

Research Article

Cordycepin Induces Ferroptosis in A549 Lung Cancer Cells via Nrf2/HO-1 Pathway Suppression: An *In Vitro* and Network Pharmacology Study

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Abstract

Background: Non-small cell lung cancer (NSCLC) remains a leading cause of cancer-related mortality worldwide, and novel therapeutic strategies are urgently needed to overcome treatment resistance. Cordycepin (Cor), a bioactive nucleoside isolated from *Cordyceps militaris*, exhibits antitumor activity in multiple malignancies. However, whether Cor induces ferroptosis in NSCLC cells and the underlying mechanisms remain unclear. Therefore, this study aimed to investigate the modulatory effects of Cor on ferroptosis in NSCLC cells and to elucidate the underlying molecular and network pharmacology mechanisms. **Methods:** Human NSCLC A549 cells were treated with Cor (25, 50, and 100 μ M). Cell viability was assessed using Cell Counting Kit-8 assays. Ferroptosis involvement was evaluated using ferroptosis inhibitors (ferrostatin-1, deferoxamine, and liproxstatin-1) and other programmed cell death inhibitors. Intracellular reactive oxygen species (ROS), Fe^{2+} accumulation, lipid peroxidation, glutathione (GSH) levels, and ferroptosis-related proteins were measured using fluorescence probes, spectrophotometric assays, and western blotting. Network pharmacology, protein-protein interaction analysis, Gene Ontology and Kyoto Encyclopedia of Genes and Genomes enrichment analyses, and molecular docking were performed to identify potential molecular targets. Nuclear factor erythroid 2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1) protein expression was further examined by western blotting. **Results:** Cor inhibited A549 cell viability in a time- and dose-dependent manner ($n = 6$). Among the tested death inhibitors, only ferroptosis inhibitors significantly reversed Cor-induced cytotoxicity ($p < 0.05$, $n = 3$). Cor treatment elevated intracellular ROS levels, Fe^{2+} accumulation, and lipid peroxidation while reducing GSH content ($p < 0.05$, $n = 3$). At the protein level, Cor downregulated glutathione peroxidase 4 (GPX4) and solute carrier family 7 member 11 (SLC7A11) expression but upregulated transferrin and acyl-CoA synthetase long-chain family member 4 (ACSL4) expression ($p < 0.05$, $n = 3$). Database screening identified 28 mutual targets linked to Cor, NSCLC, and ferroptosis. Molecular docking confirmed that Cor spontaneously binds to the Nrf2 and HO-1 proteins encoded by the key pathway genes nuclear factor erythroid 2 like 2 (*NFE2L2*) and heme oxygenase 1 (*HMOX1*), which correlated with a significant, dose-dependent downregulation of Nrf2 and HO-1 protein expression ($p < 0.05$, $n = 3$). **Conclusions:** Cordycepin induces ferroptosis in A549 NSCLC cells, characterized by oxidative stress, iron accumulation, lipid peroxidation, and suppression of antioxidant defenses. The Nrf2/HO-1 pathway may contribute to this process and represents a potential therapeutic target for NSCLC.

Keywords: cordycepin; ferroptosis; A549 cells; lung carcinoma; Nrf2; network pharmacology

1. Introduction

Cancer remains a major global public health challenge and imposes a substantial socioeconomic burden worldwide, particularly in China. Lung cancer is the leading cause of cancer incidence and mortality globally [1,2]. Non-small cell lung cancer (NSCLC) is the predominant histological subtype, accounting for approximately 80% of all lung cancer cases [3]. Despite considerable advances in diagnostic and therapeutic strategies, the overall 5-year survival rate for patients with NSCLC remains unsatisfactory at approximately 20% [4]. Early-stage NSCLC is often asymptomatic, and most patients are diagnosed only after the disease has progressed to an advanced stage, which significantly limits treatment options and contributes to poor clinical outcomes [4].

Platinum-based chemotherapy remains a cornerstone of first-line treatment for advanced NSCLC [5]. Although initially effective, its clinical benefits are frequently compromised by the development of drug resistance, treatment-related toxicities, particularly nephrotoxicity, high recurrence rates, and unfavorable long-term outcomes [6]. Consequently, increasing attention has been directed toward bioactive compounds derived from traditional medicines, which may offer both antitumor activity and broader therapeutic benefits through the modulation of multiple cellular pathways [7,8,9].

Cordycepin (Cor), a nucleoside analogue isolated from *Cordyceps militaris*, exhibits broad-spectrum antitumor activity through multiple mechanisms, including inhibition of cell proliferation [10,11], induction of apoptosis [12,13], cell-cycle arrest, and suppression of metastasis



[14,15,16]. Previous studies have demonstrated that Cor promotes the generation of reactive oxygen species (ROS) in cancer cells [17,18,19], thereby triggering apoptotic cell death in several malignancies, including esophageal cancer and lymphoma [20,21,22,23]. ROS are critical mediators of multiple forms of regulated cell death, including apoptosis, pyroptosis, and ferroptosis [24]. Erianin, a natural bibenzyl compound, has been reported to induce ferroptosis in lung cancer cells through ROS accumulation and subsequent oxidative damage [25]. These findings suggest that ROS-mediated ferroptosis may represent an important mechanism underlying the antitumor effects of natural compounds.

Ferroptosis was first described by Stockwell and colleagues in 2012 as a distinct form of regulated cell death that differs fundamentally from apoptosis, autophagy, pyroptosis, and necrosis [26]. This iron-dependent process is driven by oxidative stress and lipid peroxidation [27] and is characterized by disruption of intracellular iron homeostasis and excessive accumulation of lipid ROS (Lip-ROS) [28]. Nrf2 is a crucial transcription factor in the oxidative stress response that can induce the expression of HO-1, inhibit ROS expression, and anti-ferroptotic effects. Growing evidence indicates that ferroptosis plays a critical role in suppressing tumor progression in a variety of malignancies, including breast cancer, melanoma, hepatocellular carcinoma, and prostate cancer [29,30,31]. Consequently, pharmacological induction of ferroptosis has emerged as a promising therapeutic strategy for cancer treatment [32,33].

Despite the recognized antitumor activity of Cor, its ability to induce ferroptosis in NSCLC remains largely unexplored. Therefore, the present study used A549 cells as an *in vitro* model of NSCLC to systematically investigate the ferroptosis-inducing effects of Cor by evaluating key biochemical and molecular markers. Furthermore, network pharmacology and molecular docking analyses were employed to identify potential molecular targets and signaling pathways involved in Cor-mediated ferroptosis. These findings may provide mechanistic insights into the antitumor activity of Cor and support its potential application as a therapeutic agent for NSCLC.

2. Materials and Methods

2.1 Cell Culture

Human NSCLC A549 cells (Cat. No. CL-0016; Procell Life Science & Technology Co., Ltd., Wuhan, China) were authenticated by short tandem repeat (STR) profiling and confirmed to be free of mycoplasma contamination. Cells were cultured in RPMI-1640 medium (Cat. No. 10-040-CVRC; Corning Inc., Corning, NY, USA) supplemented with 10% fetal bovine serum (Cat. No. S9020; Solarbio, Beijing, China) and 1% penicillin–streptomycin solution in a humidified incubator containing 5% CO₂ (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C. The culture medium was refreshed regularly, and cells were

passaged every 2 days. Cells in the logarithmic growth phase were used for all subsequent experiments.

2.2 Cell Counting Kit-8 (CCK-8) Assay

A549 cells in the logarithmic growth phase were seeded into 96-well plates at a density of 5×10^3 cells/well and allowed to adhere overnight. Cells were then assigned to blank, control, and treatment groups. The blank group contained culture medium only, whereas the control group received vehicle treatment. For dose- and time-response experiments, cells were exposed to Cor (Cat. No. S31387; Yuanye Bio-Technology Co., Ltd., Shanghai, China) at final concentrations of 12.5, 25, 50, 100, and 200 µM for 24, 48, or 72 h. To investigate the mode of cell death, cells were treated with Cor (25, 50, or 100 µM) alone or in combination with the apoptosis inhibitor benzyloxycarbonyl-val-al-asp(OMe)-fluoromethylketone (Z-VAD-FMK) (10 µM; Cat. No. SJ-BP0022; Selleck Biotechnology Co., Ltd., Shandong, China), the autophagy inhibitor chloroquine (CQ; 10 µM; Cat. No. HY-17589A; MedChemExpress, Monmouth Junction, NJ, USA), the ferroptosis inhibitors ferrostatin-1 (Fer-1; 2 µM; Cat. No. F129882; Aladdin Biochemical Technology Co., Ltd., Shanghai, China), deferoxamine (DFO; 10 µM; Cat. No. HY-B0988; MedChemExpress, USA), liproxstatin-1 (Lip-1; 1 µM; Cat. No. HY-12726; MedChemExpress, USA), or the necroptosis inhibitor necrostatin-1 (Nec-1; 5 µM; Cat. No. HY-15760; MedChemExpress, USA) for 24 h. Following treatment, the culture medium was removed and replaced with 100 µL of fresh medium containing CCK-8 reagent (Cat. No. CK13; Dojindo Laboratories, Kumamoto, Japan) diluted 1:10. After incubation for 2 h at 37 °C, absorbance was measured at 450 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). Each treatment was performed in six replicate wells. Cell viability was calculated using the following formula:

$$\text{Cell viability (\%)} = \frac{[(OD_{\text{experiment}} - OD_{\text{blank}})/(OD_{\text{control}} - OD_{\text{blank}})] \times 100\%}$$

The half-maximal inhibitory concentration (IC₅₀) values of Cor were determined from dose–response curves generated using GraphPad Prism software (prism 10.1.0; October 19, 2023; GraphPad Software, Boston, MA, USA).

2.3 Detection of Intracellular ROS

A549 cells in the logarithmic growth phase were seeded into 60-mm culture dishes at a density of 1.5×10^6 cells per dish and allowed to adhere overnight. The cells were then assigned to a control group and Cor treatment groups (25, 50, or 100 µM) and incubated for 24 h. Thirty minutes before the end of the treatment period, cells were incubated with 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; Cat. No. HY-D0940; MedChemExpress, USA) at a final concentration of 10 µM. Following incu-

bation, the cells were washed three times with phosphate-buffered saline (PBS) to remove excess probe. Intracellular ROS levels were assessed by measuring green fluorescence intensity using a live-cell imaging workstation. Relative fluorescence intensity was quantified using ImageJ software (version 1.54r; NIH, Bethesda, MD, USA).

2.4 Detection of Intracellular Fe^{2+}

A549 cells in the logarithmic growth phase were seeded into 12-well plates at a density of 4×10^5 cells per well and allowed to adhere overnight. Cells were subsequently treated with Cor (25, 50, or 100 μ M) for 24 h, while untreated cells served as the control group. After treatment, the culture medium was removed, and the cells were incubated with FerroOrange fluorescent probe (Cat. No. F374; Dojindo Laboratories, Japan) at a final concentration of 1 μ M for 30 min under standard culture conditions. Following probe loading, intracellular Fe^{2+} levels were evaluated by measuring red fluorescence intensity using a live-cell imaging workstation. Relative fluorescence intensity was quantified using ImageJ software (NIH).

2.5 Detection of Intracellular Lipid Peroxidation

A549 cells in the logarithmic growth phase were seeded into 12-well plates at a density of 4×10^5 cells per well and allowed to adhere overnight. Cells were then assigned to a control group and Cor treatment groups (25, 50, or 100 μ M) and incubated for 24 h. Following treatment, cells were stained with BODIPYTM 581/591 C11 fluorescent probe (Cat. No. D3861; Thermo Fisher Scientific, USA) at a final concentration of 2 μ M for 30 min at 37 °C. After staining, the cells were washed three times with PBS to remove excess probe. Green and red fluorescence intensities were measured using a live-cell imaging workstation. Lipid peroxidation levels were assessed by calculating the ratio of green to red fluorescence intensity, and fluorescence signals were quantified using ImageJ software (NIH).

2.6 Determination of Intracellular Glutathione (GSH) Levels

A549 cells in the logarithmic growth phase were seeded into 100-mm culture dishes at a density of 6×10^6 cells per dish and allowed to adhere overnight. Cells were subsequently treated with Cor (25, 50, or 100 μ M) for 24 h, while untreated cells served as the control group. After treatment, cells were harvested, washed twice with PBS, and processed according to the manufacturer's instructions. Intracellular GSH levels were determined using a reduced glutathione assay kit (Cat. No. A006-2-1; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Absorbance was measured at 405 nm using a microplate reader (Bio-Rad Laboratories, CA, USA).

2.7 Western Blot Analysis

A549 cells in the logarithmic growth phase were seeded into 100-mm culture dishes at a density of 6×10^6 cells per dish and allowed to adhere overnight. Cells were subsequently treated with Cor (25, 50, or 100 μ M) for 24 h, while untreated cells served as the control group. Following treatment, cells were harvested and lysed in ice-cold RIPA lysis buffer supplemented with protease and phosphatase inhibitor cocktails. The lysates were incubated on ice for 30 min and centrifuged at $12,000 \times g$ for 30 min at 4 °C. The supernatants were collected, and protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Cat. No. PC0020; Solarbio, Beijing, China).

Equal amounts of protein (20 μ g per sample) were mixed with 5 $\times$ loading buffer, denatured by heating, separated by SDS-PAGE, and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 2 h at room temperature and then incubated overnight at 4 °C with the following primary antibodies: anti-Transferrin rabbit polyclonal antibody (1:1000; Cat. No. 17435-1-AP; Proteintech Group, Wuhan, China), anti-acyl-CoA synthetase long-chain family member 4 (ACSL4) rabbit polyclonal antibody (1:1000; Cat. No. A6826; ABclonal Technology Co., Ltd., Wuhan, China), anti-solute carrier family 7 member 11/cystine/glutamate antiporter (SLC7A11/xCT) rabbit polyclonal antibody (1:1000; Cat. No. 26864-1-AP; Proteintech Group), and anti-glutathione peroxidase 4 (GPX4) rabbit polyclonal antibody (1:1000; Cat. No. A1933; ABclonal Technology Co., Ltd.), anti-Nrf2 rabbit polyclonal antibody (1:1000; Cat. No. 16396-1-AP; Proteintech Group, Wuhan, China), anti-HO-1 rabbit polyclonal antibody (1:1000; Cat. No. 10701-1-AP; Proteintech Group, Wuhan, China), anti-GAPDH rabbit polyclonal antibody (1:5000; Cat. No. bs-41373R; Bioss Biotechnology Co., Ltd., Beijing, China). After washing six times with TBST, membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (1:5000; Cat. No. SA00001-2; Proteintech Group, Wuhan, China) for 1 h at room temperature. Following four additional washes with TBST, immunoreactive bands were visualized using an enhanced chemiluminescence (ECL) detection kit (Cat. No. P0018FS; Beyotime Biotechnology, Shanghai, China) and captured using a Tanon chemiluminescence imaging system (Tanon Science & Technology Co., Ltd., Shanghai, China). Band intensities were quantified using ImageJ software (NIH).

2.8 Identification of Cor-, NSCLC-, and Ferroptosis-Related Targets

Potential targets of Cor were retrieved from the BATMAN-TCM and Comparative Toxicogenomics Database (CTD). Targets with a score >10 were retained for further analysis. To identify NSCLC-related therapeutic

targets, the keyword “non-small cell lung cancer” was used to search the GeneCards and Online Mendelian Inheritance in Man (OMIM) databases. Targets with a relevance score ≥ 40 in GeneCards, together with targets retrieved from OMIM, were collected and merged after removing duplicates. Ferroptosis-related targets were obtained from the FerrDb V2 database. Targets categorized as “driver”, “marker”, “suppressor”, and “unclassified” were downloaded, and only human-derived targets were retained for subsequent analyses. The identified Cor-, NSCLC-, and ferroptosis-related targets were imported into BioinfoGP for Venn diagram analysis. The overlapping targets among the three datasets were defined as common targets and were used for subsequent network pharmacology analyses.

2.9 Construction and Analysis of the Protein–Protein Interaction (PPI) Network

The common targets identified from the intersection analysis were imported into the STRING database to construct a PPI network. The species parameter was restricted to ‘*Homo sapiens*’, while all other settings were maintained at their default values. The resulting interaction data were exported in TSV format and imported into Cytoscape software (Version 3.10.2; Institute for Systems Biology, Seattle, WA, USA) for network visualization and topological analysis. The degree value of each node was calculated, and the PPI network was constructed to identify hub targets with potentially important biological functions.

2.10 Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Enrichment Analyses

The common targets shared by Cor, NSCLC, and ferroptosis were subjected to functional enrichment analysis using the Database for Annotation, Visualization and Integrated Discovery (DAVID). GO enrichment analysis was performed to identify significantly enriched biological processes (BP), cellular components (CC), and molecular functions (MF), while KEGG pathway enrichment analysis was conducted to explore the potential signaling pathways associated with Cor-induced ferroptosis in NSCLC. The top 10 enriched GO terms and the top 20 enriched KEGG pathways were selected for visualization. Enrichment bubble plots were generated using the online bioinformatics platform available at <http://www.bioinformatics.com.cn>.

2.11 Molecular Docking Analysis

To evaluate the potential interactions between Cor and key target proteins, molecular docking analysis was performed. The three-dimensional crystal structures of the target proteins were obtained from the Protein Data Bank (PDB). The PDB ID for Nrf2 is 7X5G, and the PDB ID for HO-1 is 1N45, whereas the chemical structure of Cor was retrieved from the PubChem database. Protein structures were preprocessed using PyMOL 2.3.4 (Schrödinger, LLC, New York, NY, USA) to remove water molecules and

co-crystallized ligands. Hydrogen atoms were added, and partial atomic charges were assigned using AutoDockTools 1.5.6 (The Scripps Research Institute, La Jolla, CA, USA). Subsequently, both receptor and ligand structures were converted into PDBQT format, and Gasteiger charges were calculated for the ligand molecules.

Molecular docking simulations were performed using AutoDock Vina 1.1.2 (The Scripps Research Institute, La Jolla, CA, USA). The docking results were further analyzed using the Protein–Ligand Interaction Profiler (PLIP), and protein–ligand interaction patterns were visualized using PyMOL. Binding affinity was evaluated according to the calculated binding energy, with lower binding energy values indicating stronger binding interactions between the ligand and target protein.

2.12 Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics 26.0 (IBM Corp., Armonk, NY, USA), and graphs were generated using GraphPad Prism 10.1.0 (GraphPad Software). Data are presented as the mean $\pm$ standard deviation (SD) from at least three independent experiments. Data that met the assumptions of normality and homogeneity of variance were analyzed using one-way analysis of variance (ANOVA), followed by Dunnett’s post hoc test for multiple comparisons. For data that did not satisfy these assumptions, nonparametric analyses were performed using the Kruskal–Wallis test, followed by pairwise comparisons using the Mann–Whitney *U* test when appropriate. All statistical tests were two-sided, and a *p*-value < 0.05 was considered statistically significant.

3. Results

3.1 Effect of Cor on A549 Cell Viability

Cor reduced the viability of A549 cells in a time- and concentration-dependent manner (Fig. 1). The IC₅₀ values were 151.0 μ M (95% CI: 125.4–190.7 μ M), 53.68 μ M (95% CI: 50.41–57.19 μ M), and 43.02 μ M (95% CI: 40.46–45.72 μ M) after 24, 48, and 72 h of treatment, respectively. Based on these results, Cor concentrations of 25, 50, and 100 μ M were selected as the low-, medium-, and high-dose treatment groups, respectively, for subsequent experiments, with a treatment duration of 24 h.

3.2 Effect of Cor on the Mode of Cell Death in A549 Cells

Treatment with Cor (25, 50, and 100 μ M) significantly decreased A549 cell viability compared with the control group ($p < 0.05$). Co-treatment with the ferroptosis inhibitors Fer-1, DFO, or Lip-1 significantly attenuated Cor-induced cell death ($p < 0.05$; Fig. 2A–C). In contrast, the autophagy inhibitor CQ, the apoptosis inhibitor Z-VAD-FMK, and the necroptosis inhibitor Nec-1 failed to reverse the reduction in cell viability induced by Cor (Fig. 2D–F). These findings suggest that ferroptosis is the predominant mode of cell death induced by Cor in A549 cells.

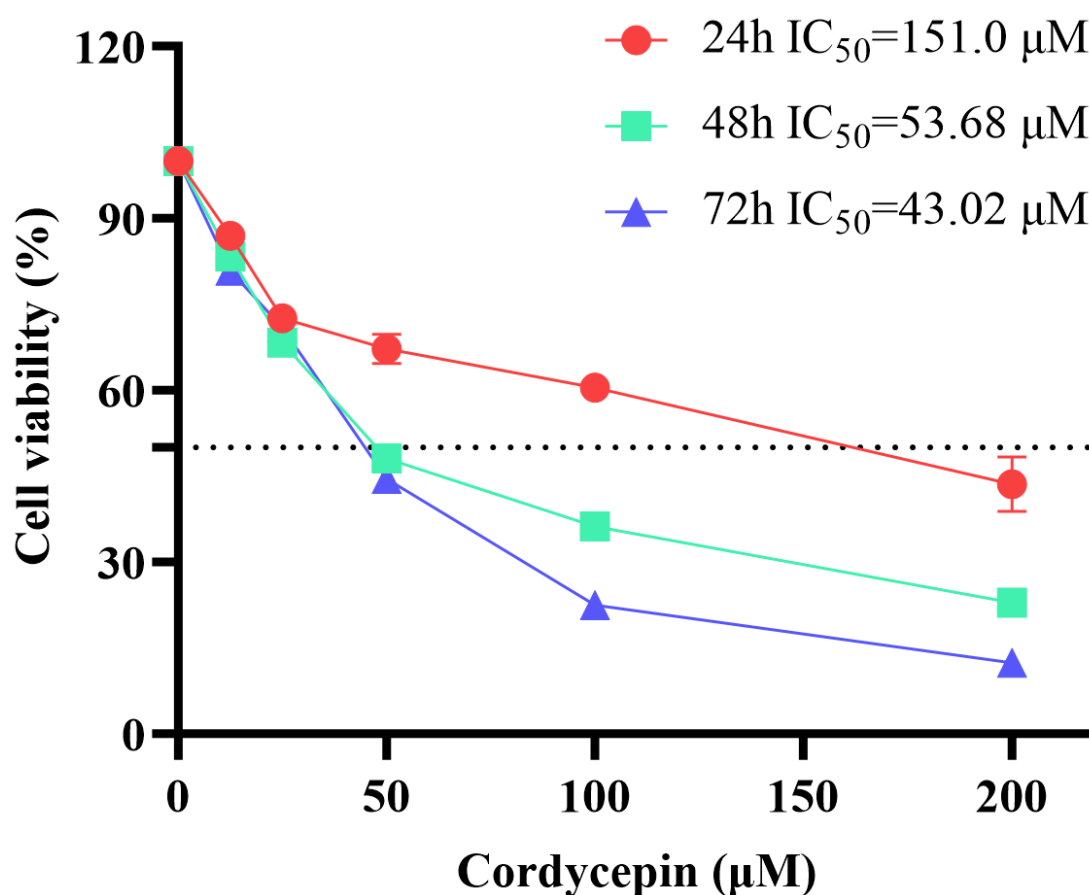


Fig. 1. Effect of cordycepin (Cor) on A549 cell viability. A549 cells were treated with increasing concentrations of Cor (12.5–200 μM) for 24, 48, or 72 h, and cell viability was determined using the cell counting kit-8 (CCK-8) assay. Cor reduced A549 cell viability in a concentration- and time-dependent manner. The calculated IC₅₀ values were 151.0 μM, 53.68 μM, and 43.02 μM after 24, 48, and 72 h of treatment, respectively.

3.3 Effects of Cor on ROS, Fe²⁺ Accumulation, Lipid Peroxidation, GSH Levels, and Ferroptosis-Related Protein Expression

Cor treatment increased intracellular ROS levels in A549 cells in a concentration-dependent manner, as indicated by enhanced green fluorescence intensity compared with the control group (Fig. 3A). Relative fluorescence intensity was significantly increased in the 50 and 100 μM Cor treatment groups ($p < 0.05$). Intracellular Fe²⁺ levels were significantly elevated following Cor treatment, as evidenced by increased red fluorescence intensity in all treatment groups (25, 50, and 100 μM) compared with the control group (Fig. 3B; $p < 0.05$). The increase in fluorescence intensity was concentration dependent.

Cor treatment also promoted lipid peroxidation in A549 cells. Compared with the control group, cells treated with Cor exhibited increased green fluorescence intensity and decreased red fluorescence intensity. Consequently, the green/red fluorescence ratio was significantly increased in the 50 and 100 μM treatment groups (Fig. 3C; $p < 0.05$).

In addition, intracellular GSH levels were significantly reduced following Cor treatment in a concentration-

dependent manner. Significant decreases were observed in the 50 and 100 μM treatment groups compared with the control group (Fig. 3D; $p < 0.05$).

Western blot analysis demonstrated that Cor significantly altered the expression of ferroptosis-related proteins in A549 cells (Fig. 3E). The protein expression levels of GPX4 and SLC7A11 were significantly decreased following Cor treatment ($p < 0.05$). In contrast, ACSL4 expression was significantly increased in all Cor-treated groups (25, 50, and 100 μM; $p < 0.05$), whereas transferrin expression was significantly elevated in the 50 and 100 μM treatment groups compared with the control group ($p < 0.05$).

3.4 Identification of Common Targets Associated with Cor, NSCLC, and Ferroptosis

A total of 414 potential targets of Cor were identified from the BATMAN-TCM and CTD databases. In addition, 1170 NSCLC-related targets were obtained from the GeneCards and OMIM databases, whereas 396 ferroptosis-related targets were retrieved from the FerrDb V2 database. Venn diagram analysis revealed 28 overlapping targets shared among Cor, NSCLC, and ferroptosis (Fig. 4A).

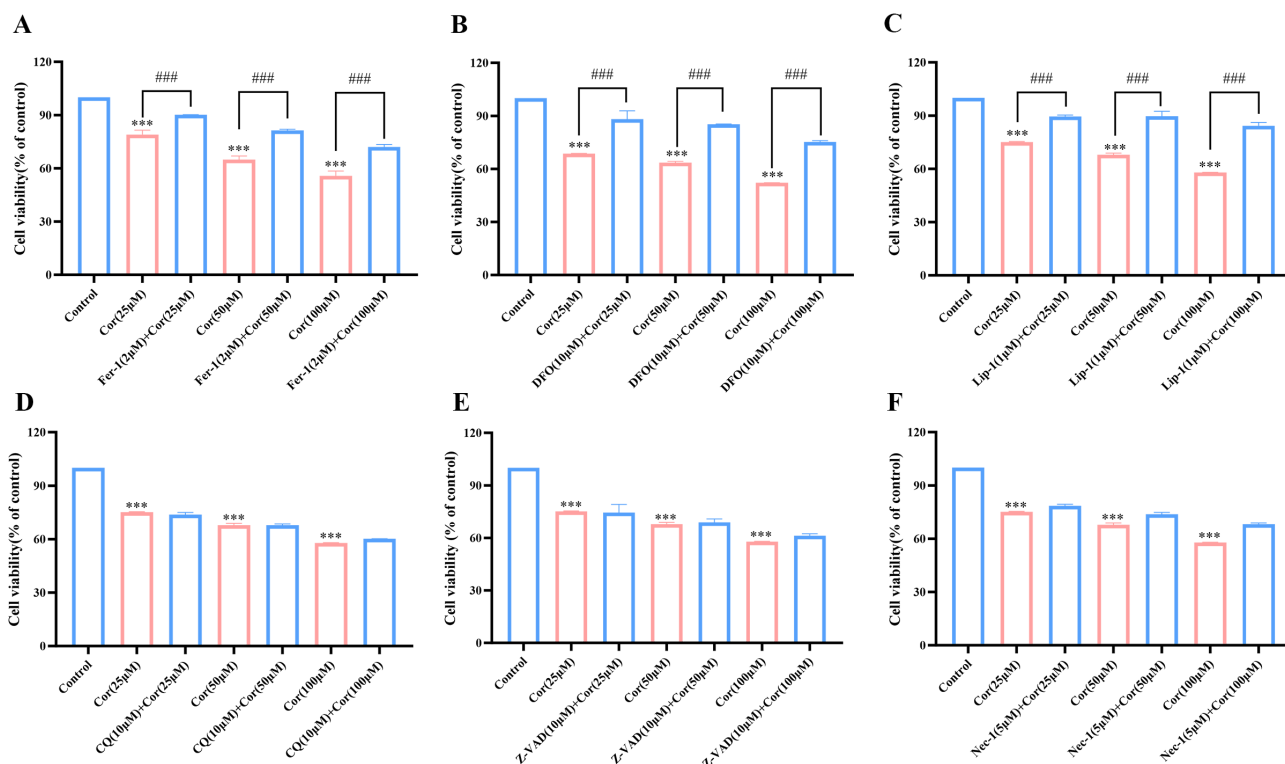


Fig. 2. Effects of cell death inhibitors on Cor-induced cytotoxicity in A549 cells. A549 cells were treated with Cor in the presence or absence of the ferroptosis inhibitors ferrostatin-1 (Fer-1) (2 μ M) (A), deferoxamine (DFO) (10 μ M) (B), and liproxstatin-1 (Lip-1) (1 μ M) (C), the autophagy inhibitor chloroquine (CQ, 10 μ M) (D), the apoptosis inhibitor Z-VAD-FMK (10 μ M) (E), or the necroptosis inhibitor Necrostatin-1 (Nec-1, 5 μ M) (F). Fer-1, DFO, and Lip-1 significantly attenuated Cor-induced cell death, whereas CQ, Z-VAD-FMK, and Nec-1 failed to reverse the cytotoxic effects of Cor. Data are presented as the mean $\pm$ standard deviation ($n = 3$). *** $p < 0.001$ versus the control group; ### $p < 0.001$ versus the corresponding Cor-treated group.

These common targets were considered potential candidates involved in Cor-mediated ferroptosis in NSCLC and were selected for subsequent analyses.

3.5 PPI Network Analysis of Common Targets

The 28 common targets were imported into the STRING database to construct a PPI network. The resulting network consisted of 28 nodes and 275 interaction edges, with a mean node degree of 19.6 and a clustering coefficient of 0.86, indicating a high degree of interconnectivity among the identified targets. The interaction data were subsequently imported into Cytoscape for network visualization and topological analysis. Node size and color intensity were scaled according to degree values, with larger and darker nodes representing higher degrees of connectivity (Fig. 4B). The targets with the highest degree values included *IL6*, *STAT3*, *TP53*, and several other hub genes. Specifically, *NFE2L2* and *HMOX1*, which encode the key components of the Nrf2/HO-1 antioxidant pathway, were among the highly connected nodes within the network, suggesting their potential involvement in Cor-mediated ferroptosis in NSCLC.

3.6 Functional Enrichment Analysis of Common Targets

To explore the potential biological functions and signaling pathways associated with Cor-induced ferroptosis in NSCLC, the 28 common targets were subjected to GO and KEGG enrichment analyses using the DAVID database. GO enrichment analysis identified 302 BP terms, 39 CC terms, and 57 MF terms, whereas KEGG analysis identified 146 significantly enriched pathways. The top 10 GO terms and top 20 KEGG pathways were visualized using bubble plots (Fig. 4C,D).

The enriched BP terms were primarily associated with the regulation of gene expression and responses to oxidative stress, hypoxia, and cellular stress. The enriched CC terms were mainly distributed in the nucleus and cytoplasm, whereas the enriched MF terms were predominantly related to protein binding, enzyme binding, and kinase activity (Fig. 4C). KEGG pathway analysis revealed significant enrichment in pathways related to cancer, the hypoxia-inducible factor 1 (HIF-1) signaling pathway, the forkhead box O (FOXO) signaling pathway, and several disease-associated pathways (Fig. 4D).

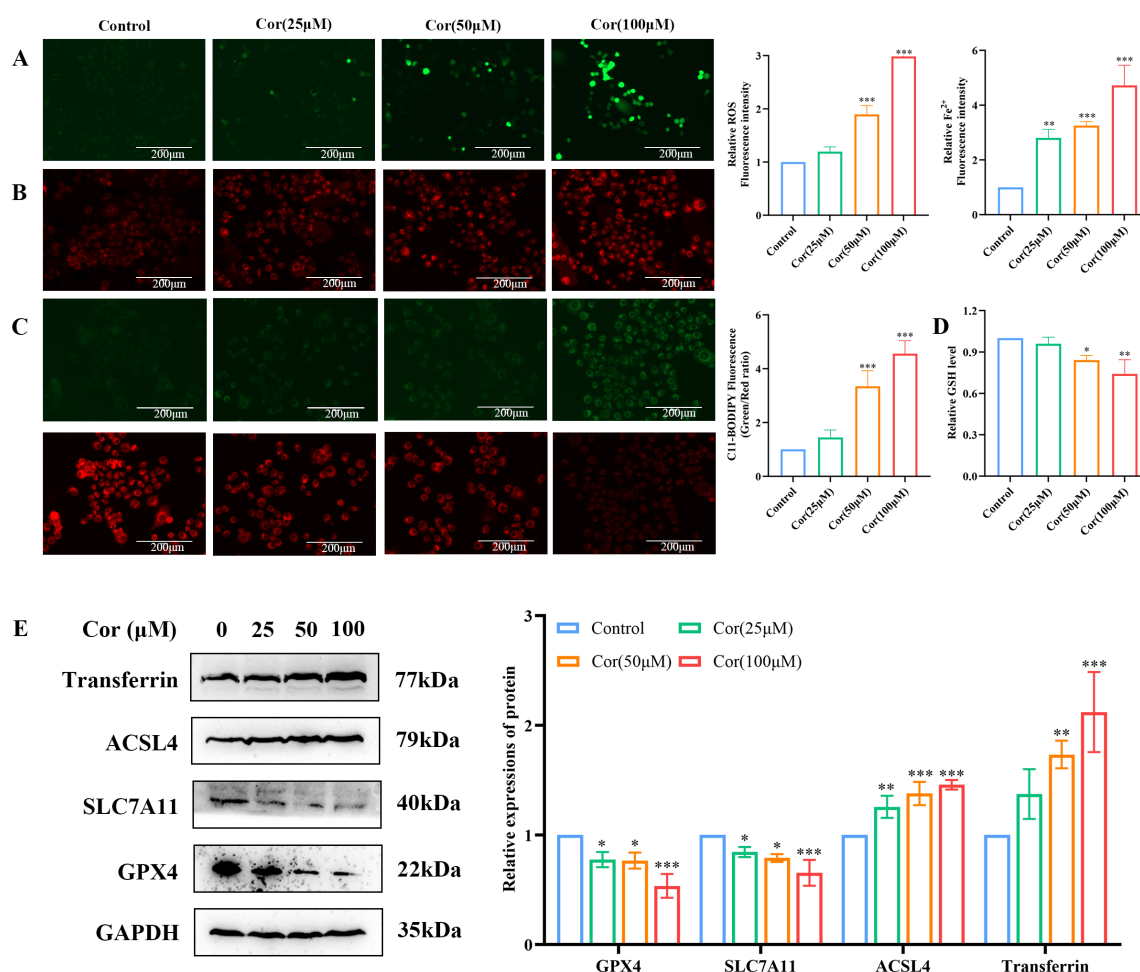


Fig. 3. Cor induces ferroptosis in A549 cells. (A) Intracellular reactive oxygen species (ROS) levels in A549 cells treated with Cor for 24 h were detected using the 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescent probe. (B) Intracellular Fe²⁺ levels were detected using the FerroOrange fluorescent probe. (C) Intracellular lipid peroxidation was assessed using the BODIPY 581/591 C11 fluorescent probe. (D) Intracellular GSH levels were measured using a spectrophotometric assay. (E) Western blot analysis of ferroptosis-related proteins (GPX4, SLC7A11, ACSL4, and transferrin) in A549 cells following 24 h of Cor treatment. Relative fluorescence intensities were quantified using ImageJ software. Scale bar = 200 μm. Data are presented as the mean ± standard deviation ($n = 3$). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus the control group.

3.7 Molecular Docking Analysis of Cor with Nrf2 and HO-1

Based on the PPI network analysis, Nrf2 and HO-1 were selected for molecular docking analysis with Cor. The docking results demonstrated favorable binding interactions between Cor and both target proteins, with binding energies below 0 kcal/mol, indicating spontaneous binding affinity. The binding energy between Cor and Nrf2 was -4.5 kcal/mol. As shown in the three-dimensional docking model (Fig. 5A), Arg499 formed hydrogen bonds with Cor, whereas Arg503 participated in hydrophobic interactions. The binding energy between Cor and HO-1 was -6.0 kcal/mol. In the docking model, Arg136, Asp140, and Asn210 formed hydrogen bonds with Cor, while Leu147, Phe167, and Asn210 contributed to electrostatic interactions (Fig. 5B). These results suggest that Cor has the potential to interact with Nrf2 and HO-1, two key components of the Nrf2/HO-1 antioxidant pathway.

tions (Fig. 5B). These results suggest that Cor has the potential to interact with Nrf2 and HO-1, two key components of the Nrf2/HO-1 antioxidant pathway.

3.8 Effects of Cor on Nrf2 and HO-1 Expression in A549 Cells

Western blot analysis demonstrated that Cor treatment (25, 50, and 100 μM) significantly reduced the protein expression levels of Nrf2 and HO-1 in A549 cells compared with the control group (Fig. 5C; $p < 0.05$). The inhibitory effects were concentration-dependent. These findings indicate that Cor suppresses the Nrf2/HO-1 antioxidant pathway and may contribute to the induction of ferroptosis in A549 cells.

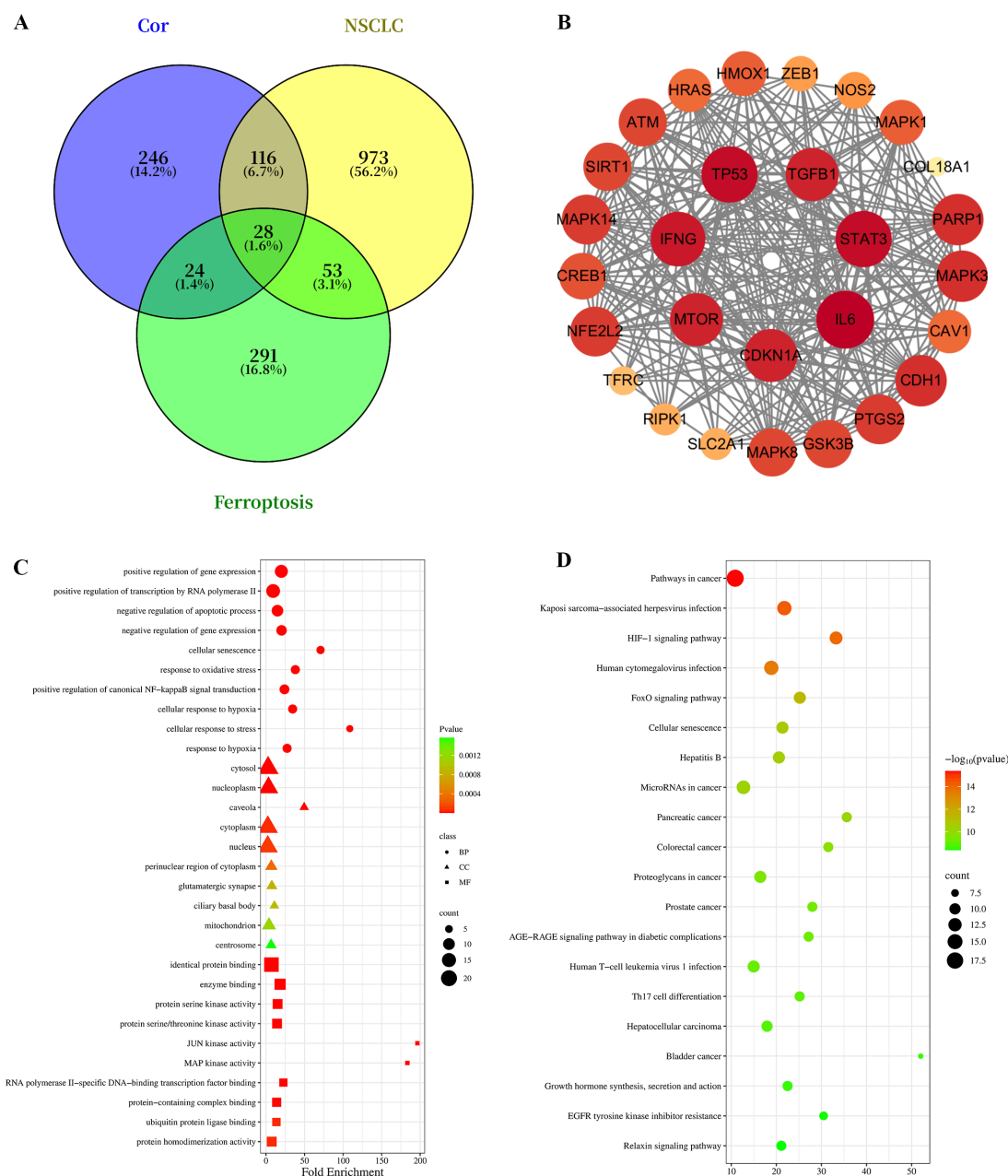


Fig. 4. Network pharmacology analysis of Cor-induced ferroptosis in non-small cell lung cancer (NSCLC). (A) Venn diagram showing the overlapping targets among Cor, NSCLC, and ferroptosis. (B) Protein-protein interaction (PPI) network of the common targets constructed using the STRING database and visualized with Cytoscape software. (C) Gene Ontology (GO) enrichment analysis of the common targets, showing the top 10 enriched terms in the biological process, cellular component, and molecular function categories. (D) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of the common targets, showing the top 20 significantly enriched pathways.

4. Discussion

For patients with advanced NSCLC, chemotherapy remains the cornerstone of treatment; however, its clinical efficacy is frequently limited by drug resistance, treatment-related toxicity, and tumor recurrence [34]. Consequently, increasing attention has been directed toward bioactive compounds derived from natural products as potential anti-cancer agents. Numerous active constituents isolated from

traditional Chinese medicines have demonstrated significant antitumor activities in both *in vitro* and *in vivo* studies, including the inhibition of tumor cell proliferation and induction of programmed cell death [35,36]. For example, shikonin, a naphthoquinone compound isolated from *Lithospermum erythrorhizon*, has been reported to promote ROS generation, suppress epidermal growth factor receptor (EGFR) and phosphatidylinositol 3-kinase/protein kinase

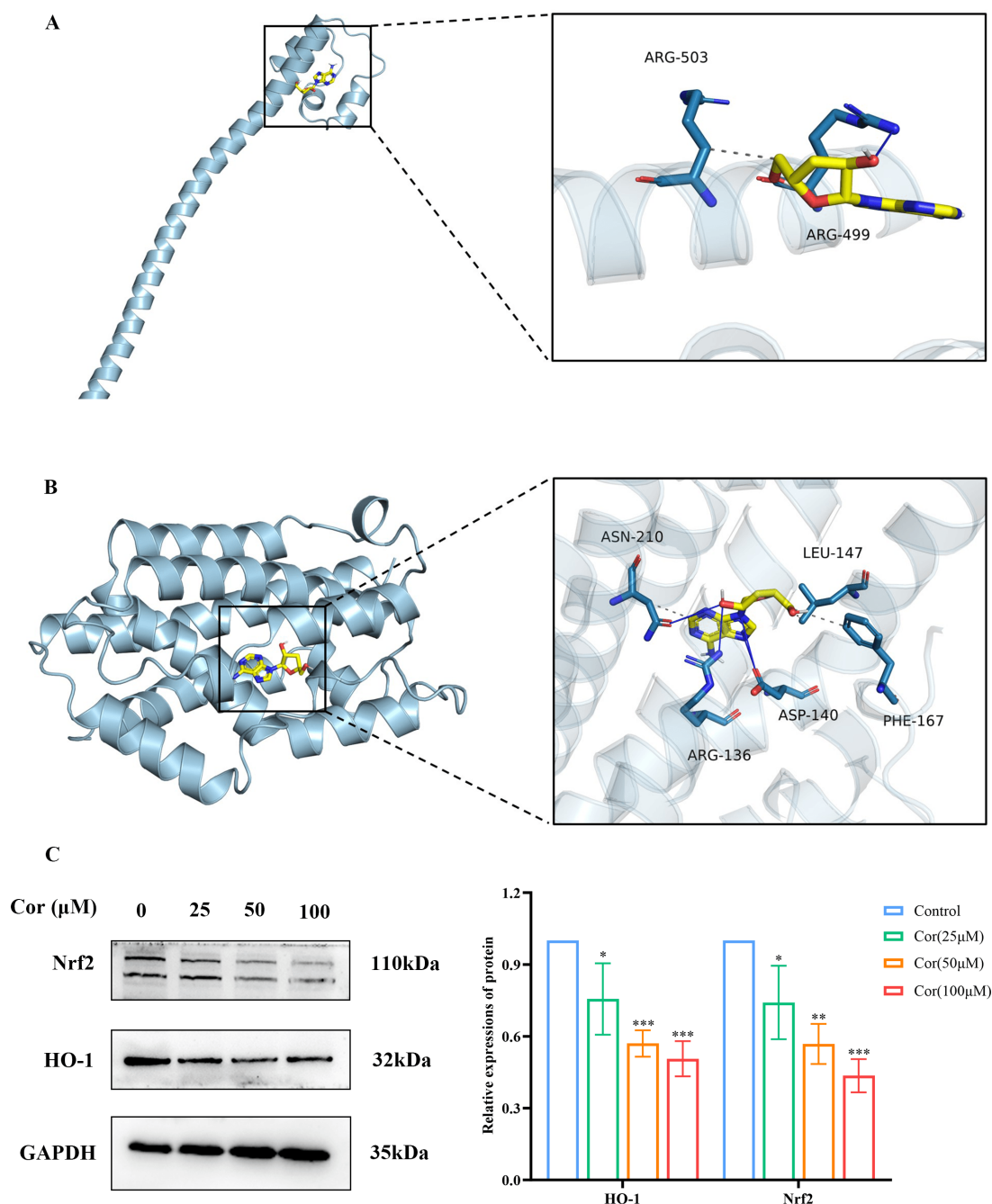


Fig. 5. Molecular docking analysis and effects of Cor on Nrf2 and HO-1 expression in A549 cells. (A) Molecular docking model of Cor with Nrf2, showing a binding energy of -4.5 kcal/mol. (B) Molecular docking model of Cor with HO-1, showing a binding energy of -6.0 kcal/mol. (C) Western blot analysis of Nrf2 and HO-1 protein expression in A549 cells following 24 h of Cor treatment. Data are presented as the mean $\pm$ standard deviation ($n = 3$). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus the control group.

B (PI3K/AKT) signaling, inhibit angiogenesis, and induce apoptosis and necroptosis in tumor cells [37,38]. In contrast, resveratrol has been shown to inhibit H_2O_2 -induced ferroptosis in alveolar epithelial A549 cells through activation of the Nrf2/GPX4 signaling pathway [39]. These findings highlight the diverse regulatory effects of natural compounds on cell death pathways and support their potential application in cancer therapy.

Cordyceps militaris is an edible medicinal fungus with a wide range of biological activities, including antioxidant, antiviral, antitumor, and immunomodulatory effects [40,41,42]. These pharmacological properties are largely attributed to its bioactive constituents, particularly nucleosides, polysaccharides, and sterols [43]. Among these compounds, Cor has attracted considerable attention because of its broad-spectrum anticancer activity [44]. Pre-

vious studies have demonstrated that Cor suppresses tumor progression through multiple mechanisms, including ROS-mediated apoptosis, cell-cycle arrest, inhibition of metastasis, and regulation of tumor suppressor pathways [17,20,45]. Moreover, the clinical efficacy of Cor in combination with the chemotherapy drug pentostatin was evaluated in patients with leukemia [46], further highlighting its therapeutic potential. Despite these advances, whether Cor can induce ferroptosis in NSCLC and whether the Nrf2/HO-1 pathway is involved in this process have remained largely unexplored. In the present study, Cor significantly reduced A549 cell viability in a concentration- and time-dependent manner. Based on the observed cytotoxic profile, concentrations of 25, 50, and 100 μ M were selected for subsequent mechanistic investigations. Importantly, our findings extend previous studies by demonstrating that Cor induces ferroptosis-associated biochemical and molecular alterations in A549 cells. Nevertheless, because only a single NSCLC cell line was evaluated, further studies using drug-resistant models, such as A549/DDP cells, are warranted to determine the therapeutic potential of Cor in overcoming chemotherapy resistance.

Accumulating evidence indicates that specific signaling pathways are closely associated with particular cancer types, and that regulated cell death pathways represent promising therapeutic targets in cancer treatment [47]. Ferroptosis, first described by Stockwell et al. [26] in 2012, is a distinct form of regulated cell death characterized by iron-dependent oxidative damage and excessive lipid peroxidation of cellular membranes. Mechanistically, ferroptosis is driven by intracellular iron accumulation and depletion of GSH, resulting in the inactivation of GPX4 and the subsequent accumulation of lipid peroxides [48]. Hallmark features of ferroptosis include reduced GSH and GPX4 levels, increased intracellular iron accumulation, elevated Lip-ROS, and enhanced lipid peroxidation [49]. A growing number of natural compounds have been reported to exert antitumor effects by inducing ferroptosis. For example, Erianin, a natural bibenzyl compound isolated from *Dendrobium chrysotoxum* Lindl., promotes ferroptosis in lung cancer cells through increased ROS production, enhanced lipid peroxidation, and depletion of intracellular glutathione [25]. Consistent with these observations, our results demonstrated that Cor treatment significantly increased intracellular ROS levels, Fe^{2+} accumulation, and lipid peroxidation while reducing GSH levels in A549 cells. In addition, Cor decreased the expression of GPX4 and SLC7A11, two key regulators of cellular antioxidant defense, and increased the expression of transferrin and ACSL4, both of which are closely associated with ferroptosis. These findings provide strong evidence that Cor induces ferroptosis in A549 cells. However, the antitumor efficacy of Cor *in vivo* and whether its anticancer activity is mediated through ferroptosis induction remain to be verified in future studies.

Hypoxia is a hallmark of the tumor microenvironment and plays a critical role in tumor progression and therapeutic resistance. Prolonged exposure of tumor cells to hypoxic conditions promotes ROS generation and oxidative stress [50,51]. Elevated ROS levels have been reported to induce various forms of cell death, including apoptosis and necrosis [52], while excessive ROS can also damage cellular membranes and promote lipid peroxidation [53,54]. In ferroptosis, intracellular iron accumulation further amplifies oxidative stress through the Fenton reaction, leading to excessive ROS production and lipid peroxidation, which ultimately drive cell death [55,56].

Previous studies have demonstrated that Cor exerts broad-spectrum antitumor activity through ROS-mediated mechanisms. In bladder cancer T24 cells, Cor induces apoptosis by promoting ROS accumulation and suppressing PI3K/AKT signaling [17]. Similarly, Cor has been shown to trigger ROS generation and inhibit the PI3K/AKT pathway in gastric cancer SGC-7901 cells [52]. Increased ROS production following Cor treatment has also been reported in cervical cancer [19], brain cancer [21], and esophageal cancer cells [23]. These findings suggest that ROS-mediated oxidative stress is a common mechanism underlying the anticancer activity of Cor and provide a rationale for investigating its role in ferroptosis induction.

To counteract excessive oxidative stress, tumor cells activate a variety of antioxidant defense mechanisms that promote cellular survival. HIF-1 is a key transcription factor that becomes stabilized under hypoxic conditions and contributes to tumor adaptation and survival in response to increased ROS levels [57,58,59]. Another important regulator of the cellular antioxidant response is Nrf2, which transcriptionally activates a range of antioxidant and cytoprotective genes, including *HO-1*, *NQO1*, *GST*, and *GPX4*, thereby limiting oxidative damage and preventing cell death [60]. Similarly, members of the FOXO family are activated under oxidative stress and enhance the expression of antioxidant enzymes, such as superoxide dismutase and glutathione peroxidase, to maintain redox homeostasis [61,62].

In the present study, network pharmacology analysis identified 28 common targets associated with Cor, NSCLC, and ferroptosis. GO enrichment analysis revealed that these targets were predominantly involved in biological processes related to oxidative stress and hypoxia responses, whereas KEGG enrichment analysis highlighted the HIF-1 and FOXO signaling pathways. These pathways play important roles in regulating cellular adaptation to oxidative stress and oxygen availability. Together, these findings suggest that Cor-induced ferroptosis in NSCLC may involve coordinated regulation of multiple targets and signaling pathways associated with redox homeostasis and cellular stress responses.

Based on the integrated results of network pharmacology analysis, functional enrichment analysis, molecular docking, and *in vitro* experiments, the Nrf2/HO-1 path-

way emerged as a potential regulatory pathway involved in Cor-induced ferroptosis in NSCLC. *NFE2L2* and *HMOX1*, which encode Nrf2 and HO-1, respectively, are key components of the cellular antioxidant defense system. Molecular docking analysis demonstrated favorable binding interactions between Cor and both Nrf2 and HO-1, suggesting that these proteins may represent potential targets of Cor. Furthermore, western blot analysis revealed significant reductions in Nrf2 and HO-1 protein expression following Cor treatment in A549 cells, consistent with the network pharmacology and molecular docking results. Collectively, these findings suggest that suppression of the Nrf2/HO-1 antioxidant pathway may contribute to the ferroptosis-inducing effects of Cor. However, the present study does not establish whether Cor directly targets Nrf2, HO-1, or both, nor does it clarify the relative contribution of each target to ferroptosis induction. Therefore, additional studies involving genetic or pharmacological modulation of Nrf2 and HO-1 are required to further elucidate the underlying mechanism.

Previous studies have shown that under oxidative stress or other exogenous stimuli, Nrf2 is released from Keap1-mediated repression, translocates to the nucleus, and activates the transcription of antioxidant and cytoprotective genes, thereby limiting oxidative damage and maintaining cellular redox homeostasis [63]. In the present study, Cor treatment markedly reduced Nrf2 and HO-1 expression, accompanied by increased ROS accumulation and decreased GPX4 and GSH levels in A549 cells. These findings are consistent with suppression of the Nrf2/HO-1 antioxidant pathway and enhanced ferroptotic signaling. Nevertheless, whether Cor regulates Nrf2 nuclear translocation, Keap1-dependent ubiquitination, or other upstream regulatory events remains unknown. Further mechanistic studies are needed to determine how Cor modulates Nrf2/HO-1 signaling and whether these effects directly contribute to ferroptosis induction in NSCLC cells.

5. Study Limitations

Several limitations of this study should be acknowledged. First, the ferroptosis-inducing effects of Cor were evaluated only in A549 cells; therefore, the findings require validation in additional NSCLC models, particularly drug-resistant cell lines such as A549/DDP. Second, the present study was limited to *in vitro* experiments, and *in vivo* studies are needed to confirm the antitumor efficacy of Cor and to evaluate its potential synergistic effects with conventional chemotherapeutic agents such as cisplatin. Third, although network pharmacology, molecular docking, and protein expression analyses implicated the Nrf2/HO-1 pathway in Cor-induced ferroptosis, direct target validation was not performed. Additional studies are required to determine whether Cor directly interacts with Nrf2, HO-1, or both. Finally, the effects of Cor on Nrf2 nuclear translocation, Keap1-dependent ubiquitination, and other upstream regu-

latory mechanisms were not investigated and warrant further exploration.

6. Conclusion

The present study demonstrates that Cor induces ferroptosis in A549 NSCLC cells, as evidenced by increased ROS production, Fe^{2+} accumulation, and lipid peroxidation, accompanied by depletion of GSH and altered expression of ferroptosis-related proteins. Network pharmacology, molecular docking, and western blot analyses collectively suggest that suppression of the Nrf2/HO-1 antioxidant pathway may contribute to the ferroptosis-inducing effects of Cor. These findings provide new insights into the antitumor mechanisms of Cor and support its potential as a therapeutic candidate for NSCLC.

Availability of Data and Materials

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

Author Contributions

YG: Conceptualization, data curation, formal analysis, investigation, and writing—original draft. JZ: Data curation, formal analysis, and investigation. YL: Conceptualization, methodology, supervision, project administration, and writing—review and editing. All authors reviewed the results, revised the manuscript critically for important intellectual content, and approved the final version of the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

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Conflicts of Interest

The authors declare no conflicts of interest.

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