Scutellarein enhances cisplatin-induced apoptotic effects by suppressing the PI3K/AKT-MDR1 pathway in human NPC/HK1 nasopharyngeal carcinoma cells

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Received October 3, 2024; Accepted January 17, 2025

DOI: 10.3892/br.2025.1938

Abstract. The combination of radiotherapy and chemotherapy has significantly improved survival rates for patients with nasopharyngeal carcinoma (NPC). Nonetheless, some patients still experience poor outcomes, potentially due to resistance to cisplatin, a widely used chemotherapeutic agent. Scutellarein, a compound extracted from *Scutellaria baicalensis*, has anticancer properties. In the present study it was examined whether scutellarein could enhance the anticancer effects of cisplatin in NPC cells. The NPC cell line, NPC/HK1, was used in the present study. Morphological assessment, MTT, ELISA, and immunoblotting assays were performed to evaluate cell membrane blebbing, viability, cytokeratin 18 fragment release, and the expression of autophagy and apoptosis markers, respectively. The results demonstrated that the combination of scutellarein and cisplatin increased cell viability inhibition, the number of membrane blebbing cells, the expression of apoptosis markers (cleaved caspase-8, cleaved caspase-7, and cleaved PARP), and the cytokeratin 18 fragment levels compared with treatments with scutellarein or cisplatin alone. Scutellarein also decreased the expression of Beclin 1 and autophagy related 3, which are markers of autophagy triggered by cisplatin. Treatment with the autophagy inhibitor, 3-methyladenine, did not enhance cisplatin-induced viability inhibition and cytokeratin 18 fragment release. Additionally, scutellarein inhibited cisplatin-induced AKT phosphorylation and multidrug resistance protein 1 (MDR1) expression, a protein linked to drug resistance. AKT phosphorylation and MDR1 expression triggered by cisplatin were inhibited by treatment with LY294002, a PI3K/AKT inhibitor. Moreover, treatment with the MDR1 inhibitor, PSC833, inhibited MDR1 expression and increased the cisplatin-induced viability inhibition and cytokeratin 18 fragment release. These findings indicated that scutellarein enhances the anticancer effects of cisplatin by inhibiting the PI3K/AKT-MDR1 signaling pathway in NPC/HK1 cells.

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Key words: scutellarein, cisplatin, nasopharyngeal carcinoma cells, viability, apoptosis

Introduction

Nasopharyngeal carcinoma (NPC) is an epithelial carcinoma originating from the mucosa of the nasopharynx (1). Risk factors for NPC include the consumption of salted fish, preserved and processed foods, tobacco smoking, betel nut use, genetic changes, and Epstein-Barr virus infection (1-3). Although the age-standardized incidence rate (ASIR) of NPC is generally low worldwide (2.12 per 100,000), certain regions, such as Hong Kong, Taiwan, and Singapore, report higher ASIRs than the global average (4,5). Radiotherapy alone or in combination with chemotherapy, a standard treatment approach, has recently been shown to improve patient survival rates (6). However, a subset of patients with NPC still faces poor prognosis due to distant metastases (7). Cisplatin, a commonly used chemotherapy drug for NPC, has been found to cause resistance in NPC cells (8,9), which may be one of the reasons for the adverse effects on some patients.

Plant extracts have shown promise as effective supplements to cisplatin therapy by enhancing its anticancer effects on cancer cells (10-14). For example, fucoidan significantly boosted cisplatin-induced apoptosis in oral cancer cells by inhibiting the PI3K/AKT pathway (10). Similarly, resveratrol, derived from grape peel residue, enhanced cisplatin-induced apoptosis in human hepatoma cells by inhibiting glutamine metabolism (11). Additionally, curcumin increased the antitumor efficacy of cisplatin in bladder cancer cell lines by modulating the ROS-ERK1/2 pathway (12). These examples underscore the potential of plant extracts to improve cisplatin-based treatments by augmenting the apoptotic response in various types of cancer.

Scutellarein, a flavonoid found in *Scutellaria baicalensis* (15), exhibits a wide range of biological activities, including anti-inflammatory, antioxidant, and neuroprotective effects (15). Notably, scutellarein also exhibits significant anticancer properties (16-18). For instance, it can induce apoptosis in human colon cancer cells through a ROS-mediated, mitochondria-dependent pathway (16). Additionally, scutellarein was revealed to promote apoptosis and inhibit proliferation, migration, and invasion in ovarian cancer cells by targeting the EZH2/FOXO1 signaling pathway (17). Furthermore, it has been shown to suppress tumor development *in vivo* (18). Given these promising results, the present study aimed to explore whether scutellarein can further enhance the anticancer effects of cisplatin in NPC cells.

Materials and methods

Materials. Scutellarein, cisplatin, dimethyl sulfoxide (DMSO), 3-methyladenine (3-MA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), PSC833, and LY294002 were obtained from MilliporeSigma. Fetal bovine serum (FBS), phosphate-buffered saline (PBS), and RPMI-1640 medium were sourced from Hyclone; Cytiva.

Cell culture. The NPC/HK1 nasopharyngeal carcinoma cell line (cat. no. iCell-h367), free from mycoplasma contamination, was obtained from Quantum Biotechnology Co., Ltd., Taiwan, R.O.C. Cells were maintained in RPMI-1640 medium supplemented with 10% FBS and cultured at 37˚C with 5% CO_2.

MTT assay. The effects of scutellarein and cisplatin on NPC/HK1 cell viability were evaluated using the MTT assay. Cells were seeded in a 6-well plate at a density of $3x10^5$ cells per well. Upon reaching ~80% confluence, the cells were treated with different concentrations of scutellarein (0.3125, 6.25, 12.5 and 25 μM) or cisplatin (4.15, 8.3, 16.6 and 33.2 μM) for 48 h. For evaluating the combined effects of scutellarein and cisplatin, cells were exposed to 12.5 μM scutellarein, 4.15 μM cisplatin, or a combination of both treatments for 48 and 72 h, respectively. Following treatment, the supernatant was discarded, and 2 ml of MTT reagent (0.5 mg/ml in PBS) was added to each well. After incubation at 37˚C with 5% CO_2 for 4 h, the supernatants were removed, and 1 ml of DMSO was added to each well to dissolve the formazan crystals. Subsequently, 100 μl of the DMSO solution from each well was transferred to a 96-well plate, and the optical density was measured at 570 nm using an ELISA reader (BMG LABTECH). Each assay was conducted in triplicate, and all experiments were repeated at least twice independently.

Morphological assessment. NPC/HK1 cells ($3x10^5$ per well) were seeded into a 6-well plate. Once the cells reached 80% confluence, they were treated without (control) or with 12.5 μM scutellarein, 4.15 μM cisplatin, or a combination of both treatments for 48 h. Cell morphology was subsequently examined and documented using light microscopy (Olympus CK 40; Olympus Corporation). Apoptosis was indicated by the presence of plasma membrane blebbing in the cells (19,20).

ELISA. NPC/HK1 cells were seeded in a 6-well plate at a density of $3x10^5$ cells per well. Once the cells reached approximately 80% confluence, they were treated with 12.5 μM scutellarein, 4.15 μM cisplatin, or their combination for 72 h. Cytokeratin 18 fragment levels in the cell culture supernatants were measured using SimpleStep ELISA kit, Human Cytokeratin 18 Fragment (cat. no. ab254515; Abcam), following the manufacturer's instructions. Each assay was conducted in triplicate, and the entire experiment was repeated at least twice independently.

Immunoblotting assay. Total protein extraction and immunoblotting were carried out as previously described (21). Primary antibodies targeting caspase-8 (cat. no. 9746; 1:1,000), cleaved caspase-8 (cat. no. 9429; 1:1,000), caspase-9 (cat. no. 9502), cleaved caspase-9 (cat. no. 9505), caspase-7 (cat. no. 9492; 1:1,000), cleaved caspase-7 (cat. no. 9491; 1:1,000), cleaved poly (ADP-ribose) polymerase (PARP) (cat. no. 9541; 1:1,000), phosphorylated (p)-AKT (cat. no. 9271; 1:1,000), AKT (cat. no. 9272; 1:1,000), Beclin 1 (cat. no. 3738; 1:1,000), multidrug resistance protein 1 (MDR1) (cat. no. 13342; 1:1,000), and GAPDH (cat. no. 97166; 1:5,000) were purchased from Cell Signaling Technology, Inc. Secondary antibodies conjugated with horseradish peroxidase, including goat anti-rabbit IgG (cat. no. 111-035-144; 1:5,000) and goat anti-mouse IgG (cat. no. 111-035-146; 1:5,000), were obtained from Jackson ImmunoResearch, Inc.

Statistical analysis. Data are presented as the mean ± standard error of the mean. Statistical analysis was conducted using SPSS software (version 17.0; SPSS, Inc.). The one-way analysis of variance followed by Tukey's post hoc test was used for comparisons among multiple groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Scutellarein enhances cisplatin-induced viability inhibition in NPC/HK1 cells. The effects of scutellarein and cisplatin on NPC/HK1 cell viability were first assessed. The cells were treated with various concentrations of scutellarein and cisplatin, respectively. Cell viability was examined using the MTT assay. Scutellarein significantly reduced cell viability at concentrations of 6.25, 12.5, and 25 μM as revealed in Fig. 1A. In addition, cisplatin markedly decreased cell viability at concentrations of 4.15, 8.3, 16.6, and 33.2 μM as shown in Fig. 1B. The IC_{50} values for scutellarein and cisplatin were

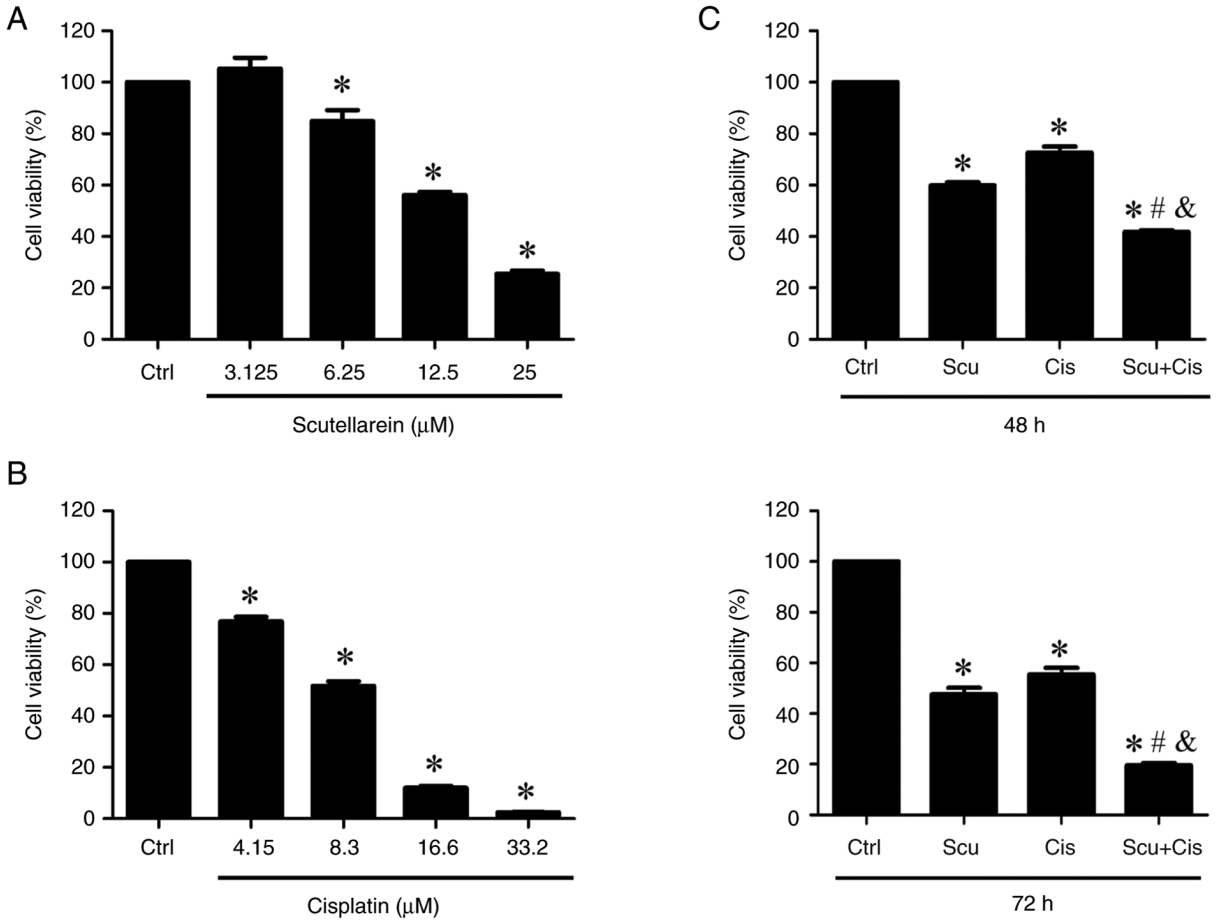

Figure 1. Scutellarein enhances cisplatin-induced viability inhibition in NPC/HK1 cells. NPC/HK1 cells were treated without (Ctrl) or with different concentrations of (A) scutellarein and (B) cisplatin. (C) NPC/HK1 cells were treated without (Ctrl) or with 12.5 μM scutellarein, 4.15 μM cisplatin, or their combination for 48 and 72 h. Cell viability was assessed using the MTT assay. Statistical significance was indicated as follows: *P<0.05 vs. Ctrl; $^{\#}$P<0.05 vs. Cis alone; and $^{\&}$P<0.05 vs. Scu alone; n=3. Ctrl, control; Scu, scutellarein; Cis, cisplatin.

revealed to be 23.5 and 12.8 μM, respectively. Subsequently, the combined effects of scutellarein and cisplatin (12.5 μM scutellarein plus 4.15 μM cisplatin) on NPC/HK1 cell viability were examined. The results of the MTT assay revealed that the combination of scutellarein and cisplatin significantly reduced cell viability more effectively than scutellarein or cisplatin alone (Fig. 1C), suggesting that scutellarein enhances the efficacy of cisplatin in inhibiting the viability of NPC/HK1 cells.

Scutellarein enhances cisplatin-induced apoptotic effects in NPC/HK1 cells. Next, it was investigated whether the combined treatment of scutellarein and cisplatin enhances apoptotic effects. Morphological analysis revealed that this combination reduced the number of attached cells and increased the number of cells exhibiting membrane blebbing, a hallmark of apoptosis (19,20) (Fig. 2A and B), compared with treatment with scutellarein or cisplatin alone. The expression of several key apoptotic protein markers, including cleaved caspase-8, cleaved caspase-7, and cleaved PARP (22,23), were also examined using an immunoblotting assay. The results showed that treatment with scutellarein or cisplatin alone slightly increased the levels of these apoptosis markers compared with the control (Fig. 2C). By contrast, the combined treatment markedly elevated the expression of cleaved caspase-8, cleaved caspase-7, and cleaved PARP (Fig. 2C). Additionally, the release of cytokeratin 18 fragments, another indicator of apoptosis (24), was assessed using ELISA. As revealed in Fig. 2D, treatment with scutellarein or cisplatin alone led to a slight increase in cytokeratin 18 fragment release compared with the control. By contrast, the combination of scutellarein and cisplatin significantly enhanced the release of cytokeratin 18 fragments from NPC/HK1 cells compared with either treatment alone. These results indicated that scutellarein enhances the apoptotic effects induced by cisplatin in NPC/HK1 cells.

Inhibition of autophagy does not affect the anticancer effects induced by cisplatin in NPC/HK1 cells. Autophagy is a cellular mechanism that degrades and recycles damaged organelles and proteins (25,26). This process is essential in regulating cancer cell survival or death in response to anticancer treatments (27,28). For example, inhibition of autophagy by autophagy inhibitors can enhance anticancer agent-induced apoptotic effects (10). Therefore, it was investigated whether scutellarein affects autophagy and consequently influences the anticancer effects of cisplatin in NPC/HK1 cells. Beclin 1 and autophagy related 3 (Atg3) are well-established autophagy markers (29). The immunoblotting results demonstrated that cisplatin markedly increased the protein expression of Beclin 1 and Atg3 (Fig. 3A), suggesting that cisplatin induces autophagy in NPC/HK1

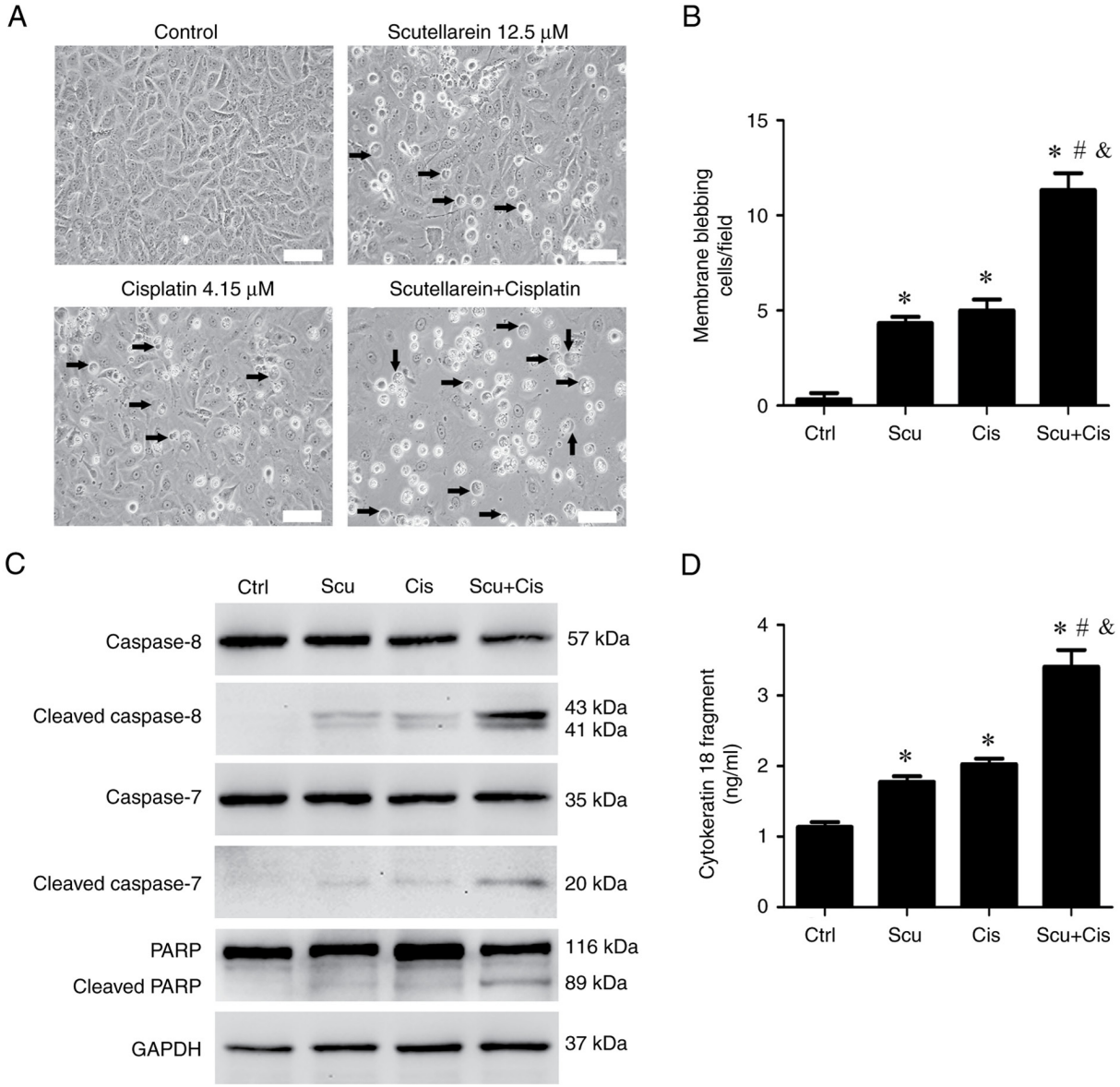

Figure 2. Scutellarein enhances cisplatin-induced apoptotic effects in NPC/HK1 cells. NPC/HK1 cells were treated without (Ctrl) or with 12.5 μM scutellarein, 4.15 μM cisplatin, or their combination for 48 h. (A) The microscopic observations of membrane blebbing cells. Cell morphology was observed using light microscopy (scale bar, 100 μm; arrowheads indicate the membrane-blebbing cells). (B) The average numbers of membrane blebbing cells per field were evaluated. (C) Protein expression of cleaved caspase-8, cleaved caspase-7, PARP, and cleaved PARP was examined using an immunoblotting assay. (D) NPC/HK1 cells were treated without (Ctrl) or with 12.5 μM scutellarein, 4.15 μM cisplatin, or their combination for 72 h. Cytokeratin 18 fragment levels in the cell culture supernatants were measured using ELISA. Statistical significance was indicated as follows: *P<0.05 vs. Ctrl; $^{\#}$P<0.05 vs. Cis alone; and $^{\&}$P<0.05 vs. Scu alone; n=3. Ctrl, control; Scu, scutellarein; Cis, cisplatin.

cells. However, scutellarein treatment markedly inhibited the cisplatin-induced expression of Beclin 1 and Atg3 (Fig. 3A), suggesting that autophagic effects induced by cisplatin were suppressed by scutellarein. To evaluate whether autophagy inhibition could potentiate the anticancer effects of cisplatin, NPC/HK1 cells were treated with 3-MA, an autophagy inhibitor. Then, cell viability and apoptotic effects were assessed using MTT and ELISA assays, respectively. As revealed in Fig. 3B and C, co-treatment with cisplatin and 3-MA did not significantly alter cell viability (Fig. 3B) or cytokeratin 18 fragment levels compared with cisplatin treatment alone (Fig. 3C). These results suggest that autophagy inhibition may not contribute to the anticancer effects of scutellarein-enhanced cisplatin in NPC/HK1 cells.

Scutellarein enhances cisplatin-induced inhibition of cell viability and release of cytokeratin 18 fragments by inhibiting the PI3K/AKT-MDR1 pathway in NPC/HK1 cells. The PI3K/AKT pathway is crucial in advancing cancer progression and mediating cisplatin resistance (29-31). Activation of PI3K/AKT has been reported to upregulate the expression of MDR1 (32,33), a membrane protein that plays a crucial role in reducing the effectiveness of cisplatin (34,35). The immunoblotting results revealed that cisplatin treatment markedly induced the expression of p-AKT (PI3K/AKT activation) and MDR1 compared with the control (Fig. 4A). Co-treatment with scutellarein and cisplatin markedly reduced p-AKT and MDR1 expression compared with cisplatin alone (Fig. 4A), suggesting that scutellarein can inhibit cisplatin-induced

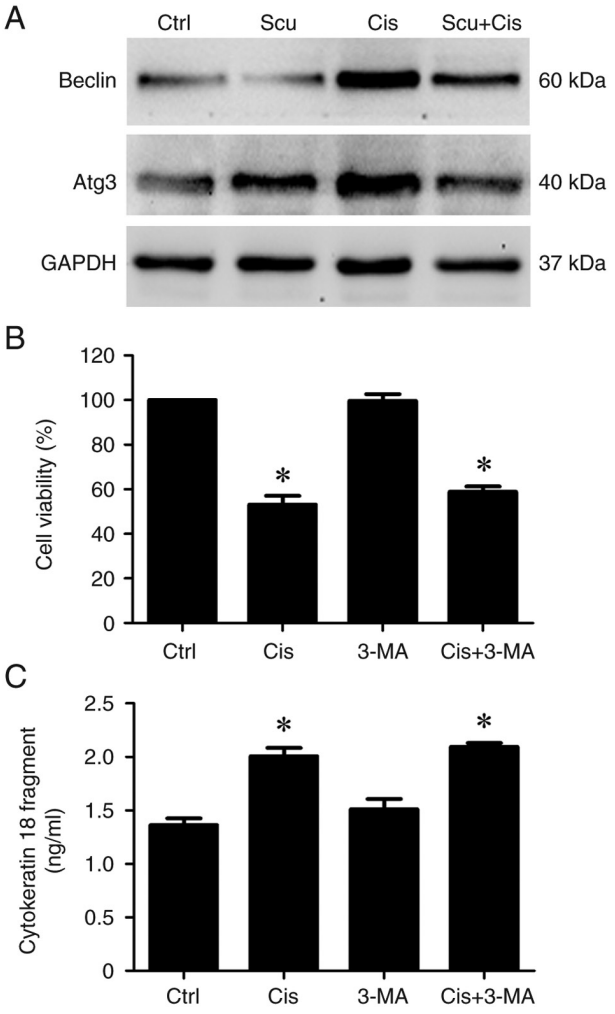

Figure 3. Inhibition of autophagy does not affect the anticancer effects induced by cisplatin in NPC/HK1 cells. (A) NPC/HK1 cells were treated without (Ctrl) or with 12.5 μM scutellarein, 4.15 μM cisplatin, or their combination for 48 h. Protein expression of Beclin 1, Atg3, and GAPDH was examined using an immunoblotting assay. (B) NPC/HK1 cells were treated without (Ctrl) or with 4.15 μM cisplatin, 10 μM 3-MA, or their combination for 72 h. Cell viability was assessed using the MTT assay. (C) Cytokeratin 18 fragment levels in the cell culture supernatant were measured using ELISA. Statistical significance was indicated as follows: *P<0.05 vs. Ctrl; n=3. Ctrl, control; Scu, scutellarein; Cis, cisplatin; Atg3, autophagy related 3; 3-MA, 3-methyladenine.

PI3K/AKT activation and MDR1 expression. To assess whether MDR1 is a downstream target of the PI3K/AKT pathway in NPC/HK1 cells, LY294002, a PI3K/AKT inhibitor, was used. Immunoblotting analysis revealed that LY294002 effectively blocked cisplatin-induced AKT activation and MDR1 expression (Fig. 4B). Furthermore, the results obtained using ELISA showed that LY294002 also enhanced the cisplatin-induced release of cytokeratin 18 fragments (Fig. 4B). These findings indicated that the PI3K/AKT pathway regulates MDR1 expression, which may be involved in the resistance of cisplatin-induced apoptosis. Subsequently, the role of MDR1 in cisplatin-induced anticancer effects was investigated in NPC/HK1 cells. NPC/HK1 cells were co-treated with cisplatin and PSC833, an MDR1 inhibitor. Then, the expression of MDR1, cell viability, and cytokeratin 18 fragment levels were measured using immunoblotting, MTT assay, and ELISA, respectively. The results demonstrated that PSC833 not only reduced cisplatin-induced MDR1 expression (Fig. 4C) but also enhanced cisplatin-induced inhibition of cell viability (Fig. 4C) and increased the release of cytokeratin 18 fragments (Fig. 4C). These findings indicated that scutellarein may enhance the anticancer efficacy of cisplatin by inhibiting the PI3K/AKT-MDR1 pathway in NPC/HK1 cells.

Discussion

Cisplatin is commonly used in conjunction with radiotherapy in the treatment of NPC (36). However, resistance to cisplatin can develop in some NPC cells (8,9), potentially leading to a poor prognosis for these patients. Previous studies have demonstrated that certain plant extracts, such as fucoidan and curcumin, can enhance the anticancer effects of cisplatin (10,12). The present study investigated whether scutellarein, an extract from *Scutellaria baicalensis*, can similarly improve the efficacy of cisplatin. The MTT assay demonstrated that scutellarein significantly reduced cell viability at concentrations of 6.25, 12.5, and 25 μM (Fig. 1A), reflecting its inherent cytotoxic properties. Cisplatin also reduced cell viability in a dose-dependent manner, with effective concentrations ranging from 4.15 to 33.2 μM (Fig. 1B). Notably, when scutellarein was combined with cisplatin at 12.5 and 4.15 μM, respectively, the combination led to a more substantial reduction in cell viability compared with cisplatin alone (Fig. 1C). This indicates that scutellarein not only has significant anticancer activity on its own but also enhances the cytotoxic effects of cisplatin. These findings suggest that incorporating scutellarein into cisplatin-based treatments could improve therapeutic outcomes for NPC/HK1 cells by boosting the overall efficacy of cisplatin in reducing cancer cell viability.

Scutellarein can increase the number of membrane blebbing cells induced by cisplatin (Fig. 2B) and enhance the cisplatin-induced caspase-8 and caspase-7 activation, PARP cleavage (Fig. 2C), and cytokeratin 18 fragment release (Fig. 2D), suggesting that scutellarein may promote the apoptotic effects of cisplatin in NPC/HK1 cells. Apoptosis can be triggered through extrinsic and intrinsic pathways (22), with caspase-8 and caspase-9 as the initiator caspases for these pathways, respectively (22). The findings of the present study suggest that scutellarein primarily facilitates cisplatin-induced apoptosis through the extrinsic pathway, as indicated by its predominant activation of caspase-8 (Fig. 2C) rather than caspase-9 (data not shown). Caspase-7, an effector caspase-activated downstream of caspase-8 (22,23), plays a critical role in the cleavage of PARP and cytokeratin 18 (22,23,37). The observed increases in PARP cleavage and cytokeratin 18 fragment release suggest a possible role for the involvement of the caspase-8/caspase-7 axis in the enhancement by scutellarein of cisplatin-induced apoptosis.

Autophagy, an essential cellular mechanism for degrading and recycling damaged organelles and proteins (25,26), is crucial in regulating cancer cell survival or death in response to anticancer agents (27,28). The present study investigated whether scutellarein influences autophagy and thereby affects the anticancer efficacy of cisplatin in NPC/HK1 cells. It was observed that cisplatin treatment markedly elevated the levels of autophagy markers Beclin 1 and Atg3 (Fig. 3A), indicating

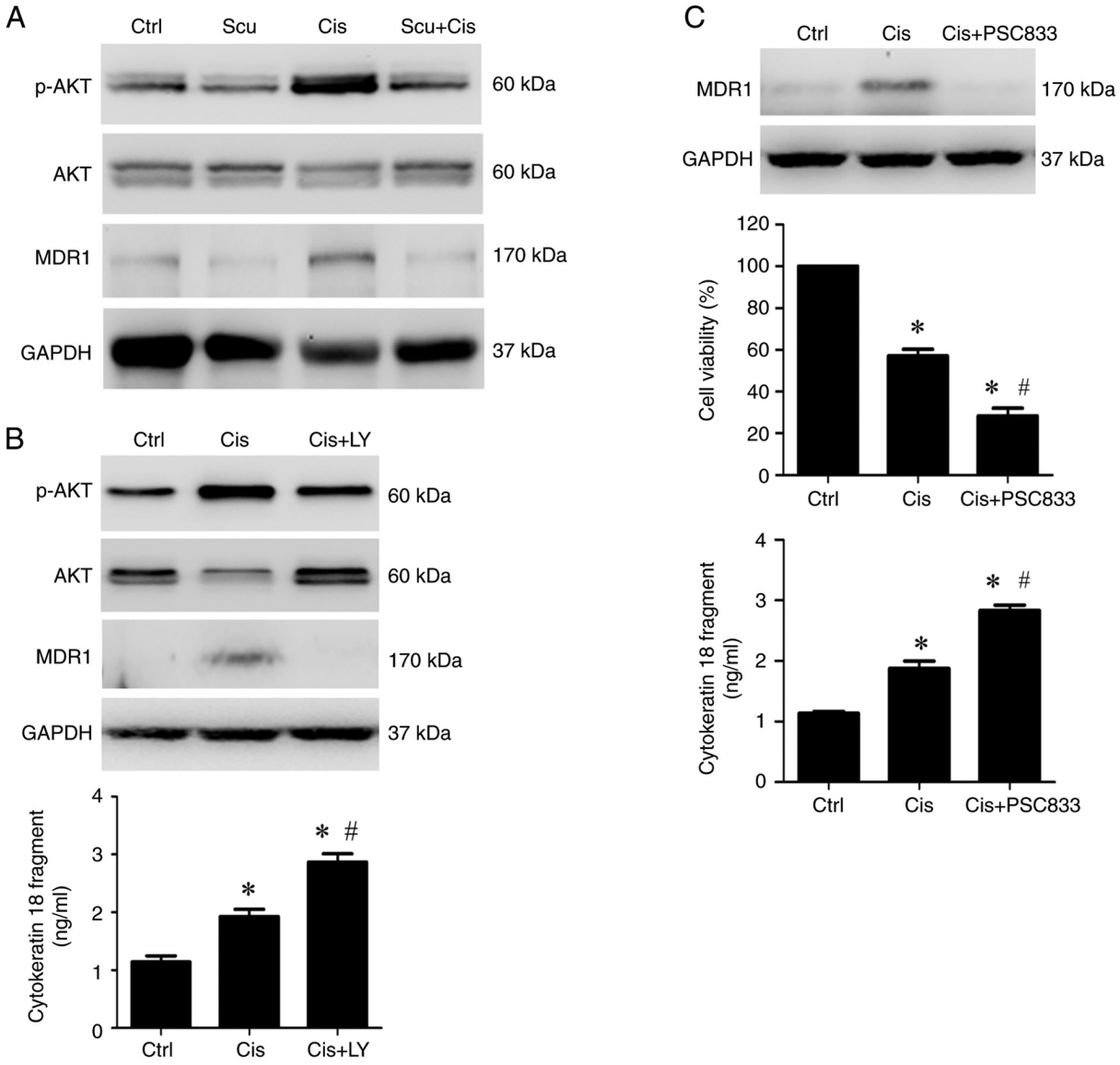

Figure 4. Scutellarein enhances cisplatin-induced inhibition of cell viability and release of cytokeratin 18 fragments by inhibiting the PI3K/AKT-MDR1 pathway in NPC/HK1 cells. (A) NPC/HK1 cells were treated without (Ctrl) or with 12.5 μM scutellarein, 4.15 μM cisplatin, or their combination for 48 h. Protein expression of p-AKT, AKT, MDR1, and GAPDH was examined using an immunoblotting assay. (B) NPC/HK1 cells were treated without (Ctrl) or with 4.15 μM cisplatin, or 4.15 μM cisplatin plus 10 μM LY294002 for 48 h. Upper panel, protein expression of p-AKT, AKT, MDR1, and GAPDH was examined using an immunoblotting assay. Lower panel, cytokeratin 18 fragment levels in the cell culture supernatant were measured by ELISA. (C) NPC/HK1 cells were treated without (Ctrl) or with 4.15 μM cisplatin, or 4.15 μM cisplatin plus 10 μM PSC833 for 48 h. Upper panel, protein expression of MDR1 was examined using an immunoblotting assay. Middle panel, cell viability was assessed using the MTT assay. Lower panel, cytokeratin 18 fragment levels in the cell culture supernatant were measured by ELISA. Statistical significance was indicated as follows: *P<0.05 vs. Ctrl; and $^{\#}$P<0.05 vs. Cis alone; n=3. p-, phosphorylated; MDR1, multidrug resistance protein 1; Ctrl, control; Scu, scutellarein; Cis, cisplatin; LY, LY294002.

an induction of autophagy. Notably, scutellarein inhibited the cisplatin-induced expression of these autophagic markers (Fig. 3A), suggesting that scutellarein disrupts the autophagic response triggered by cisplatin. To assess whether inhibition of autophagy could enhance the anticancer effects of cisplatin, NPC/HK1 cells were co-treated with cisplatin and 3-MA, an autophagy inhibitor. However, the MTT and ELISA assays revealed no significant changes in cell viability (Fig. 3B) or cytokeratin 18 fragment levels (Fig. 3C) compared with cisplatin treatment alone. These results suggest that blocking autophagy with 3-MA does not improve the anticancer efficacy of cisplatin in this cell model. Previous studies have shown that autophagy inhibition can enhance the anticancer effects of cisplatin in lung and ovarian cancer cells (27,38). However, in the present study, autophagy inhibition did not produce the same effect in NPC/HK1 cells. It is suggested that differences in cellular backgrounds may influence whether autophagy inhibition enhances the anticancer efficacy of cisplatin. The PI3K/AKT signaling pathway is a well-established cancer progression driver and a key cisplatin resistance mediator (30-33). This activation of this pathway has been shown to upregulate MDR1 (32,33), a membrane protein that diminishes the effectiveness of cisplatin by exporting it out of the cell. The immunoblotting results support these findings, demonstrating that cisplatin treatment leads to a marked increase in p-AKT and MDR1 expression

(Fig. 4A), highlighting the role of this pathway in mediating cisplatin resistance. Notably, co-treatment with scutellarein and cisplatin resulted in a marked reduction in both p-AKT and MDR1 levels compared with cisplatin treatment alone (Fig. 4A), suggesting that scutellarein effectively inhibits PI3K/AKT activation and subsequent MDR1 expression. Additionally, it was found that treatment with LY294002, a PI3K/AKT inhibitor, inhibited AKT phosphorylation and suppressed cisplatin-induced MDR1 expression (Fig. 4B), indicating that the PI3K/AKT pathway is upstream of MDR1 expression. Furthermore, a previous study has shown that inhibiting MDR1 expression can increase cisplatin sensitivity in bladder cancer cells (34). In the present study, it was also observed that inhibition of MDR1 expression by PSC833 (Fig. 4C), an MDR1 inhibitor, not only enhanced the ability of cisplatin to inhibit cell viability (Fig. 4C), but also increased the release of cytokeratin 18 fragments (Fig. 4C), indicating that inhibition of MDR1 expression also enhances cisplatin sensitivity in NPC cells. The present study revealed that scutellarein enhances the anticancer effects of cisplatin by inhibiting the PI3K/AKT-MDR1 pathway, which may be one of the potential therapeutic strategies for overcoming cisplatin resistance in NPC.

Acknowledgements

Not applicable.

Funding

This study was funded by the National Science and Technology Council (grant nos. MOST-107-2320-B-471-001 and MOST-110-2635-B-214-001), Pingtung Veterans General Hospital (grant nos. PTVGH-E-11305 and PTVGH-11301), E-Da Hospital (grant no. EDPJ110059), and I-Shou University (grant nos. ISU113-IND012-007 and ISU-113-IUC-12).

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

The present study was designed by YTW, LCC, HHW, YJC and YYL. HHW, CXH, CHC, CCT and YL performed all the experiments. Data were collected and analyzed by YTW, LCC and HHW. YTW, LCC, and YYL wrote the initial manuscript. YJC and YYL revised the manuscript. YJC and YYL confirm the authenticity of all the raw data. The final manuscript was read, reviewed and approved by all authors, confirming the accuracy and integrity of the work.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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