

Worenine enhances the sensitivity of non-small cell lung cancer A549/DDP cells to cisplatin *via* HIF-1 α /BNIP3-mediated mitophagy

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Abstract. Mitophagy has been implicated in chemoresistance in various cancers. Worenine, an active compound derived from *Coptidis rhizome*, has exhibited anti-tumor activity. We aimed to clarify the impacts of worenine on regulating the cisplatin (DDP) sensitivity and mitophagy in DDP-resistant non-small cell lung cancer (NSCLC) cells. The DDP-resistant NSCLC cell line (A549/DDP) were established *via* exposure to escalating DDP concentrations. Its resistance characteristics were confirmed by CCK-8 and flow cytometry assays. Moreover, the effects of worenine and/or DDP on the A549/DDP cells viability, colony formation abilities, apoptosis, and mitophagy-related proteins were evaluated. The A549/DDP cells were successfully established, with markedly higher IC₅₀ value than A549 cells (25.67 μ M vs. 9.555 μ M). We noted that the HIF-1 α /BNIP3-mediated mitophagy was clearly activated in A549/DDP cells. Worenine decreased A549/DDP cell viability and colony formation abilities, promoted apoptosis, and suppressed HIF-1 α /BNIP3-mediated mitophagy. Importantly, worenine enhanced the responsiveness of A549/DDP cells to DDP. Besides, the suppressive impacts of worenine on A549/DDP cells were reversed by HIF-1 α overexpression. Our findings indicate that worenine may enhance the sensitivity of A549/DDP cells to DDP *via* suppressing HIF-1 α /BNIP3-mediated mitophagy. Targeting mitophagy may be a promising therapeutic strategy for addressing chemoresistance in NSCLC.

Key words: Worenine — Cisplatin — Non-small cell lung cancer — Mitophagy — HIF-1 α

Introduction

Non-small cell lung cancer (NSCLC) is a widespread form of lung cancer with increasing incidence (Slatore et al. 2022). Due to the lack of early clinical presentations, patients with NSCLC are often diagnosed at advanced or metastatic stages, resulting in a poor prognosis (Mithoowani et al. 2022). Chemotherapy in conjunction with new therapies currently represents the leading approach for managing NSCLC. Thereinto, cisplatin (DDP)-based chemotherapy is the most

frequently employed and effective in clinical settings (Chen et al. 2024). However, resistance to DDP is frequently seen in NSCLC patients receiving chemotherapy and related to poor prognosis (Min et al. 2021; Li et al. 2024). Thus, investigating the molecular mechanisms of DDP resistance in NSCLC is essential for developing additional therapeutic strategies for its management.

Coptis chinensis Franch (Goldthread), a traditional Chinese medicine (TCM), is commonly utilized for treating inflammation, diarrhea, and various other ailments (Wu et al. 2019; Xiao et al. 2022). The dried rhizome of *Coptis chinensis* is effective against multiple types of human cancer, including lung cancer, breast cancer, and colon cancer (Kang et al. 2005; Meng et al. 2018). Worenine, an isoquinoline alkaloid, is one of the active compounds derived from *Coptidis rhizoma* (Chen et al. 2010). Previous studies have shown that

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worenine can inhibit various diseases, such as psoriasisiform dermatitis (Lu et al. 2024) and colon cancer (Ji et al. 2021). For examples, Wang et al. (2023) found that the active compounds of *Coptis chinensis*, including worenine, inhibited weight loss, hyperglycemia/dyslipidemia, and significantly alleviated lung inflammation in diabetic mice. Moreover, Ji et al. (2021) indicated that worenine can inhibit colorectal cancer cell growth, proliferation, and cell cycle progression by negatively regulating HIF-1 α . However, the anti-tumor activity of worenine in NSCLC is largely unknown.

Mitochondria, as the cellular powerhouses, present a multifaceted biological landscape in biosynthesis and intracellular/intercellular signaling pathways (Al Amir Dache et al. 2023). They play important roles in control of different pathological processes, especially functions related to cancer growth and metastasis (Al-Faze et al. 2024). Reportedly, most cancer treatments induce mitochondrial dysfunction, leading to the release of ROS and cytochrome c into the cytoplasm, which in turn activates caspase and ultimately triggers apoptosis (Chiu et al. 2020). In this case, mitochondria can be removed by selective autophagy (called mitophagy). Mitophagy is a cellular adaptive mechanism designed to protect cancer cells from treatment-induced mitochondrial apoptosis by removing dysfunctional mitochondria, which eventually contributes to the development of anticancer treatment resistance (Deepak et al. 2024). In particular, commonly used chemotherapeutic agents such as DDP, paclitaxel, and doxorubicin are resistant due to mitophagy, often leading to therapeutic failure (Oun et al. 2018). For example, Cav-1/parkin-mediated mitophagy contributes to DDP resistance in the NSCLC cell line A549, whereas inhibition of Cav-1 restores DDP sensitivity and promotes apoptosis (Liu et al. 2020). Hence, the therapeutic strategy of targeting mitophagy to overcome cancer chemoresistance such as DDP is an encouraging approach and has the potential to significantly increase sensitivity to chemotherapy and improve treatment outcomes (Mukherjee et al. 2023). Recent evidence suggests that HIF-1 α as a major regulator that initiates the transcriptional program of mitophagy and plays a key role in the regulation of mitophagy by controlling the expression of its downstream gene BNIP3, a receptor protein on the outer mitochondrial membrane (Xin et al. 2023). Besides, worenine has been found to inhibit colon cancer cell growth by regulating HIF-1 α expression (Ji et al. 2021). However, the relationship between worenine, HIF-1 α /BNIP3-mediate mitophagy, and DDP resistance in NSCLC is unclear.

Here, we aimed to investigate the impacts of worenine on regulating the DDP sensitivity and mitophagy of DDP-resistant lung cells. Our work will provide a theoretical basis for designing therapeutic strategies to reverse DDP resistance in NSCLC.

Materials and Methods

Cell culture and construction of DDP-resistant cells

NSCLC cell line A549 was obtained from Guangdong Bohui Biological Technology Co., Ltd. (Guangdong, China) and maintained in Ham's F-12K medium (Gibco, USA) containing 1% penicillin/streptomycin and 10% FBS under a 5% CO₂ environment (37°C).

Then, we established DDP-resistant A549 cells (A549/DDP) by a concentration escalation method. DDP (Beyotime) was ultrasonically dissolved in dimethylformamide (DMF, Shanghai Yuanye Biotechnology Co., Ltd., China). A549 cells were subjected to medium supplemented with 0.5 μ M DDP for 48 h. Subsequently, cells were further induced with increasing DDP concentrations, ultimately reaching a concentration of 6 μ M. When the cells were able to survive normally in 6 μ M DDP for 2 months, they were considered resistant. The cells were then incubated in medium with 2 μ M DDP to maintain their DDP-resistant phenotype. A549 and A549/DDP cells were treated with 0, 2, 4, 8, and 16 μ M DDP to evaluate their sensitivity to the drug.

Cell treatment

Worenine (purity > 97%, Shanghai Yuanye Bio-Technology, China) was dissolved in dimethyl sulfoxide (DMSO). Then, 5 μ M (Wor-L), 10 μ M (Wor-M), and 20 μ M (Wor-H) worenine was utilized to treat A549/DDP cells for 24 h to evaluate their effects on A549/DDP cells. Moreover, A549/DDP cells were co-cultured with 4 μ M DDP and 20 μ M worenine to investigate the influence of worenine on their sensitivity to DDP.

Cell transfection

The sequences for HIF-1 α overexpression (oe-HIF-1 α) and corresponding negative control (NC) sequences were designed using the NCBI website. The oe-HIF-1 α and oe-NC plasmids were cloned into pLVX-puro vector, and then transfected into HEK293T cells with Lenti-Mix using Lipofectamine™ 2000 (Invitrogen). A549/DDP cells (2×10^5) were inoculated into a plate (6-well) and then lentiviral particles (1×10^8 U/ml) were introduced to the cells. After 24 h, complete medium with 2.5 g/ml puromycin was utilized to select stable transfected cell lines. Meanwhile, the transfection efficiency was confirmed by Western blot detection of HIF-1 α expression in cells.

CCK-8 assay

After different treatments, A549 and A549/DDP cells were collected, digested, and re-suspended with fresh complete

medium. Cell suspension (100 μ l, 2000 cells/well) was then inoculated into the 96-well plate and maintained in a 37°C, 5% CO₂ incubator. After reaction for 24 h, CCK-8 solution (10 μ l; C0037, Beyotime, Jiangsu, China) was added to each well and an additional 2 h of incubation was conducted. Subsequently, the absorbance (OD450) was measured with DR-3518G microplate reader.

Cell apoptosis detection

After different treatments, the digested A549 and A549/DDP cells were collected for detecting cell apoptosis using Annexin V-FITC kit (C1062M, Beyotime). The apoptotic cells were detected utilizing the CytoFLEX S flow cytometry (Beckman Coulter, Fullerton, CA) and analyzed with CELL Quest software.

Colony formation assay

After 48 h of different treatments, A549/DDP cells were collected, digested, and re-suspended with fresh complete medium. A549/DDP cells (400 cells) were inoculated into the 6-well plate and incubated for 7 days in a 37°C, 5% CO₂ incubator. When visible clones appeared, absolute methanol was applied to fix the cells for 15 min. Following, clones were stained with crystal violet (Beyotime) for 20 min and then the colonies were counted.

Western blot assay

Proteins from cells were collected by RIPA lysis buffer and quantitated by BCA Protein Assay Kit. Samples were separated using 10% SDS-PAGE, followed by transferred onto PVDF membranes. The membranes were incubated overnight at 4°C with the following primary antibodies: HIF-1 α (1:1,000; #36169, Cell Signaling Technology, Danvers, Massachusetts, USA), BNIP3 (1:1,000; ab109362, Abcam, Cambridge, Massachusetts, USA), LC3B I/II (1:2,000; ab192890, Abcam), and GAPDH (1:10,000; ab181602, Abcam). The membranes were subjected to three washes with 1 $\times$ TBST, followed by incubation with secondary antibodies (goat anti-rabbit IgG H&L, 1:10,000; Abcam) at 25°C for 1 h. Using the enhanced chemiluminescence (ECL) kit, the protein bands were detected, and their gray values were determined using the ImageJ software.

Immunofluorescence assay

After different treatments, cells were fixed using 4% paraformaldehyde. They were treated with 0.5% Triton X-100 for 20 min for permeabilization, followed by blocking with normal goat serum for 30 min. Next, the prepared cells were incubated at 4°C with LC3B and Mitotracker antibodies

overnight and subsequent treated with FITC-labeled secondary antibodies for 1 h at 37°C in the dark. Finally, the nuclei were stained with DAPI (C1005; Beyotime) for 5 min. Images were captured using a fluorescent microscope.

Quantitative PCR (qPCR)

Total RNA was isolated from cells utilizing Trizol reagent (Invitrogen, USA) and then reverse transcribed into cDNA using the FastKing-RT SuperMix (Tiangen Biochemical Technology Co., Ltd., Beijing, China). The HIF-1 α mRNA expression in cells was then quantified *via* qPCR with SYBR Green PCR Master Mix (Lifeint, Xiamen, China). The primer sequences for gene amplification were provided as follows: HIF-1 α (128 bp), 5'-GAGCGTTCTCCGAGTGTCAA-3' (forward), 5'-CCTGCCCCCTGTTGTGGTTAT-3' (reverse); GAPDH (72 bp), 5'-CCTGCACCACCAACTGCTTA-3' (forward), 5'-TGAGTCCTTCCACGATACCA-3' (reverse). The relative HIF-1 α mRNA expression was determined utilizing the 2^{- $\Delta\Delta$ CT} method, with GAPDH served as the internal reference.

Data statistics

Statistical analyses were completed using GraphPad Prism 8.0 software. The differences of data (mean $\pm$ standard deviation) between groups were analyzed using one-way ANOVA (multiple groups) or Student's *t*-test (two groups), with *p* < 0.05 as statistical significance threshold.

Results

The DDP-resistant cells were successfully established, and mitophagy was activated in A549/DDP cells

As demonstrated in Figure 1A, DDP at 4, 8, or 16 μ M significantly decreased A549 cell viability and the IC₅₀ for A549 was 9.555 μ M (*p* < 0.01). However, only 16 μ M DDP dramatically reduced A549/DDP cell viability, and the IC₅₀ for A549/DDP cells was 25.67 μ M (*p* < 0.01; Fig. 1B). As expected, after treatment with different concentrations (0, 8, and 16 μ M) of DDP for 24 h, only 16 μ M of DDP significantly inhibited the apoptosis rate of A549/DDP cells compared to A549 cells (*p* < 0.01; Fig. 1C). The data demonstrated that A549/DDP cells exhibited reduced DDP sensitivity compared to A549 cells, confirming the successful establishment of DDP-resistant cells.

To determine whether mitophagy played a role in the DDP resistance, we conducted Western blot assays to examine the BNIP3, HIF-1 α , and LC3B II/I expression. A549/DDP cells exhibited significantly increased levels of BNIP3, HIF-1 α , and LC3B II/I proteins compared to A549 cells (*p* < 0.05;

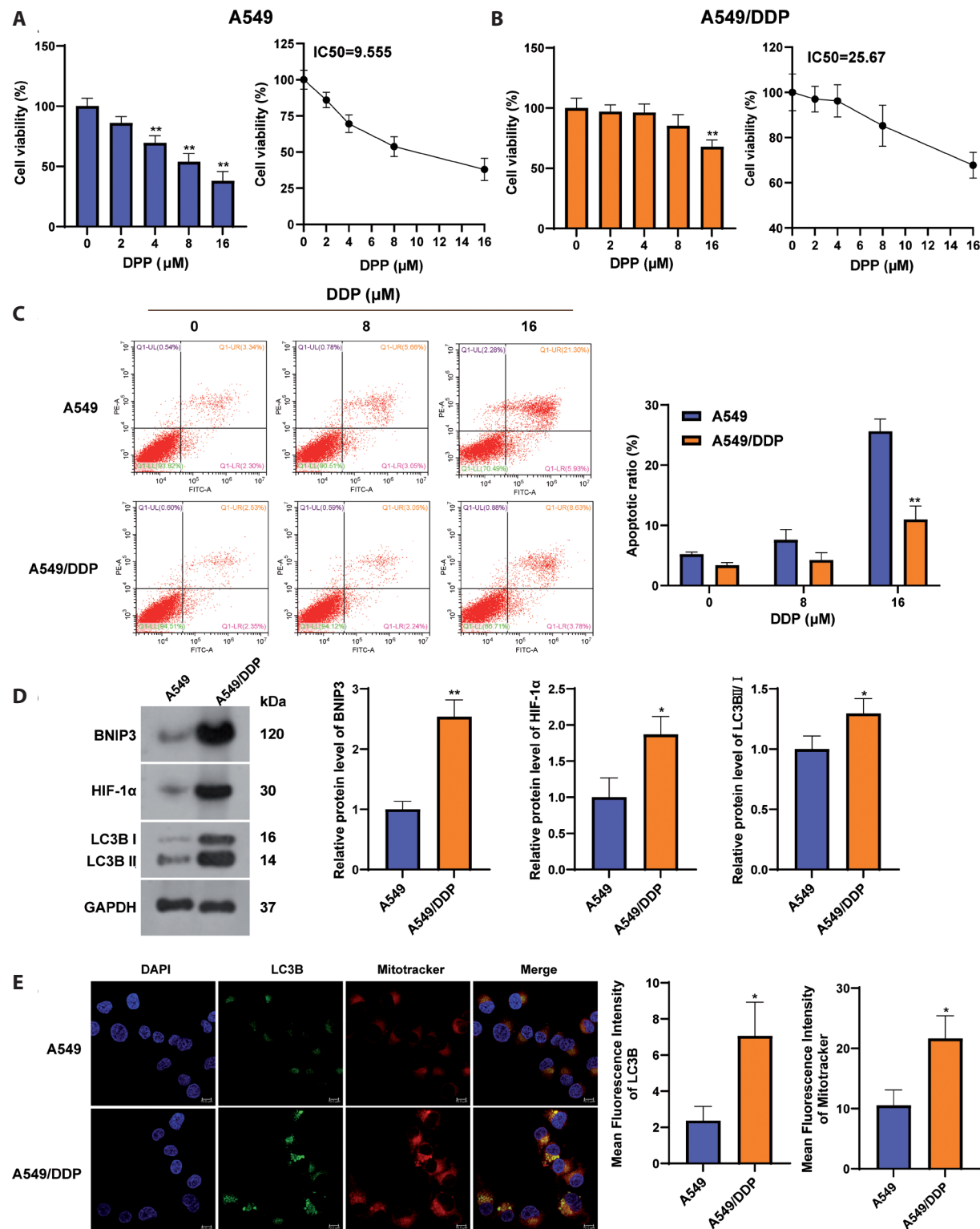


Figure 1. The effects of DDP on the viability, apoptosis, and HIF-1α/BNIP3-mediated mitophagy in A549 and A549/DDP cells. **A.** The viability of A549 cells and the IC₅₀ of DDP in A549 cells. **B.** The viability of A549/DDP cells and the IC₅₀ of DDP in A549/DDP cells. ** $p < 0.01$ compared with control (0 μM DDP). **C.** The apoptosis of A549 and A549/DDP cells after DDP treatment. **D.** Western blot assay showed the BNIP3, HIF-1α, and LC3B II/I protein expression in A549 and A549/DDP cells after DDP treatment. **E.** Immunofluorescence assays revealed the expression levels of LC3B and mitotracker in A549 and A549/DDP cells after DDP treatment. * $p < 0.05$ and ** $p < 0.01$ compared with A549 cells.

Fig. 1D). Besides, immunofluorescence assays revealed that LC3B and mitotracker were elevated expressed in A549/DDP cells ($p < 0.05$; Fig. 1E). These data indicated that the HIF-1 α /BNIP3-mediated mitophagy was activated in A549/DDP cells.

Worenine inhibited A549/DDP cell proliferation, promoted apoptosis, and suppressed mitophagy

After 24 h of worenine treatment, the A549/DDP cell viability was obviously decreased by worenine at concentrations of 10 or 20 μ M ($p < 0.05$; Fig. 2A). Additionally, the HIF-1 α protein expression was remarkably inhibited by 10 or 20 μ M worenine ($p < 0.01$; Fig. 2B), revealing the inhibitory impact of worenine on HIF-1 α expression. Because 20 μ M worenine treatment resulted in less than 50% cell viability, this concentration was selected for subsequent assays. Further functional experiments showed that worenine significantly reduced the colony formation abilities while induced apoptosis rate of A549/DDP cells ($p < 0.05$; Fig. 2C,D). Following, Western blot assay demonstrated that worenine remarkably inhibited the expression of mitophagy-related proteins (BNIP3, HIF-1 α , and LC3B II/I) in A549/DDP cells ($p < 0.05$; Fig. 2E). Immunofluorescence assay also revealed the decreased LC3B and mitotracker expression in A549/DDP cells ($p < 0.05$; Fig. 2F). Overall, worenine suppressed A549/DDP cell proliferation, induced apoptosis, and suppressed mitophagy.

Worenine enhanced the DDP sensitivity of A549/DDP cells

As illustrated in Figure 3A, co-treatment with 20 μ M worenine and 4 μ M DDP led to a significant decrease in A549/DDP cell viability compared to DDP treatment alone ($p < 0.01$). Moreover, the combination of worenine and DDP significantly promoted A549/DDP cell apoptosis compared to treatment with DDP alone or worenine alone ($p < 0.01$; Fig. 3B). These results provide preliminary evidence that worenine can suppress the malignant behavior of A549/DDP cells and may help reverse DDP resistance in NSCLC.

The impacts of worenine on A549/DDP cells were reversed by HIF-1 α overexpression

The HIF-1 α expression was dramatically increased in A549/DDP cells after transfection with oe-HIF-1 α ($p < 0.01$; Fig. 4A). Additionally, compared to worenine treatment alone, co-treatment with 20 μ M worenine and oe-HIF-1 α enhanced A549/DDP cell viability ($p < 0.01$; Fig. 4B) and suppressed apoptosis ($p < 0.05$; Fig. 4C). Furthermore, co-treatment with worenine and oe-HIF-1 α remarkably increased BNIP3, HIF-1 α , and LC3B II/I expression in A549/DDP cells relative

to worenine treatment alone ($p < 0.05$; Fig. 4D). Immunofluorescence assay also revealed the increased LC3B and mitotracker expression in A549/DDP cells after co-treatment with worenine and oe-HIF-1 α in comparison to worenine treatment alone ($p < 0.05$; Fig. 4E). Taken together, the suppressive impacts of worenine on A549/DDP cells were reversed by HIF-1 α overexpression.

Discussion

DDP, as the first-line anti-tumor drug, is extensively utilized to treat various malignancies including NSCLC (Kim et al. 2017). However, the development of chemoresistance remains a major hurdle for its clinical use (Sun et al. 2018; Wang et al. 2021; Lu et al. 2022). Tackling this chemoresistance is the key to enhancing patient survival and overall outcomes. Multiple mechanisms contributing to DDP resistance have been reported, such as DNA repair and inactivation of apoptosis pathways (Fang et al. 2017). Unfortunately, the targeted treatment strategies to reverse DDP resistance are still lacking. In this study, we found that worenine effectively reduced A549/DDP cell proliferation, promoted apoptosis, and suppressed HIF-1 α /BNIP3-mediated mitophagy, and could enhance the DDP sensitivity of A549/DDP cells. Meanwhile, HIF-1 α was identified as an important molecule in this process.

DDP treatment has been shown to elevate the levels of autophagy-related proteins including LC3 in lung cancer cells, indicating the activation of autophagy (Bui et al. 2022). Mitophagy, a mitochondrial-specific autophagy, serves as a crucial mitochondrial quality control mechanism that selectively removes excess or damaged mitochondria through autophagy. Mitophagy plays a dual role in selectively degrading excess or damaged mitochondria (Yan et al. 2018). Mitophagy can enhance the survival of various cancer cells under cytotoxic stress by degrading damaged mitochondria and lowering mitochondrial ROS levels (Yan et al. 2017). Conversely, severely damaged mitochondria may lead to metabolic disturbances and push cancer cells toward autophagic cell death (Villa et al. 2017). Of concern, long-term chemotherapy induces mitosis, which promotes tolerance and confers stem cell properties to tumor cells, ultimately leading to chemoresistance (Visconti et al. 2017). During this period, mitophagy contributes to the induction of cancer drug resistance by selectively removing impaired mitochondria (Kong et al. 2022; Mubthasima et al. 2024). Kong et al. (2022) observed that mitophagy was prominently enhanced in paclitaxel-resistant NSCLC cells, whereas resveratrol treatment enhanced the sensitivity of cancer cells to paclitaxel by reducing mitophagy. Mubthasima et al. (2024) emphasized that EPHA2 knockdown enhanced mitochondrial fusion and reduced mitophagy, effectively strengthen-

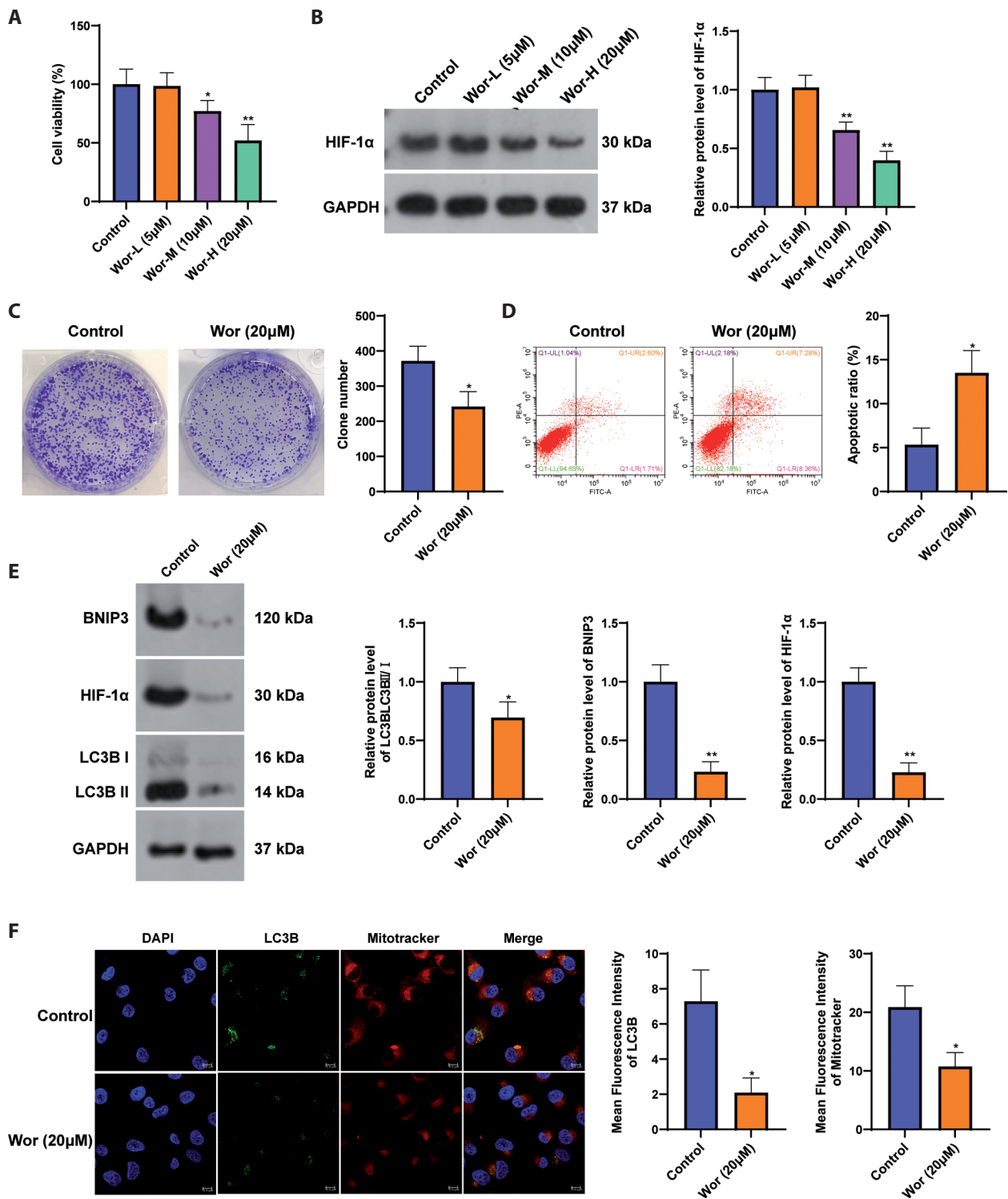


Figure 2. Worenine inhibited A549/DDP cell proliferation, promoted apoptosis, and suppressed mitophagy. The viability of A549/DDP cells (A) and the HIF-1α protein expression (B) after different concentrations of worenine (5, 10 and 20 μM). The colony formation rate (C) and the apoptosis (D) of A549/DDP cells after worenine treatment (20 μM). E. Western blot assay showed the BNIP3, HIF-1α, and LC3B II/I protein expression in A549/DDP cells after worenine treatment (20 μM). F. Immunofluorescence assays revealed the LC3B and mitotracker expression in A549/DDP cells after worenine treatment (20 μM). * $p < 0.05$ and ** $p < 0.01$ compared with control. Wor, worenine.

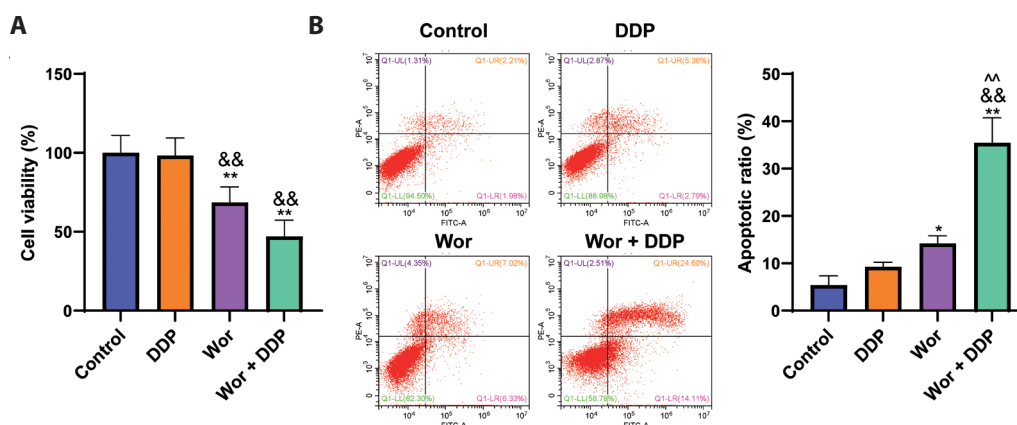


Figure 3. Worenine enhanced the sensitivity of A549/DDP cells to DDP. The effects of 20 μ M worenine and 4 μ M DDP on the viability (A) and on the apoptosis (B) of A549/DDP cells were evaluated. * $p < 0.05$ and ** $p < 0.01$ compared with control; && $p < 0.01$ compared with DDP; ^^ $p < 0.01$ compared with worenine.

ing the sensitivity of cervical cancer to DDP. These studies support that inhibition of mitophagy as a promising strategy to overcome chemotherapy resistance and improve cancer treatment. In addition, numerous studies have revealed that hypoxia remarkably enhances DDP-induced autophagy in a manner reliant on HIF-1, which decreases DDP sensitivity and contributes to resistance (Wu et al. 2015). HIF-1 α -induced autophagy activation leads to chemo/radioresistance in tumor cells (Rohwer et al. 2011; Minassian et al. 2019). Furthermore, BNIP3 is a transcriptional target of HIF-1 and can target mitochondria for autophagosomal degradation (Chourasia et al. 2015). Blocking the HIF-1 α /BNIP3 pathway has been demonstrated to reduce autophagy and enhance the sensitivity to gemcitabine in bladder cancer cells in hypoxic environments (Yang et al. 2018). We observed that the HIF-1 α /BNIP3-mediated mitophagy was activated in A549/DDP cells, confirming the key role of mitophagy in regulating DDP resistance.

Accumulating studies have identified several compounds of TCM, such as shenmai injection (Chen et al. 2021) and asiatic acid (Cheng et al. 2022) for their ability to combat DDP resistance in A549/DDP cells *via* multiple molecular mechanisms. The exploration of active compounds of TCM and their anticancer mechanisms have gained increasing attention. Our results indicated that worenine decreased A549/DDP cell proliferation, promoted apoptosis, and suppressed HIF-1 α /BNIP3-mediated mitophagy, and could enhance the DDP sensitivity of A549/DDP cells. Worenine, a major active component of *Coptidis Rhizoma* has been found to have a similar chemical structure to coptisine (Liu et al. 2016). Research indicates that worenine suppress colon cancer cell proliferation *via* the regulation of HIF-1 α (Ji et al. 2021). Our results also found that overexpression of HIF-1 α diminished the impact of worenine on the prolifera-

tion, apoptosis, and mitophagy of A549/DDP cells. We thus conclude that worenine may boost the DDP sensitivity of A549/DDP cells *via* suppressing HIF-1 α /BNIP3-mediated mitophagy.

This study was the first to demonstrate that worenine can aggrandize the DDP sensitivity of A549/DDP cells, paving the way for new combination therapies that may enhance treatment outcomes for patients with resistant NSCLC. However, this research also has several limitations. First, the use of a single cell line in this research may limit the robustness of our results. Other NSCLC cell lines should be applied to verify our results before reaching a final conclusion. Second, we did not perform *in vivo* assays to clarify the anti-tumor effect and molecular mechanisms of worenine in DDP-resistant contexts. Notably, drug development is a long process that requires many basic experiments from drug discovery to drug entry into clinical trials. In future studies, we will focus on elucidating the specific anti-cancer mechanism of worenine and exploring the optimal dosage and *in vivo* safety of worenine combined with DDP, so as to provide reliable pre-basic support for the clinical application of worenine and thus accelerate its clinical anti-tumor application.

Conclusion

Worenine may improve the DDP sensitivity of A549/DDP cells *via* inhibiting HIF-1 α /BNIP3-mediated mitophagy. Our findings demonstrate the potential of worenine as a complementary treatment for DDP-resistant NSCLC, underscoring the crucial role of mitophagy in chemoresistance. Focusing on mitophagy may offer a promising approach to mitigate chemoresistance in NSCLC and improving the effectiveness of current chemotherapy regimens.

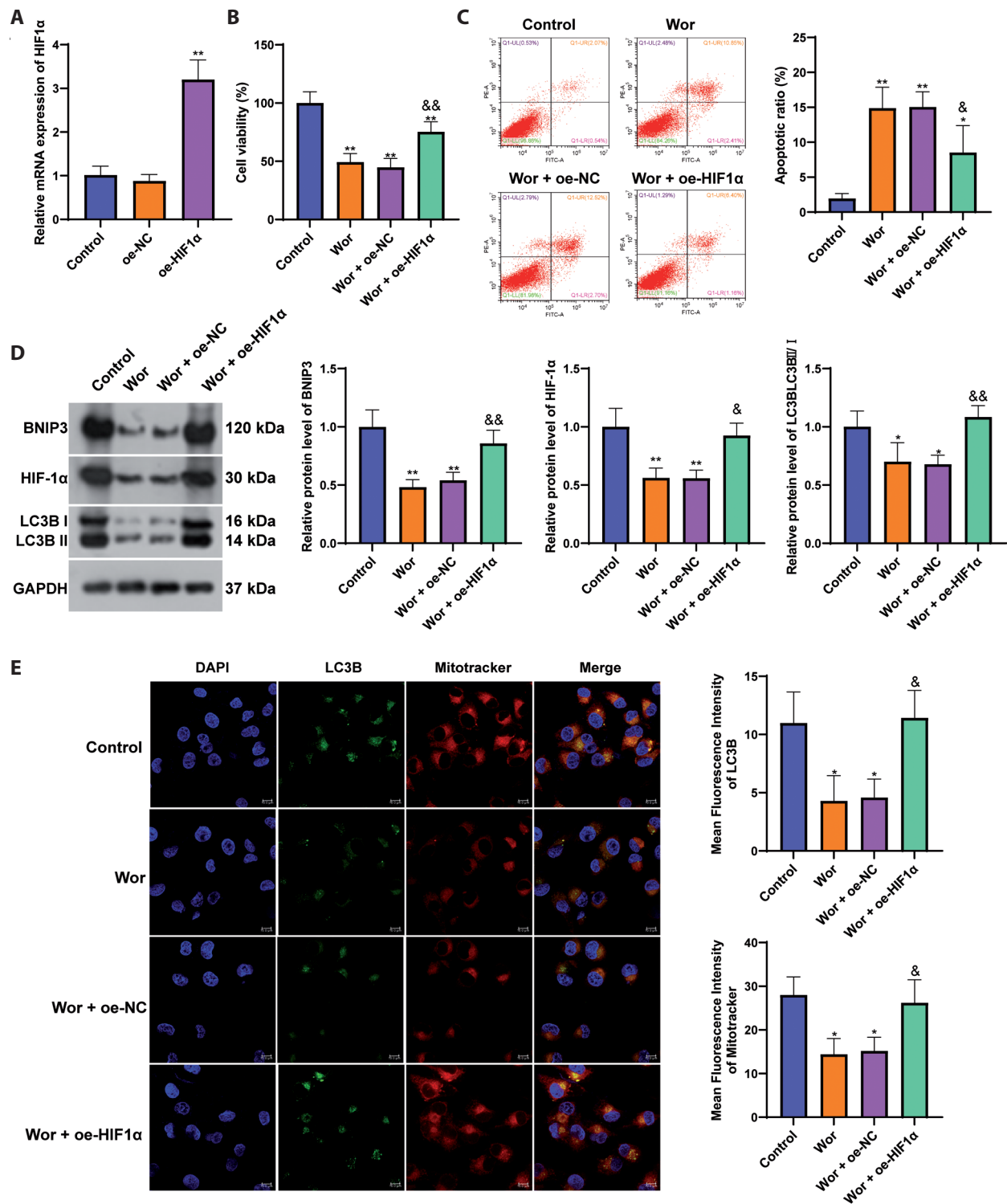


Figure 4. The effects of worenine on A549/DDP cells were reversed by HIF-1α overexpression. **A.** The HIF-1α mRNA expression in A549/DDP cells after transfection with oe-HIF-1α. The viability (**B**) and apoptosis (**C**) of A549/DDP cells after different treatments (for the group involving the worenine, its concentration was 20 μM). **D.** Western blot assay showed the BNIP3, HIF-1α, and LC3B II/I protein expression in A549/DDP cells after different treatments. **E.** Immunofluorescence assays revealed the LC3B and mitotracker expression in A549/DDP cells after different treatments. * $p < 0.05$ and ** $p < 0.01$ compared with control; & $p < 0.05$ and && $p < 0.01$ compared with worenine.

Conflicts of interest. The authors report there are no competing interests to declare.

Contributions. GY: conceptualization, investigation, writing – original manuscript; MX: investigation, formal analysis; MW: supervision; YZ: investigation, formal analysis; FY: conceptualization, project administration, writing – review and editing.

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