has a high mortality rate and is the sixth most common type of cancer to be diagnosed.[1] Hepatocellular carcinoma (HCC), comprising 75%–85% of cases, is associated with multiple risk factors. The determinants are viral hepatitis (mainly infection by HBV) and aflatoxin contamination in China.[2] At present, the commonly used treatment methods include hepatectomy, liver transplantation, immunotherapy, radiotherapy, chemotherapy, etc.[3] However, most of the treatment regimens have obvious adverse reactions, which will affect the quality of life of patients. In addition, drug resistance induced by treatment strategies primarily based on targeted drugs, chemotherapeutic agents, and combination therapies has become a major challenge in HCC therapy. Therefore, it is necessary to find new drugs for overcoming resistance to HCC and reveal their action mechanisms against Multidrug resistance (MDR).

Drug resistance manifests in two primary forms: intrinsic (pre-

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existing resistance prior to chemotherapy) and acquired resistance (developed during treatment).[4] MDR is a kind of acquired resistance which can be an outcome of various cellular and molecular responses including enhanced drug efflux, suppression of apoptosis, activation of protective autophagy, mutations in genes, gene amplification, and aberrant signaling cascades.[5] The predominant drug resistance mechanism involves ATP-dependent ABC transporters (e.g., ABCB1 and ABCG2) that actively pump out drugs and are overexpressed in tumors.[6] Notably, research shows the PI3K/AKT signaling pathway plays a key role in liver cancer drug resistance, it drives MDR by coordinating key mechanisms like autophagy, apoptosis, and ABC transporters regulation,[7] that reduce drug effectiveness, collectively contributing to chemotherapeutic failure, making cancer treatment more challenging. Targeting this pathway may help overcome MDR.

Baicalin, a flavonoid extracted from Scutellariae Radix, has various kinds of pharmacological activities. As a natural product, baicalin exhibits multi-targeted mechanisms, low toxicity compared to conventional chemotherapeutics, and broad-spectrum bioactivity. Baicalin exhibits potent anti-cancer activity against various carcinomas, including HCC. Several studies show that baicalin can effectively inhibit the progression of HCC by indirectly inducing autophagy or directly promoting apoptosis, etc.[8] Baicalin has been shown to enhance the chemotherapy sensitivity of breast cancer to doxorubicin.[9] Additionally, it can enhance the effect of 5-Fluorouracil (5-FU) in gastric cancer by promoting intracellular ROS generation.[10] However, the molecular mechanisms underlying baicalin's ability to reverse MDR in HCC remain poorly understood and require further investigation.

This study aims to investigate whether baicalin reverses MDR in BEL-7402/5-FU cells by modulating the PI3K/AKT signaling and restoring dysregulated apoptosis-autophagy. We propose that baicalin-induced autophagy activation synergizes with promotion of apoptosis which contributes to overcoming MDR, which may provide a new therapeutic strategy for refractory HCC.

2. Materials and methods

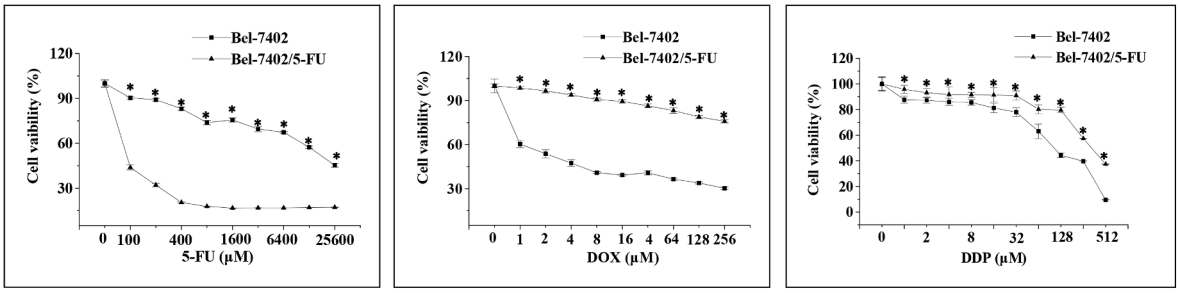
2.1. Materials and reagents

Baicalin (purity ≥ 98%, B20570) was purchased from Shanghai Yuanye Biotech. Co. (Shanghai, China) and dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution of 10 mM. Doxorubicin (KGA8184) and Rhodamine-123 staining kit (KGA217) were obtained from Nanjing Keygen Biotech. Co. (Nanjing, China). Penicillin–streptomycin (15140122), trypsin-EDTA (25200-072), and fetal bovine serum (10099141) were obtained from Gibco (Grand Island, NY, USA). RPMI 1640 (C11875500BT) was purchased from Invitrogen (Carlsbad, CA, USA). The Enzo Life Sciences, Inc. (Farmingdale, Maine, USA) provided the Cyto-ID autophagy detection kit (ENZ-51031-K200). P-glycoprotein (P-gp)/ABCB1 (YT3692), Multidrug Resistance-associated Protein 1 (MRP1)/ABCC1 (YT0885), Breast Cancer Resistance Protein (BCRP)/ABCG2 (YT0053), GAPDH (YM3029), and β-actin (YM3028) antibodies were purchased from Immunoway (Plano, TX, USA). Cell Signaling Technology (Danvers, MA, USA) provided the AKT (60203-2), p-AKT (66444-1), and PI3K (60225-1) while antibodies to SQSTM1/p62 (18420-1-AP), LC3 (14600-1-AP), and BAX (50599-2) were obtained from ProteinTech Group (Chicago, IL, USA). 0.740-YP (B5246-1) was obtained from APExBIO (Houston, USA).

2.2. Cell culture

The human HCC cell line Bel-7402 (KG022) and its resistant cells Bel-7402/5-FU (KG125) were purchased from Nanjing Keygen Biotech. Co. (Nanjing, China). The Bel-7402/5-FU resistant cell line was developed through cyclical drug exposure combining sustained low-dose adaptation and intermittent high-concentration pulse over 10 months. Stable resistant cells were maintained with periodic drug-free recovery, cryopreserved after three passages, and validated post-thaw.[11] The cells were

A



B

Drug	IC_{50} (μM)		RI
	Bel-7402	Bel-7402/5-FU	
5-FU	3.51	25228.00	7187.46
DOX	3.48	435.00	125.00
DDP	150.46	387.66	2.58

Fig. 1. Bel-7402/5-FU showed cross-resistance to different chemotherapeutic drugs. (A) Cell viability. (B) RI of Bel-7402/5-FU to different chemotherapeutic drugs. 5-FU: 5-fluorouracil, DOX: doxorubicin, DDP: cisplatin, RI: Resistance index. Mean ± SD, $n = 8$. *$P < 0.05$, in contrast to untreated control cells.

cultured in RPMI-1640 complete medium supplemented with 10% FBS and 1% penicillin-streptomycin in a 5% CO_2 incubator at 37 °C. Before subsequent experiments were carried out, Bel-7402/5-FU-resistant cells needed to be cultured in RPMI-1640 complete medium containing 20 μg/mL 5-FU for at least 2 weeks to maintain the 5-FU resistance phenotype.

2.3. Cross-resistance assay

The cytotoxicity of chemotherapeutic drugs was detected via MTT assay.[12] Cells were inoculated in 96-well plates at the density of 5×10^3 per well and cultured in an incubator for 24 h. The cells were treated for 48 h with chemotherapeutic agents at the following concentrations: 5-FU (100-25600 μM), doxorubicin (2–512 μM), cisplatin (2–512 μM). All compounds were diluted in RPMI-1640 medium. These concentration ranges were selected based on preliminary half-maximal inhibitory concentration (IC_{50}) values which derived from dose-response assays in Bel-7402/5-FU cells. Eight replicate wells were set up in each group. The nonlinear regression approach was used to calculate the IC_{50} of various anticancer medications. The resistance index (RI) was calculated through dividing the IC_{50} of the MDR cells by the IC_{50} of parental cells.

2.4. Intrinsic cytotoxicity assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for cytotoxic effects of baicalin. As described in section 2.3, the cells were cultured for 24, 48, and 72 h, respectively, and various doses of baicalin were added. DMSO solubilized baicalin in final doses of 5, 10, 20, 40, 80, and 160 μM, respectively. The same volume of DMSO was used as a negative control group. Using an ELISA plate reader at OD570 to detect the cell viability.

2.5. Intracellular accumulation of doxorubicin

As described by Shi et al.,[13] the intracellular accumulation of doxorubicin was detected. Baicalin at the doses of 0, 20, 40, 80, and 160 μM, treated cells for 48 h, respectively. Then the concentration of the cells was adjusted to 1×10^6/mL after digestion with trypsin-EDTA, and the cells were added doxorubicin until the final concentration of doxorubicin was 5 μg/mL, and then were incubated for 30 min at 37 °C protected from light. The flow cytometry with an excitation wavelength of 488 nm and emission wavelength of 575 nm was used to detect the average fluorescence intensity of doxorubicin in cells.

2.6. Efflux assay of rhodamine-123

As described by Shi et al.,[13] the intracellular accumulation of Rho123 was detected. As described in section 2.5, cells were resuspended in 5 μg/mL Rho123, and the cells were incubated for 30 min at 37 °C. Using pre-cooled PBS to wash the cells and cells were resuspended in PBS after being cultured for another 60 min incubation at 37 °C. The flow cytometry with an excitation wavelength of 488 nm and emission wavelength of 525 nm was used to detect the average fluorescence intensity of Rho123 in cells.

2.7. Detection of apoptosis

Becton Dickinson (San Jose, CA, USA) provided the Annexin V-FITC/PI apoptosis detection kit. Following the manufacturer's protocol, we detected Bel-7402/5-FU apoptosis after baicalin treatment at various doses for 48 h. The percentage of early apoptosis (Annexin V-positivity and PI-negativity) versus late apoptosis (Annexin V-positivity and PI-positivity) was used as the apoptosis rate.

2.8. Cyto-ID autophagy detection

Cells were treated as described in 2.5. Bel-7402/5-FU was collected by trypsin-EDTA digestion and centrifugation, and the cell density was adjusted to 1×10^6 cells. 2 mL of RPMI-1640 medium was supplemented with 1 μL of Cyto-ID reagent. Using 0.5 mL of diluted Cyto-ID reagent to resuspend the cells. After incubation for 30 min at 37 °C and protected from light, the average fluorescence intensity of FL1 channel was assessed by flow cytometry.

2.9. Western blot analysis

The cell was treated as described in 2.5. Furthermore, the cell was treated with an 80 μM dose of baicalin and 740Y-P for 48 h. Following a PBS wash, RIPA buffer containing protease inhibitors was added to cells for lysing. Bel-7402/5-FU was collected and then centrifuged (12,000 rpm at 4 °C, 20 min), the supernatant was aspirated and 5 × loading buffer was added and stored at −80 °C for storage. Thermo Fisher Scientific, Inc. (Waltham, MA, USA) provided the BCA Protein Assay Kit (23227) which was used to measure the protein concentration. After electrophoresing the total protein (50 μg) with 10% SDS-PAGE, it was transferred to a PVDF membrane. Proteins were blocked by incubation with TBST containing 5% skimmed milk for 2 h at room temperature. Washing the membranes with TBST three times. Corresponding primary antibodies (1:1000) were added and incubated

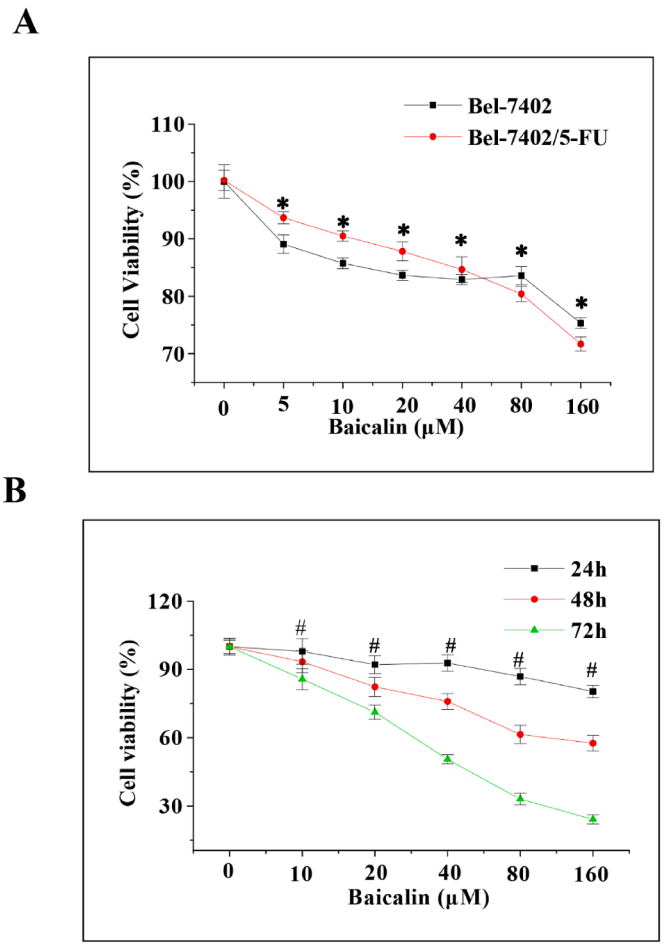


Fig. 2. Baicalin inhibited cell viability of Bel-7402/5-FU and the parental cells. (A) Baicalin inhibited cell viability. (B) Baicalin inhibited Bel-7402/5-FU cells viability in both concentration- and time-dependent manners. Mean ± SD, $n = 8$. $^*P < 0.05$, in contrast to untreated control cells. $^{\#}P < 0.05$ for comparisons between time points at the same baicalin concentration.

overnight in a shaker at 4 °C, and secondary antibodies (1:5000) were added and incubated for 2 h at room temperature. After antibody recovery, the membranes were washed four times with TBST for 5 min each time. The proteins were detected using ECL chemiluminescent reagents, with β-actin or GAPDH as an internal reference. Band intensities were quantified using ImageLab. Target protein signals were normalized to β-actin or GAPDH. All data from three independent experiments are expressed as mean ± SD.

2.10. Statistical analysis

All the statistical analyses were analyzed by SPSS 24.0. The difference between groups used the student's *t*-test, one-way ANOVA was used for three or more groups. A *P*-value of <0.05 was considered statistically significant.

3. Results

3.1. Resistance to drugs in Bel-7402/5-FU cells

RI is used as the basis for evaluating drug resistance of drug resistant cells. RI < 5 is no or low-grade drug resistance, RI < 15 is moderate drug resistance, RI > 20 is high-grade drug resistance. The results of MTT assay are as shown in Fig. 1. Compared to the parental cells, Bel-7402/5-FU cells exhibited greater resistance to 5-FU, doxorubicin, and cisplatin. Furthermore, Bel-7402/5-FU showed more resistance to 5-FU and doxorubicin, and RI was 7187.46 and 125.00, respectively. But Bel-7402/5-FU showed less resistant to cisplatin, and RI was 2.58. Together, all the results indicated that to various chemotherapeutic drugs, Bel-7402/5-FU exhibited cross-resistance.

3.2. Baicalin increased cytotoxicity of Bel-7402 and Bel-7402/5-FU cells

MTT assays were detected to evaluate the impact of baicalin on cell viability of cells. As shown in Fig. 2, baicalin inhibited cell viability. Baicalin inhibited the viability of both Bel-7402 and Bel-7402/5-FU cells across all tested concentrations. At concentrations exceeding 80 μM, Bel-7402/5-FU cells exhibited greater sensitivity compared to parental Bel-7402 cells. Parental Bel-7402 cells showed viabilities of 83.58% ± 1.60% at 80 μM and 75.33% ± 0.94% at 160 μM, while Bel-7402/5-FU cells demonstrated 80.42% ± 1.31% and 71.68% ± 1.23% viability at corresponding concentrations. Furthermore, baicalin decreased cell viability of Bel-7402/5-FU cells in a way that was dependent on both time and dose. Cell viability showed slight inhibition at 24 h, significantly decreased by 48 h, and reached the lowest level after 72 h of baicalin at various concentrations.

3.3. Baicalin inhibited drug efflux of Bel-7402/5-FU cells

An important index to evaluate drug resistance is the function of drug efflux pump. As shown in Fig. 3A and C, and Fig. 3B and D, compared to Bel-7402 cells, the intracellular accumulation of doxorubicin and Rho123 was decreased in Bel-7402/5-FU cells, suggesting that the MDR cells promoted drug efflux, but baicalin inhibited the efflux pump and significantly increased the buildup of doxorubicin and Rho123 in Bel-7402/5-FU cells, respectively (both $P < 0.05$). The ABC

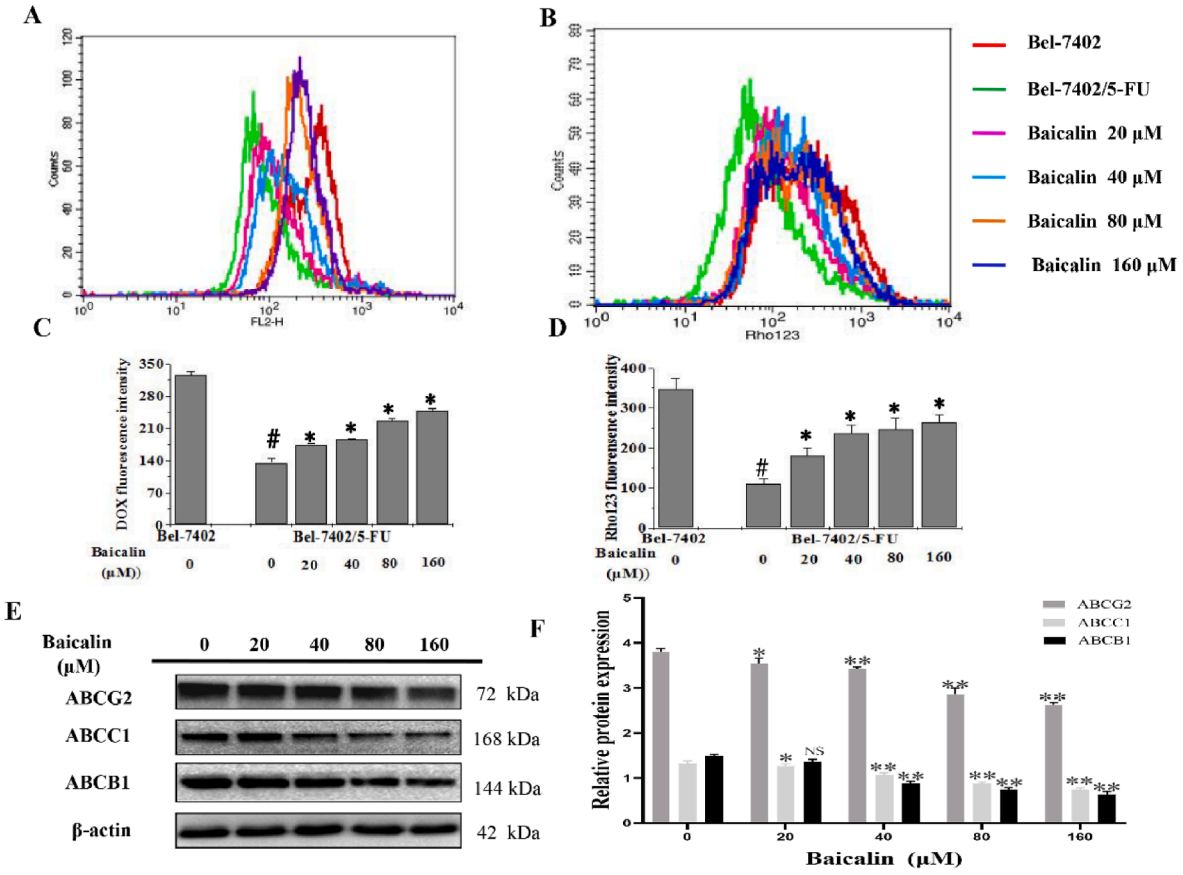


Fig. 3. Baicalin inhibited drug efflux of Bel-7402/5-FU cells. Flow cytometry measured the accumulation of DOX (A) and Rho123 (B). Mean fluorescence intensity of DOX (C) and Rho123 (D) quantitative analysis. (E) Representative Western blot image. (F) Quantitative analysis of drug resistance-related protein expressions. $^{\#}P < 0.05$, compared to the Bel-7402 cells group; $^{*}P < 0.05$, $^{**}P < 0.001$, compared to untreated Bel-7402/5-FU cells. Mean ± SD, $n = 3$.

transporter family (e.g., P-gp/ABCB1, BCRP/ABCG2, MRP1/ABCC1) reduces intracellular drug accumulation and diminishes therapeutic efficacy to mediate MDR. Baicalin decreased the expression of ABCG2 and ABCC1 at 20 μM ($P < 0.05$). At concentrations exceeding 20 μM markedly reduced the expression of efflux-related proteins ABCB1, ABCC1, and ABCG2 (all $P < 0.05$) as shown in Fig. 3E and F.

3.4. Baicalin promoted cell apoptosis of Bel-7402/5-FU

Following the manufacturer's protocol, baicalin's impact on Bel-7402/5-FU apoptosis was assessed. According to Fig. 4A, various quadrants displayed living cells (lower left), damaged cells (upper left), early apoptotic cells (lower right), and late apoptotic cells (upper right). Therefore, the sum of early and late apoptosis can be used to calculate the amounts of apoptotic cells. As shown in Fig. 4 A–C, Bel-7402/5-FU treated at various doses of baicalin had a higher apoptosis rate in contrast to control group ($P < 0.05$). Bax was closely related to cell apoptosis. As shown in Fig. 4D and E, the protein expression of Bax increased by 1.91-fold (20 μM), 3.29-fold (40 μM), 4.38-fold (80 μM), and 5.29-fold (160 μM) compared to the control group ($P < 0.05$ for all concentrations), demonstrating baicalin's potent apoptosis-inducing activity ($P < 0.05$).

3.5. Baicalin inhibited cell autophagy of Bel-7402/5-FU

The quantity of fluorescent cells can be determined by flow cytometry using the Cyto-ID autophagy detection kit, which selectively marks accumulated autophagic vesicles as fluorescent. As shown in Fig. 5A and B, baicalin treatment promoted cell autophagy of Bel-7402/5-FU. In addition, baicalin regulated autophagy-related protein expression. According to Fig. 5C–F, LC3-II/LC3-I ratio and Beclin-1 were upregulated. In comparison with the untreated control cells, LC3-II/LC3-I ratio increased by 12.86-fold at 40 μM, 20.43-fold at 80 μM, and 43.86-fold at 160 μM. Meanwhile, Beclin-1 increased by 1.22-fold (40 μM), 1.54-fold (80 μM), and 1.89-fold (160 μM), $P < 0.05$ for all concentrations. However, p62 was reduced to a certain extent, compared to control ($P < 0.05$).

3.6. Baicalin suppressed the activation of PI3K/AKT pathway in Bel-7402/5-FU cells

We detected the activation of PI3K/AKT by Western blot analysis. According to Fig. 6A and B, baicalin inhibited the expression of PI3K protein in the cells, and p-AKT/AKT ratio was down-regulated at 40 μM and 80 μM concentrations ($P < 0.05$). Compared with untreated control cells, baicalin treatment induced downregulation of PI3K expression, showing 0.86-fold suppression at 20 μM, which progressively intensified to 1.65-, 1.76-, and 3.96-fold reductions at concentrations of 40, 80, and 160 μM, respectively ($P < 0.05$). To validate baicalin's function on PI3K/AKT signaling pathway, we selected an 80 μM concentration of baicalin to treat cells according to the experimental results and activated PI3K/AKT signaling pathway using agonist 740Y-P. Compared to the control group, the levels of phosphorylation of PI3K and p-AKT/AKT ratio in agonist 740Y-P group were significantly increased ($P < 0.05$). Compared to the 740Y-P group, co-treatment with baicalin suppressed the 740Y-P induced increases, reducing PI3K protein expression and the p-AKT/AKT ratio to levels 2.39-fold and 2.18-fold lower, respectively. According to

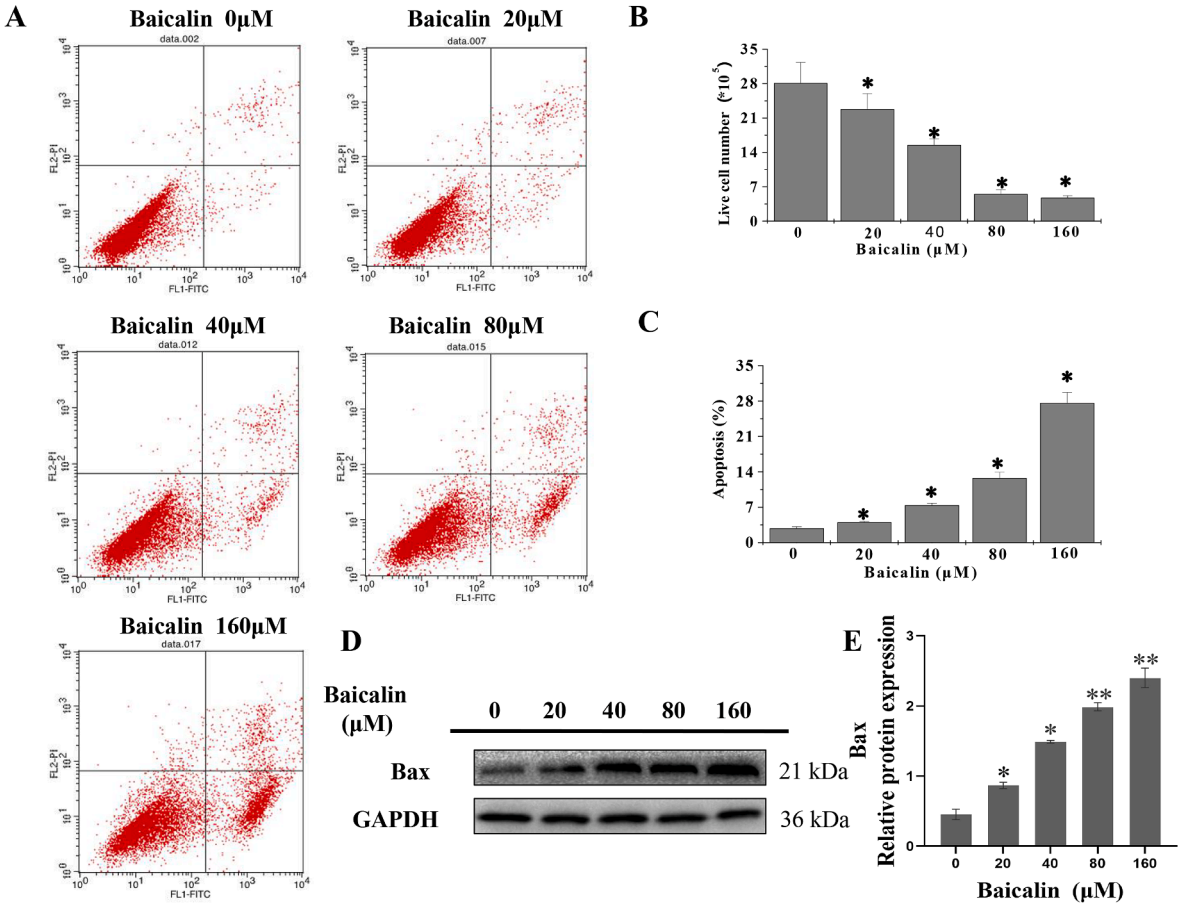


Fig. 4. Baicalin induced cell apoptosis of Bel-7402/5-FU. (A) Apoptosis was detected by flow cytometry. (B) Live cell number. (C) Quantification of fluorescence activated cell sorting (FACS) analysis. (D) Representative Western blot image. (E) Quantitative analysis of Bax protein expressions. *$P < 0.05$, **$P < 0.001$, compared to untreated control cells. Mean ± SD, $n = 3$.

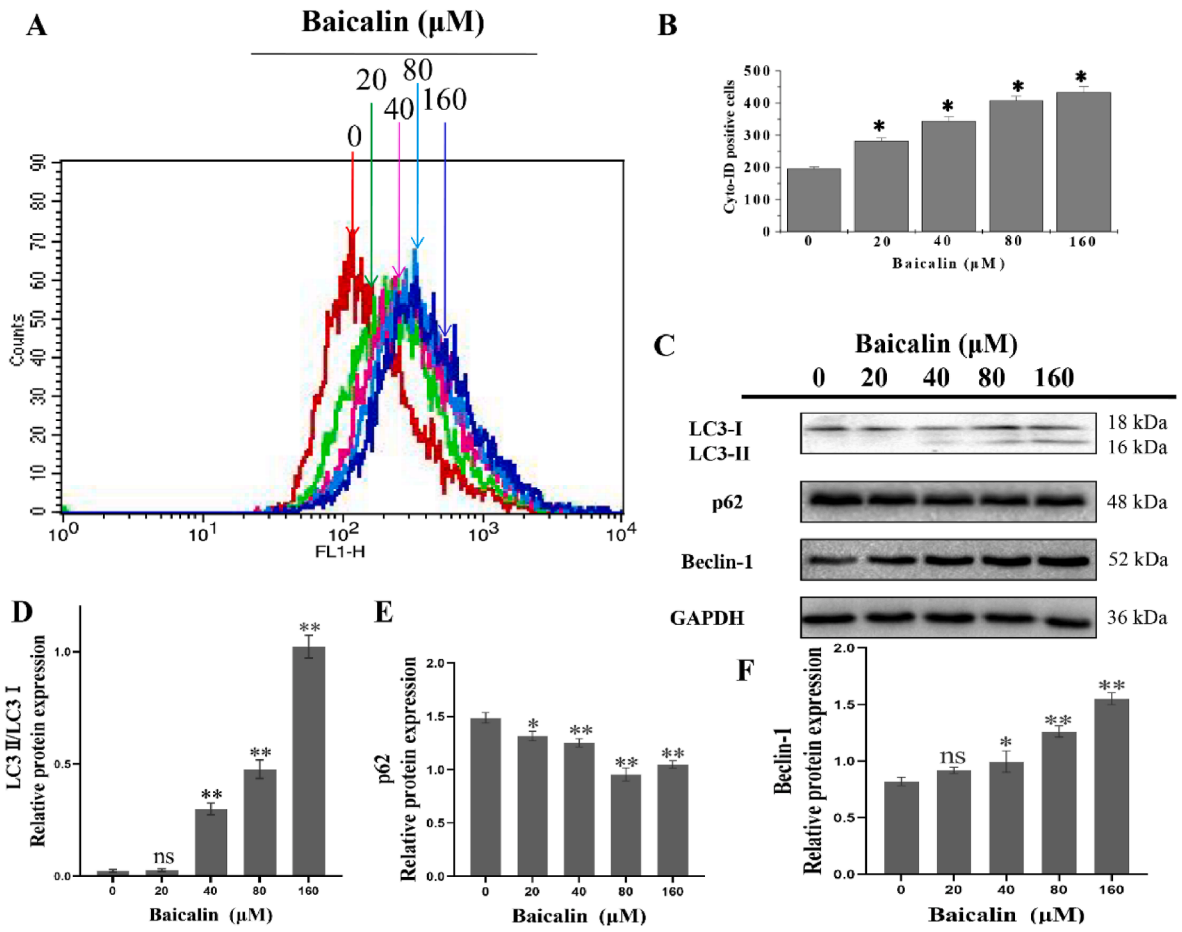


Fig. 5. Baicalin induced cell autophagy of Bel-7402/5-FU. (A) Autophagy was detected by flow cytometry. (B) Quantification of FACS analysis. (C) Representative Western blot image. Quantitative analysis of LC3 (D), p62 (E), and Beclin-1 (F) protein expressions. Mean ± SD, $n = 3$. Compared to untreated control cells, $^{*}P < 0.05$, $^{**}P < 0.001$.

these findings, baicalin may prevent Bel-7402/5-FU cells from activating PI3K/AKT ($P < 0.05$).

3.7. Baicalin reverses drug resistance via PI3K/AKT inhibition and enhanced apoptosis/autophagy

We used Western blot to check the levels of Bax protein, the LC3-II/LC3-I ratio, and ABCG2 protein. Fig. 7A shows that Bax protein was weakly expressed in Bel-7402/5-FU cells. Compared to untreated Bel-7402/5-FU cells, those treated with 740Y-P showed lower Bax expression ($P > 0.05$), while cells treated with baicalin had much higher Bax levels ($P < 0.05$). Additionally, when baicalin was applied to Bel-7402/5-FU cells treated with the agonist 740Y-P, it still significantly increased Bax expression ($P < 0.05$). As shown in Fig. 7B, compared to untreated Bel-7402/5-FU cells, those treated with 740Y-P showed a lower LC3-II/LC3-I ratio ($P > 0.05$), while baicalin treatment significantly increased the LC3-II/LC3-I ratio ($P < 0.05$). As shown in Fig. 7C, compared to untreated Bel-7402/5-FU cells, 740Y-P increased ABCG2 protein expression ($P > 0.05$), while baicalin treatment significantly decreased ABCG2 protein levels ($P < 0.05$). Together, these results show that baicalin reverses drug resistance by inhibiting the PI3K/AKT signaling pathway, promoting apoptosis and autophagy in Bel-7402/5-FU cells.

4. Discussion

Hepatocellular carcinoma is among the most malignant digestive tumors, and chemotherapy is an important therapeutic strategy, but long-term use of chemotherapeutic drugs is likely to lead to decreased sensitivity of HCC to chemotherapeutic drugs, which may lead to drug resistance or even multidrug resistance, and the bad prognosis of the patients will result from the influence of the therapeutic effect. Baicalin is the primary active ingredient of *Scutellaria baicalensis* Georgi. Research indicated that baicalin could induce autophagy and apoptosis of tumor cells.[14] Nevertheless, the molecular mechanism of baicalin reversing MDR in HCC has not been reported. Therefore, we detected the reversal impact of baicalin on Bel-7402/5-FU in research. In our research, the RI of Bel-7402/5-FU for 5-fluorouracil, doxorubicin, and DDP were 7187.46, 125, and 2.58, which means that the cells are multidrug resistant, suggesting they exhibit cross-resistance to various chemotherapeutic drugs.

Tumor MDR is generated through multiple mechanisms, and one of the primary mechanisms is the ABC family-mediated drug efflux pump.[15] Drug efflux reduces the accumulation of chemotherapeutic drugs in cells, which affects the therapeutic effect. The breast cancer resistance protein (BCRP/ABCG2), P-glycoprotein (P-gp/ABCB1), and multidrug resistance protein (MRP1/ABCC1) are considered to be crucial drug efflux transporters.[16] Studies have demonstrated that baicalin can directly inhibit the transport activity of the drug transporters BCRP and MRP3,[17] suggesting its potential role as a competitive inhibitor that interacts with drug-binding sites. In this study, we detected how baicalin affected the drug effluent in Bel-7402/5-FU. DOX is among the first-line chemotherapy drugs, and it has the ability to get through the cell membrane and accumulate in the cells with red fluorescence, so it can observe the accumulation of drugs in the cell by detecting the fluorescence intensity of doxorubicin. After baicalin treatment, the fluorescence of DOX was markedly enhanced in cells, indicating a rise in

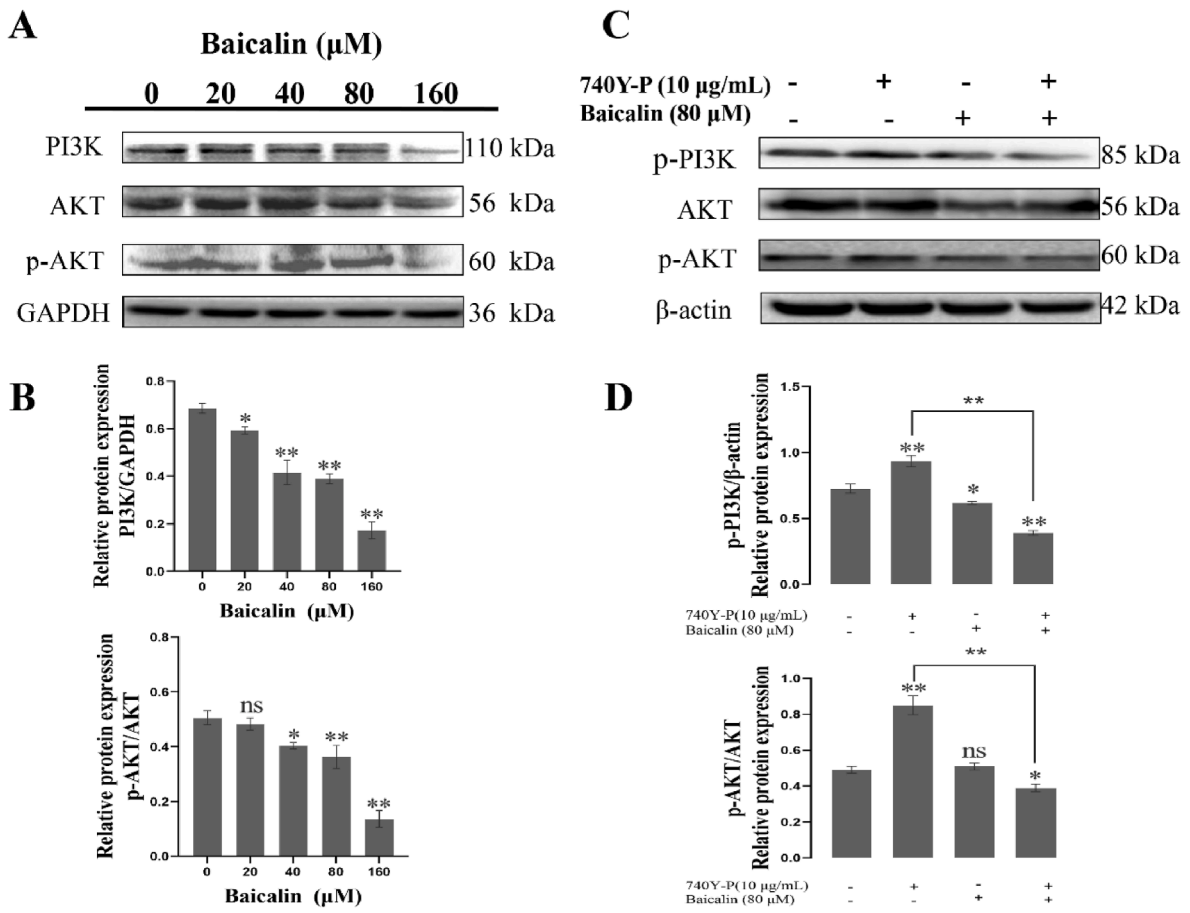


Fig. 6. Baicalin suppressed the activation of PI3K/AKT signaling pathway in BEL-7402/5-FU cells. (A) Western blot images of relative proteins affected by baicalin at varying concentrations. (B) Densitometric analysis of PI3K and p-AKT/AKT expression levels. (C) Representative Western blot images of relative proteins affected by 740Y-P and baicalin. (D) Densitometric analysis of p-PI3K and p-PI3K/AKT expression levels. Mean ± SD, $n = 3$. Compared to the untreated control group, $^{*}P < 0.05$, $^{**}P < 0.001$.

DOX intracellular accumulation and a decrease in efflux. In addition, Rho123 acts as a specific substrate for the drug transporter P-gp, and detecting the fluorescence intensity of intracellular Rho123 can also indirectly reflect the P-gp drug pump's function. This finding indicated that Rho123 accumulated in Bel-7402/5-FU was slightly stronger than that in parental cells. In addition, the accumulation of Rho123 in Bel-7402/5-FU was greatly increased after baicalin treatment, and those results indicated that baicalin inhibited the P-gp's drug pump function in a way that depended on concentration. Meanwhile, the expression of the key protein of drug transport was further detected. Our finding indicated that baicalin reduced the expression of ABCG2, ABCB1, and ABCC1 in Bel-7402/5-FU, suggesting that baicalin can suppress the drug efflux of cells and build up the accumulation of chemotherapeutic agents in cells, thus reversing drug resistance.

Apoptosis is among the primary mechanisms of killing tumor cells by chemotherapeutic drugs; however, it also mediates MDR in tumor cells, leaving them immune to multiple apoptosis-inducing chemotherapeutic drugs.[18] Studies have shown that inducing apoptosis could enhance the chemosensitivity and overcome multidrug resistance in tumor cells.[19,20] When MDR occurs, the proapoptotic BCL-2 family members usually have deregulated.[21] Additionally, Bax is a crucial protein in the process of apoptosis, and it is among the central effects of apoptosis. When Bax is activated by pro-apoptotic proteins, Bax undergoes a conformational change and oligomerizes, forming a pore in the mitochondrial membrane, while BCL-2 normally binds to the pore so that the pore closes.[22] The mitochondrial outer membrane permeabilization (MOMP) is the last typical pathway of cell-intrinsic apoptotic cell death.[23] Upregulation of Bax in cancer caused mitochondrial apoptosis, and overexpression of BCL-2 in tumor would stop apoptosis. Therefore, the BCL-2/Bax ratio can dual modulate apoptosis and drug resistance. Zhao et al.[24] have shown that baicalin could significantly increase Bax expression while downregulating anti-apoptotic Bcl-2, shifting the BCL-2/Bax ratio to favor MOMP and caspase activation in blood-brain barrier. Those results indicated that baicalin significantly enhanced Bax-mediated apoptosis in a dose-dependent manner, which showed that baicalin would regulate Bax and promote mitochondrial apoptosis, and this may be among the crucial mechanisms of baicalin reversing MDR in HCC.

The function of autophagy in regulating cellular homeostasis is critical in stressful environments such as the tumor microenvironment. Methods of targeting autophagy to treat tumors include inhibiting the cellular effects of autophagy and inducing autophagy-mediated cancer cell death.[25] Research has indicated that cell autophagy is strongly related to tumor drug resistance, that autophagy has dual properties of oncogenic and tumor suppressor, and that aberrant autophagic states can promote or inhibit tumor drug resistance.[26] Autophagy prevents the accumulation of chemotherapeutic agents in cells and thus can promote the occurrence of MDR, but autophagy activity in the cellular environment can promote the efficiency of antitumor drugs.[27] Previous studies have shown that autophagic cell death can be caused in MDR cells as a cell death mechanism.[28,29] Therefore, autophagy can also be used as one of the targets of action to enhance the MDR's therapeutic effect. LC3 is involved in formatting the autophagosome membranes, including two mutually transforming forms: LC3-II and LC3-I. The ratio of LC3-II/LC3-I or the LC3-II protein's expression is closely associated with the number

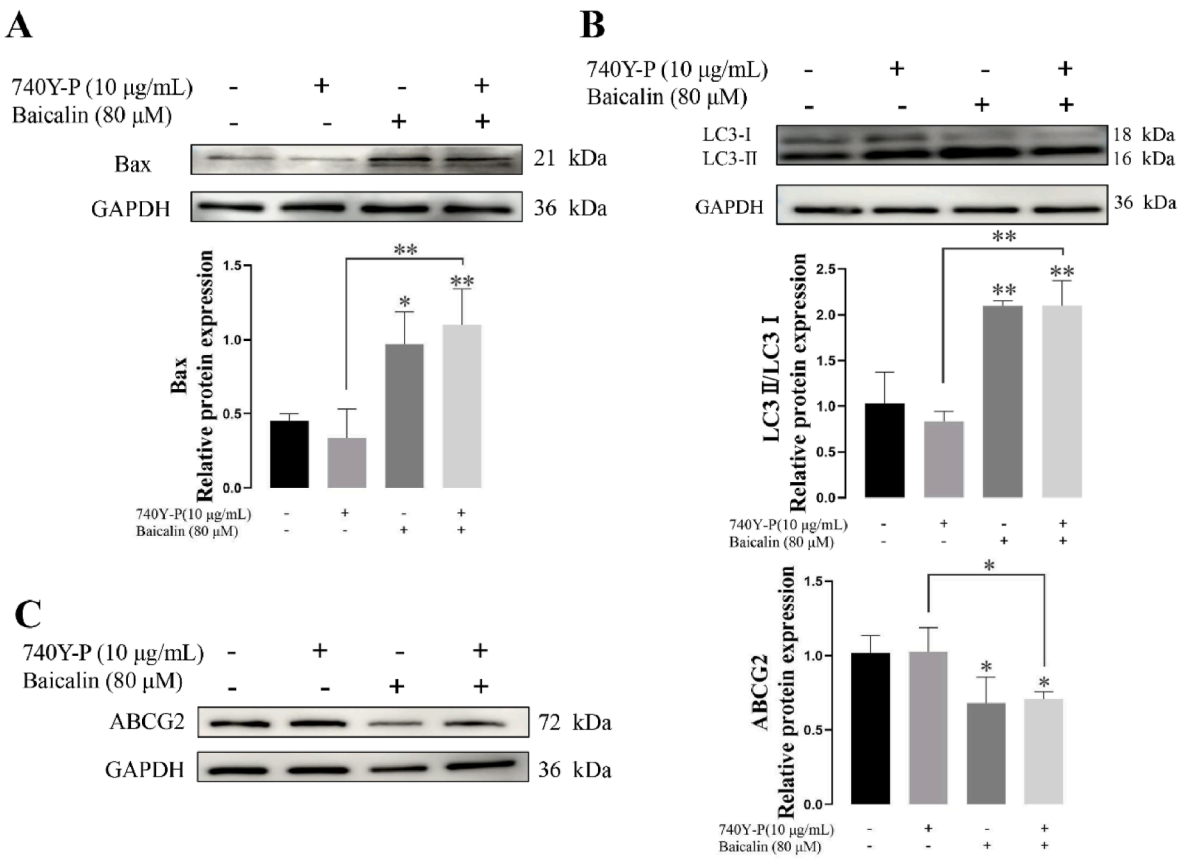


Fig. 7. Baicalin reverses drug resistance via PI3K/AKT inhibition and enhanced apoptosis/autophagy in Bel-7402/5-FU cells. (A) Western blot images of Bax protein and densitometric analysis of its expression levels under different treatments in Bel-7402/5-FU cells. (B) Western blot images of the LC3-II/LC3-I ratio and densitometric analysis of their expression levels under different treatments in Bel-7402/5-FU cells. (C) Western blot images of ABCG2 protein and densitometric analysis of its expression levels under different treatments in Bel-7402/5-FU cells. Mean ± SD, $n = 3$. Compared to the untreated control group, $^{*}P < 0.05$, $^{**}P < 0.001$.

of autophagosomes, which typically respond to cellular autophagy levels.[30,31] The expression level of LC3-II is closely related to tumor drug resistance. In chemotherapy resistance, LC3-II can not only reflect the activation of protective autophagy,[32] but also indicate the malignant progression caused by autophagy deficiency. p62 (sequestosome-1, SQSTM1) is one of the autophagy-selective substrates, binding to LC3 and ubiquitinated substrates, which are then incorporated into autophagosomes and broken down in autophagic lysosomes.[30] Therefore, the expression of p62 decreases when autophagy is induced.[33] Beclin-1 is an autophagy marker protein involved in autophagosome formation process.[34] Beclin-1 is typically reported to be inhibited in HCC, which is considered one of the excellent prognostic markers, as it enhances the chemosensitivity and tumor response to chemotherapeutic treatments.[35] These results indicated that baicalin could increase the intracellular autophagic flux in Bel-7402/5-FU. Furthermore, baicalin would increase the expression of Beclin-1 and LC3-II/LC3-I ratio, but decrease the expression of p62 to a certain extent. Autophagy is a dynamic process. LC3-II/LC3-I and Beclin-1 levels primarily reflect autophagosome formation, while p62 degradation depends on lysosomal activity. The results showed that the baicalin promoted the formation of autophagosomes in Bel-7402/5-FU. However, the expression of p62 was decreased, suggesting autophagic flux is blocked. Increased LC3-II/LC3-I ratio reflects enhanced autophagosome formation, while sustained p62 accumulation indicates impaired lysosomal degradation. These results indicated that baicalin would promote Bel-7402/5-FU cell autophagy. In brief, autophagy may be used as a mode to induce cell death of MDR cells, which can reverse the drug resistance of hepatoma cells.

The PI3K/AKT is among the most commonly activated signaling pathways in human cancer, with activation observed in nearly 50% of HCC patients, which can regulate cell growth, apoptosis, metabolism, and proliferation.[36,37] Notably, the PI3K/AKT pathway serves as a central hub in MDR mechanism by orchestrating crosstalk with multiple downstream targets.[38] Studies found that blocking PI3K/AKT could reverse MDR while also improving cancer cells' sensitivity to chemotherapeutic drugs through upregulating the expression of ABC transporters. It exhibits intricate interactions with both apoptosis and autophagy in MDR. The aberrant activation of PI3K/AKT can suppress chemotherapy-triggered apoptosis by enhancing the activity of anti-apoptotic genes and inhibiting the expression of pro-apoptotic genes.[39] Xu et al. provide compelling evidence that PI3K/AKT pathway inhibition reestablishes the apoptosis-autophagy equilibrium in sorafenib-resistant HCC.[40] Research has shown that blocking PI3K/AKT would induce cell apoptosis and autophagy, which could kill tumor cells.[41,42] The abnormal activation of AKT promotes cell survival via inhibiting pro-apoptotic genes like Bax and stimulating anti-apoptotic genes like Bcl-2, to modulate MDR.[43] Furthermore, targeting PI3K/AKT pathway-mediated autophagy enhances tumors' chemosensitivity.[44] In our study, to clarify whether PI3K/AKT is involved in baicalin-induced autophagy and apoptosis in Bel-7402/5-FU, we detected it at various doses of baicalin. Those results indicated that baicalin of different concentrations could inhibit the expression of PI3K protein and p-AKT/AKT ratio in Bel-7402/5-FU in contrast to untreated group. Those results showed that baicalin could block the activation of PI3K/AKT signaling pathway in cells. In the follow-up experiments, we further activated PI3K/AKT pathway using PI3K agonist(740Y-P), and selected an 80 μM concentration of baicalin combined with the agonist to treat cells. According to the experimental results, the expressions of PI3K and AKT phosphorylation were elevated in the agonist 740Y-P group, suggesting that PI3K/AKT was activated in Bel-7402/5-FU. When 740Y-P was combined with baicalin, PI3K/AKT was inhibited.

We further examined pathway-related proteins. The results showed that activating the PI3K/AKT pathway with the agonist 740Y-P in Bel-7402/5-FU cells reduced Bax protein levels, decreased the LC3-II/LC3-I ratio, and slightly increased ABCG2 expression, indicating that 740Y-P suppressed apoptosis and autophagy. In contrast, 80 μM baicalin inhibited ABCG2 expression, increased Bax levels, and reduced the LC3-II/LC3-I ratio. When 740Y-P was combined with baicalin, it strongly suppressed ABCG2 expression, promoted Bax expression, and markedly increased the LC3-II/LC3-I ratio. Baicalin suppressed the PI3K/AKT pathway, leading to induced apoptosis and autophagy in Bel-7402/5-FU cells, ultimately reversing drug resistance.

5. Conclusion

In summary, baicalin reverses drug resistance in Bel-7402/5-FU cells by blocking the PI3K/AKT pathway, which promotes apoptosis and autophagy through regulating key proteins. However, whether baicalin can reverse drug resistance by inhibiting the PI3K/AKT pathway to promote apoptosis and autophagy in Bel-7402/5-FU cells under *in vivo* conditions requires further validation.

CRediT authorship contribution statement

Fei Li: Writing – original draft, Formal analysis, Data curation. **Zilin Lan:** Data curation. **Weiwei Jiang:** Formal analysis. **Jianheng Zhou:** Software, Methodology. **Jiumao Lin:** Supervision, Resources, Project administration. **Jinyan Zhao:** Writing – review & editing, Resources.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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