Reversal of Cisplatin Resistance in Non-small Cell Lung Cancer through Autophagy Modulation by Ailanthone

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Abstract [**Objectives**] To investigate whether Ailanthone (AIL) could reverse cisplatin resistance in non-small cell lung cancer (NSCLC) by modulating autophagy pathways in A549/DDP cells. [**Methods**] Cisplatin-resistant A549/DDP cells were treated with AIL (0.6 μmol/L), cisplatin (50 μg/mL), or their combination. Cell proliferation was assessed by MTT, EdU and colony formation assays; migration by Transwell and wound healing assays; autophagy markers (P62, LC3B, Beclin1, ATG5) by Western blot; LC3B puncta by immunofluorescence; with rescue experiments using rapamycin. [**Results**] The AIL-cisplatin combination synergistically inhibited proliferation and migration, while downregulating P-gp and MVP. AIL significantly increased P62 accumulation while decreasing LC3B-II, Beclin1 and ATG5. Rapamycin reversed these effects, restoring viability and resistance markers. [**Conclusions**] AIL reverses cisplatin resistance in NSCLC by inhibiting autophagy through P62/LC3B regulation, offering a promising therapeutic strategy for refractory NSCLC.

Key words Ailanthone, Non-small cell lung cancer, Cisplatin resistance, Autophagy

1 Introduction

According to global cancer statistics, lung cancer is the leading cause of cancer-related deaths globally (contributing 18.4% of all cancer-related deaths), causing considerable social strain and economic impact[1-2]. Non-small cell lung cancer (NSCLC) is the most prevalent subtype of lung cancer, comprising 85% of all newly diagnosed cases, with a 5-year survival rate of only 15%[3]. Current clinical management of NSCLC encompasses surgery, radiotherapy, chemotherapy, targeted therapy, and immunotherapy, with combination regimens as the mainstay. Platinum-based agents remain the chemotherapeutic cornerstones[4-5]. Development of chemoresistance compromises therapeutic efficacy and worsens the prognosis of NSCLC, necessitating the elucidation of resistance mechanisms and chemosensitization strategies. Cisplatin resistance is mediated by multifactorial processes, including impaired drug accumulation, enhanced efflux, DNA repair pathway activation, and apoptosis evasion[5]. Recent research has discovered that cisplatin resistance is closely related to autophagy[6]. Autophagy, a conserved catabolic process, mediates metabolic adaptation during development and nutrient deprivation and is, essential for homeostatic maintenance[7]. Autophagy exhibits dual tumor-modulatory roles, suppressing carcinogenesis through oncogenic component degradation in normal cells, while paradoxically sustaining cancer cell survival and inducing chemoresistance during malignancy progression[8].

Ailanthus altissima is a plant from the Simaroubaceae family, and ailanthone (AIL) is a small molecule extracted from its bark. Studies addressing melanoma, leukemia, bladder, liver, gastric, and lung cancers, have found that besides treating inflammation, AIL also exhibits anti-tumor activity, offering strong scientific support for its application in cancer treatment[9]. Furthermore, research indicates that AIL has the potential to reverse tumor drug resistance[9-12]. These discoveries not only enhance the understanding of AIL's antitumor mechanisms but also offer new research directions for overcoming tumor drug resistance.

This study investigated the ability of AIL to reverse cisplatin resistance in NSCLC through modulation of autophagy.

2 Materials and methods

2.1 Cell culture The cisplatin-resistant A549/DDP NSCLC cell line (Shanghai Meixuan Biotechnology Co., Ltd.) was cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 5% antibiotic-antimycotic solution (penicillin-streptomycin) (Biological Industries) under standard conditions (37 ℃, 5% CO_2). To maintain chemoresistance, the cells were exposed periodic 2 μg/mL cisplatin (MCE) every 4 weeks. Prior to the experiments, cells were cultured in drug-free medium for ≥ 7 d to eliminate residual drug effects. AIL (Shanghai Tongtian) was applied at predetermined concentrations.

Experimental groups comprised: control (drug-free), AIL (0.6 μmol/L, 24 h), DDP (50 μg/mL, 24 h), AIL + DDP (co-treatment). Post-treatment samples were subjected to molecular (RT-qPCR/WB) and functional (proliferation/apoptosis) analyses.

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2.2 MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay for cellular viability assessment The 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used to assess the cell resistance and viability after various treatments. Cells were seeded in 96-well plates and treated for 24 h, and then incubated with MTT solution for 4h. After incubation with DMSO lysis buffer, cell viability was determined by measuring the absorbance at 490 nm.

2.3 The 5-Ethynyl-2'-deoxyuridine test 5-Ethynyl-2'-deoxyuridine (EdU) working solution was prepared, EDU labeled, fixed, washed, and permeabilized, and the nuclei were labeled with blue fluorescence (DAPI), photographed, and analyzed using a fluorescence microscope (Olympus).

2.4 Clone formation assay A specific amount of single-cell suspension (1×10^3 cells/well) was grown on 6-well plates to allow the cells to be fully adherent. After washing with PBS (Gibco), different treatments were performed and the cells were stained with 0.25% crystal violet after 14 d. Colonies containing > 50 cells were counted under a microscope.

2.5 Transwell Migration Assay A549/DDP cells (30 000) were seeded in Transwell upper chambers (24-well) with 200 μL RPMI-1640 (1% FBS), while the lower chamber contained 600 μL RPMI-1640 supplemented with 20% FBS to establish a chemoattractant gradient. After 24-h treatment (AIL/DDP/combination), migrated cells were fixed (4% PFA), stained (0.1% crystal violet), and quantified via five random fields/membrane (200 ×).

2.6 Wound healing assay A549/DDP cells (~90% confluent in 6-well plates) were serum-starved in 1% FBS medium, scratched with a sterile pipette tip, and treated with AIL/DDP/combination for 24 h. Wound images (0 h/8 h, 100 ×) were captured, and closure rates (%) were calculated using ImageJ by comparing widths at both timepoints.

2.7 Immunofluorescence analysis Cells were cultured on glass 24-well plates (1.2×10^4 cells/well), fixed in 4% formaldehyde for 30 min, and permeabilized with 1% Triton X-100 in PBS for 10 min at room temperature. The glass pieces were then blocked for 1 h containing 0.05 g BSA powder + 0.125 mL goat serum in 2.5 mL PBS buffer and incubated at 4 ℃ overnight with a rabbit anti-microtubule-associated protein light-chain 3B (microtubule-associated protein light chain 3 B, LC3B (Abcam, 1 μg/mL) monoclonal antibody. After three washes with PBS, the glass pieces were incubated with the secondary antibody for 1 h at room temperature. Finally, DAPI was added for nuclear staining and observed under a fluorescence microscope.

2.8 Western blot Proteins were extracted using RIPA buffer containing PMSF (1 ∶ 100). Lysates (20 μg) were separated via 10% SDS-PAGE, transferred to PVDF membranes, blocked with 5% non-fat milk, and probed with primary antibodies: P-gp (1 ∶ 2 000), MVP (1 ∶ 2 000), MMP-2 (1 ∶ 600), MMP-9 (1 ∶ 800), LC3 (1 ∶ 1 000), p62 (1 ∶ 10 000), Beclin1 (1 ∶ 2 000), ATG5 (1 ∶ 2 000), and β-actin (1 ∶ 10 000). HRP-conjugated secondary antibodies (1 ∶ 5 000) were applied before ECL detection (Bio-Rad).

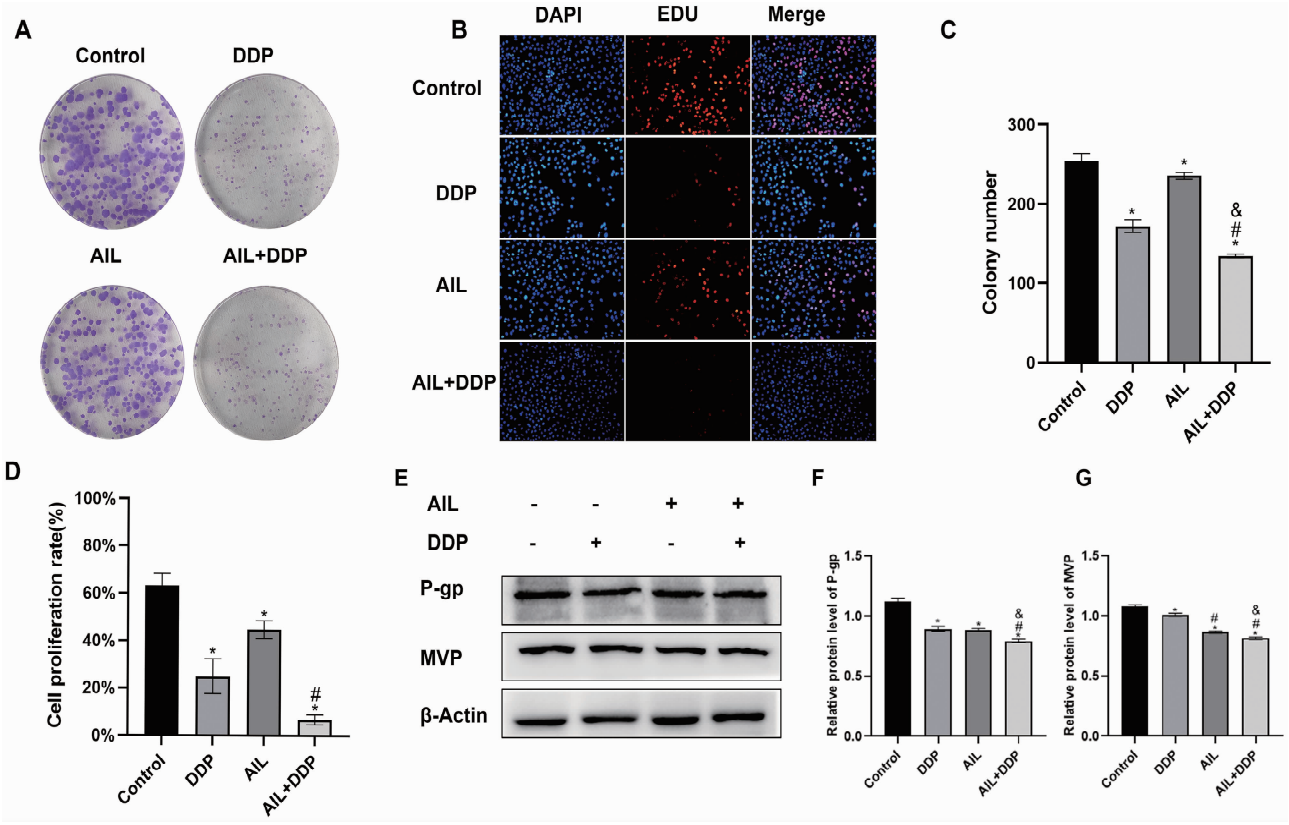
2.9 Statistical analysis All experiments were performed in triplicate. Data analysis used GraphPad Prism 8 (plotting) and SPSS 22 (statistics), with results expressed as Mean ± *SD*. Group comparisons used one-way ANOVA with post-hoc *LSD* or Dunnett *T*3 tests based on normality/homogeneity of variance. Factorial design assessed drug interactions; $P < 0.05$ defined statistical significance.

3 Results and analysis

3.1 AIL suppresses the growth of NSCLC A549/DDP cells and enhances the sensitivity of A549/DDP cells to cisplatin AIL sensitizes cisplatin-resistant A549/DDP cells. Colony formation/EDU assays (Fig. 1A-D) demonstrated synergistic growth/proliferation suppression by AIL + DDP. Western blot analysis (Fig. 1E-G) revealed reduced P-gp/MVP protein levels in AIL-treated groups, with maximal reduction in the combination group. In summary, AIL suppressed the proliferation of A549/DDP NSCLC cells and increased their responsiveness to cisplatin.

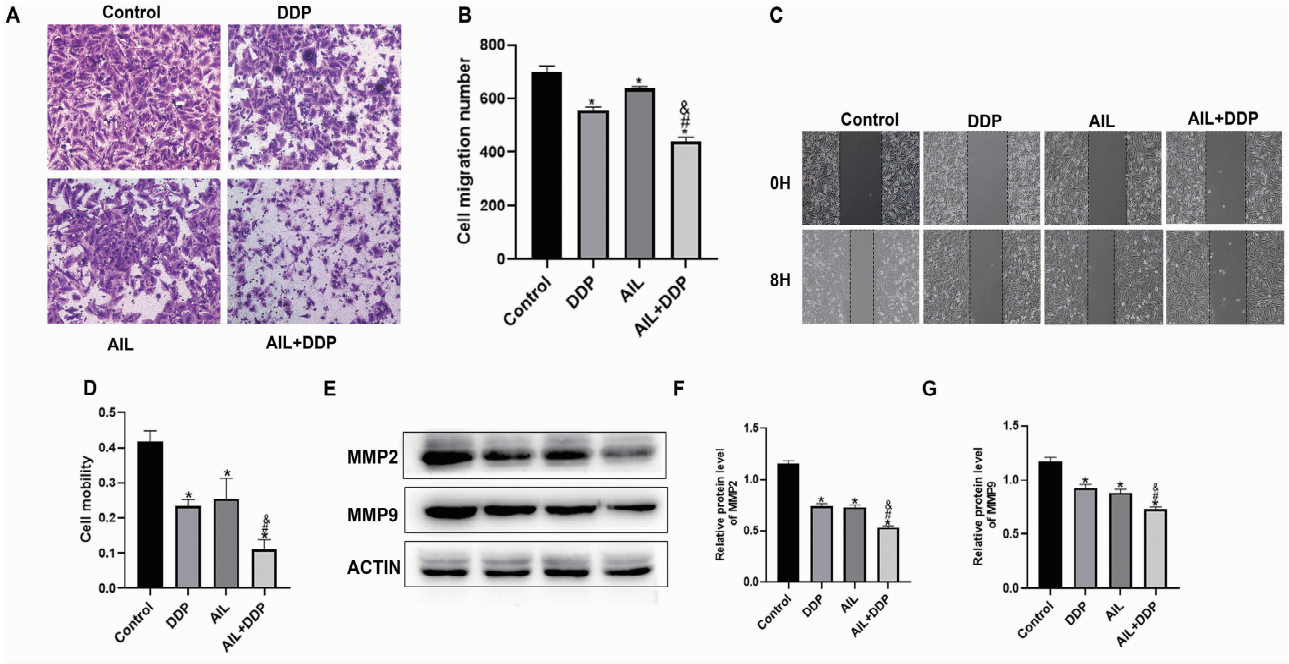
3.2 AIL inhibits the migration of NSCLC A549/DDP cells Transwell and wound healing assays revealed that AIL + DDP synergistically inhibited A549/DDP cell migration, with reduced migrated cell numbers (Fig. 2A-B) and delayed wound closure (Fig. 2C-D). Western blot analysis (Fig. 2E-G) showed combined treatment downregulated MMP2/MMP9 expression, suggesting suppression of metastasis-related proteins. In summary, combined treatment with AIL and DDP not only sensitizes A549/DDP cells to cisplatin but also effectively inhibited their migratory potential, providing a novel mechanism for their anti-tumor effects.

3.3 AIL reverses cisplatin resistance in A549/DDP NSCLC cells through autophagy suppression It is well known that most cancer treatments can induce autophagy, which is closely related to tumor cell survival and cancer drug resistance[13]. AIL reversed cisplatin resistance in NSCLC A549/DDP cells via autophagy inhibition. Western blot (Fig. 3A-B) showed increased p62 and decreased LC3B/Beclin1/ATG5 in AIL/AIL + DDP groups compared with control/DDP. Immunofluorescence (Fig. 3C-D) confirmed reduced LC3B intensity in AIL-treated cells. Recovery experiments (Fig. 3E-H) with rapamycin (autophagy activator) restored cell viability and P-gp/MVP expression in AIL + DDP + RAPA compared with AIL + DDP groups, as shown by MTT/Western blot analysis. In summary, AIL reversed cisplatin resistance in A549/DDP cells by inhibiting autophagy.



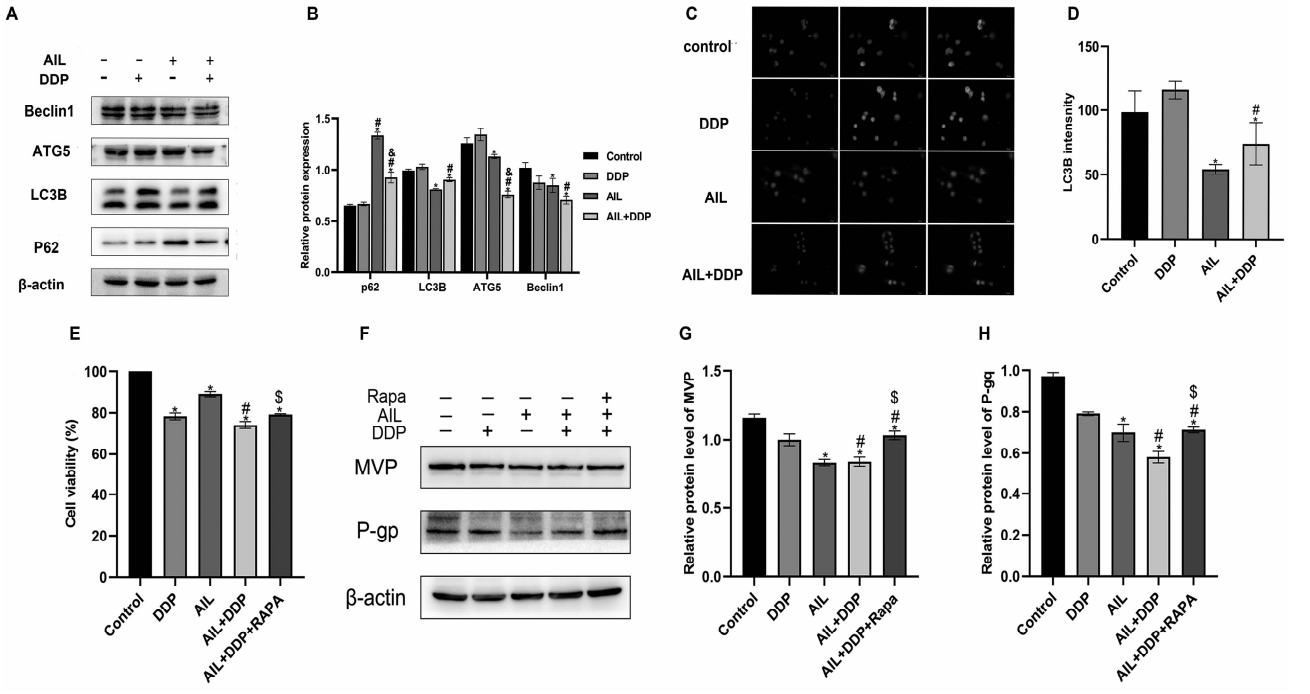
NOTE A. Colony formation assay post-treatment; B. EDU assay for proliferation rates; C-D. Quantification of colony/EDU results; E-G. Western blot analysis and quantification of P-gp/MVP expression after AIL + DDP treatment. ＊: compared with the control group, $P<0.05$; #: compared with the DDP, $P<0.05$; &: compared with AIL, $P<0.05$.

Fig. 1 AIL + DDP inhibits A549/DDP cell proliferation and cisplatin resistance



NOTE A-B. Transwell migration assay and quantification post-treatment; C-D. Wound healing assay and closure rate analysis; E-G. MMP2/MMP9 expression by Western blot and quantification. ＊: compared with the control group, $P<0.05$; #: compared with the DDP, $P<0.05$; &: compared with AIL, $P<0.05$.

Fig. 2 AIL + DDP suppresses A549/DDP cell migration



NOTE A-B. Western blot analysis and quantification of autophagy markers (p62, LC3B, Beclin1, ATG5) post-treatment; C-D. LC3B immunofluorescence intensity and quantification; E. MTT assay showing restored cell viability with rapamycin (autophagy activator); F-H. Western blot analysis and quantification of resistance proteins (P-gp/MVP). *: compared with the control group, $P<0.05$; #: compared with the DDP, $P<0.05$; &: compared with AIL, $P<0.05$.

Fig. 3 AIL + DDP inhibits autophagy and reverses cisplatin resistance in A549/DDP cells

4 Discussion

This study elucidates the critical involvement of AIL in counteracting cisplatin resistance in NSCLC through autophagy regulation, thereby offering novel perspectives for therapeutic resistance reversal strategies.

Current clinical management of non-small cell lung cancer (NSCLC) combines post-surgical radiotherapy with chemotherapy, primarily using platinum-based agents like cisplatin (DDP)[5]. FDA-approved since 1978, DDP remains pivotal for advanced NSCLC, demonstrating early efficacy that diminishes with prolonged use due to rising resistance[14].

Mechanistic studies link DDP resistance to autophagy, a process maintaining cellular homeostasis under physiological conditions but displaying dual roles in cancer. Autophagy adaptively responds to stressors like chemotherapy, exhibiting context-dependent pro-survival or pro-death effects in tumor progression[6]. Autophagy, a regulated cellular process, exhibits dual roles in cancer: suppressing tumor initiation yet sustaining cancer cell survival under stress and mediating therapy resistance via adaptation. Deciphering its regulatory mechanisms, particularly in chemoresistance, is critical for developing targeted oncology therapies[15-16].

To address tumor resistance, researchers increasingly explore mechanisms and traditional Chinese medicine (TCM) as a solution. TCM's multi-target effects may combat tumors, reduce inflammation, alleviate chemotherapy side effects, and improve quality of life. Identifying high-efficacy, low-toxicity agents to enhance chemotherapy sensitivity remains crucial. Notably, TCM monomers like AIL demonstrate potent anti-tumor activity. For instance, Yang[17] found that AIL can promote apoptosis and autophagy in human vestibular schwannoma cells by downregulating miR-21. Wang[18] showed that AIL could reverse chemotherapy resistance in gastric cancer cells by regulating the expression of P23.

Our prior work showed AIL induces cell cycle arrest and apoptosis in A549/DDP cells, enhancing DDP sensitivity in cisplatin-resistant NSCLC. This study explores how AIL regulates cisplatin resistance mechanisms in these cells.

Our findings indicated that AIL reduced cisplatin resistance in A549/DDP cells. Downregulation of resistance-associated proteins P-gp and MVP by AIL (alone or combined with cisplatin) suggests its potential to reverse resistance, though underlying mechanisms require further investigation.

Studies have shown that AIL can induce autophagy in tumor cells such as human promyelocytic leukemia cells HL-60, NSCLC cells, and human vestibular schwannoma cells, thereby inhibiting tumor progression[19-21]. Besides, cisplatin can also induce autophagy in NSCLC cells[21-23]. Autophagy is essential for the survival of NSCLC cells and may lead to chemotherapy resistance. Among autophagy-related markers, Beclin1 is a critical factor involved in autophagosome formation and plays a pivotal role in managing intracellular autophagy activity after binding with ligands[24]. LC3B-II, a structural protein of autophagosomes, attaches to the autophagosome membrane and can be used as a reliable marker for quantifying intracellular autophagy levels[25]. P62, on the other hand, has been confirmed as a substrate of selective autophagy,

and changes in its expression can serve as a sensitive indicator for detecting the degree of autophagy[26]. Additionally, BNIP3L is a key protein that mediates mitophagy. It promotes autophagy by recruiting LC3, interacting with Parkin, and increasing the amount of free Beclin1 in the cytoplasm[27]. To verify the expression of related proteins, we used immunofluorescence to observe LC3B fluorescence intensity after AIL and DDP treatment. Consistent with previous studies, the fluorescence intensity of LC3B was increased in the DDP-treated group. However, after AIL treatment, the fluorescence intensity of LC3B was significantly decreased, indicating that autophagy was inhibited in A549/DDP cells. To further study the effect of AIL on autophagy in A549/DDP cells, this research used Western Blotting was performed to analyze the protein levels of autophagy-related genes in A549/DDP cells. These results indicated that AIL inhibited autophagy in A549/DDP cells. On the other hand, studies have shown that AIL can not only induce autophagy and apoptosis of Lewis cells in non-small cell carcinoma but also affect apoptosis and autophagy in human vestibular Schwannoma cells. Therefore, this study investigated how AIL affects cisplatin resistance in NSCLC and its relationship with autophagy[28-29]. This study explored how AIL affects cisplatin resistance in NSCLC and its relationship with autophagy.

To explore whether AIL regulates autophagy, we added the autophagy activator rapamycin and measured changes in cell viability and drug resistance markers after adding RAPA. These results show that the combined use of AIL and DDP can significantly reduce the expression of autophagy-related markers, indicating that AIL can inhibit autophagy. Additionally, MTT and Western Blot results further confirmed that AIL reverses cisplatin resistance through autophagy, but the exact mechanism remains unknown. These findings enrich our understanding of the mechanism of action of AIL in the treatment of NSCLC and provide strong support for its development as a potential therapeutic drug.

5 Conclusions

This study demonstrates that AIL reverses cisplatin resistance in NSCLC by regulating autophagy in A549/DDP cells, restoring drug sensitivity and exhibiting antitumor effects. While these findings highlight AIL's potential as a novel anticancer agent, limitations include the exclusive use of resistant cells without sensitive counterparts. Future studies should validate these findings in sensitive cell lines and explore additional resistance mechanisms to fully elucidate AIL's therapeutic potential.

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pothesize that Ardisacrispin B may pharmacologically inhibit the development of CRC by regulating 11 correlative biotargets. However, further research is needed to validate this hypothesis.

5 Conclusions

In short, we would like to emphasize that Ardisiacrispin B is a valuable compound for the development of a safe and effective multi-targeted anticancer medicament. Further studies have confirmed its anticancer effect on CRC *in vitro*, and screened 11 correlative biotargets of Ardisacrispin B in relation to CRC. This work provides novel insights into the perspectives and challenges associated with the Ardisiacrispin B research and its clinical application in CRC for future investigations.

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