Cordycepin Reverses Cisplatin Resistance in Non-Small Cell Lung Cancer by Activating AMPK and Inhibiting the AKT Signaling Pathway

Xiao-Zhong Liao
Department of Oncology, The First Affiliated Hospital, Guangzhou University of Chinese Medicine
Ying Gao
Laboratory of General Surgery, Sun Yat-sen University First Affiliated Hospital
Hong-Wei Zhao
Department of Oncology, the First Affiliated Hospital, Guangzhou University of Chinese Medicine
Mi Zhou
Department of Oncology, The First Affiliated Hospital, Sun Yat-Sen University
Dan-Lei Chen
Department of Oncology, The First Affiliated Hospital, Sun Yat-Sen University
Lan-Ting Tao
Department of Oncology, The First Affiliated Hospital, Guangzhou University of Chinese Medicine
Wei Guo
Department of Oncology, The First Affilited Hospital, Guanghzou University of Chinese Medicine
Ling-Ling Sun
Department of Oncology, The First Affiliated Hospital, Guangzhou University of Chinese Medicine
Chu-Ying Gu
Department of Oncology, The First Affiliated Hospital, Guangzhou University of Chinese Medicine
Han-Rui Chen
Department of Oncology, The First Affiliated Hospital, Guangzhou University of Chinese Medicine
Zhi-Wei Xiao
Department of Oncology, The First Affiliated Hospital, Guangzhou University of Chinese Mediine
Jia-Xing Zhang
Department of Oncology, The First Affiliated Hospital, Guangzhou University of Chinese Medicine
Mei-Fang He
Laboratory of General Surgery, The First Affiliated Hospital, Guangzhou University of Chinese Medicine
lizhu lin (✉ lizhulin26@yahoo.com)
the First Affiliated Hospital,Guangzhou University of Chinese Medicine https://orcid.org/0000-0001-8283-6554

Research

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Abstract

Background: Cisplatin (DDP) is the firs-line chemotherapeutic agent for the treatment of NSCLC. However, DDP resistance limits their usage to maximize the antineoplastic effect. The aims of this study were to investigate whether cordycepin (Cor) could reverse multidrug resistance (MDR) in NSCLC and to explore the underlying mechanisms.

Methods: Cell proliferation and apoptosis were analyzed in NSCLC cell lines in vitro and in vivo, parental and DDP-resistant A549 cells, treated with DDP alone or combination with Cor. Proteins of different signaling pathways were investigated between DDP-sensistive and -insensitive A549 cell lines by GO terms and KEGG analysis, and perturbations of the MAPK and PI3K-AKT signaling pathways were evaluated by western blot.

Results: Our data showed that Cor enhanced DDP inhibition of cell proliferation and promotion of apoptosis markedly compared to DDP alone group in both A549 and A549DDP. The synergic actions were associated with activation of AMPK and inhibition of AKT, mTOR and downstream P709S6K, S6 phosphorylation in the AKT pathway.

Conclusion: Cor/DDP combination has synergistic effect on inhibiting proliferation and promoting apoptosis of NSCLC cells in the presence or absence of DDP resistance.

Background

Lung cancer is the most common cancer and the leading cause of cancer-related death worldwide, and Non-small-cell lung cancer (NSCLC) is the major (85%) histological subtype of the disease cases of which is (1). In most patients, the disease is diagnosed at an advanced stage. Cisplatin (DDP) is the firs-line chemotherapeutic agent for the treatment of NSCLC attributing to its effective anticancer activity. DDP reacts with DNA to induce its characteristic biological effects leading to the DNA damage or activation of the irreversible apoptotic program (2). However, it has been widely reported DDP resistance limits their usage to maximize the antineoplastic effect (3). Most advanced NSCLCs acquire DDP resistance leading to tumor growth or distant metastasis over time. Several molecular mechanisms of drug resistance are explored including heterogeneitic cancer cells, tumor microenvironment and drug targets. One imperative mechanism is the alterations in drug metabolism by upregulating intracelluar glutathione (GSH) level (2). Moreover, the increasing content of GSH is a major contributing factor to cisplatin resistance by binding to drugs, interacting with ROS and participating in DNA repair processes (4).

Cordycepin (3'-deoxyadenosine) is the main active compound of the traditional herbal medicine *Cordyceps militaris*, which has effect on anti-inflammortory, antiviral and anti-tumor (5–7). A serious of studies have presented that Cor has anti-tumor activities including induction of cell apoptosis, inhibition of cell proliferation and migration (8, 9). Moreover, Cor was revealed to resensitize drug-resistant cancer cells in various tumors, including glioblastoma, ovarian cancer cell and lung cancer cells (10–13). And it

was reported that Cor had the inhibitory effect on NSCLC cells (10). However, the effects of Cor reversing DDP resistance in NSCLC are unclear.

AMP-activated protein kinase (AMPK) as a stress-response molecule is closely correlated to the tumor–suppressive functions by suppressing the activity of Akt and downstream effect mammalian target of rapamycin (mTOR), leading to cell growth inhibition and cell cycle arrest (14). Meanwhile, AMPK activation could inhibit p53 and p27 to exert tumor suppressor function. AMPK has been reported to directly phosphorylate p53 on serine 15 that is the common phosphorylation site by DNA damage response kinases (15). Notably, recent numerous studies proved that AMPK is a major regulator of drug metabolism closely correlated to drug resistance (14, 16). Shell *et al*. presented that metformin administration could significantly increase cancer chemosensitivity associated with mTOR inhibition following AMPK activation (17). Previous studies indicate that dysregulation of AKT is a prominent feature of various tumors including NSCLC (18, 19). In addition, *AKT* amplification and the mTOR signal pathway also play an important role in mediating DDP resistance in lung cancer (20).

The aim of this study was to identify the effect of Cor combination with DDP on cell proliferation and apoptosis in both DDP-sensitive and –insensitive NSCLC cells *in vivo* and *in vitro*. Furthermore, we explored underlying mechanisms for acquiring DDP resistance and provide potential targets of Cor for reversing DDP resistance in NSCLC cells. The present study will provide a potential novel therapeutic target for overcoming DDP resistance in patients with NSCLC.

Materials And Methods

Cell culture and reagents

Human lung cancer cell line A549 was gifted from the State Key Laboratory of Oncology in South China and cultured in RPMI1640 (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) (Invitrogen Corp, Carlsbad, CA, USA) and 100 U/mL streptomycin/penicillin (Gibco, Carlsbad, CA, USA) at 37 °C and 5% CO_2. A549DDP cells (DDP-resistant human lung cancer cells) were cultured in RPMI 1640 medium containing 10% FBS, penicillin and streptomycin. Moreover, DDP was added to the medium of A549DDP cells to final concentration to 2 μg/mL. Cordycepin and DDP were purchased from Sigma (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO). Cordycepin was dissolved in DMSO to make a 1 mM stock solution and stored at -20 °C for use. DDP was dissolved in physiological saline to make a 10 mM stock solution and stored at -20 °C for use.

Cell viability assay

The cell viability of NSCLC cell lines with Cordycepin and DDP were measured by CCK-8 kit (Dojindo Laboratories, 119 Kumamoto, Japan). A549 and A549DDP (8.0×10^3 cells per well) were seeded into 96-well plates. Cells were treated with different concentration of Cordycepin and DDP. After 24 hr, 10 μL CCK-8 solution was added into each well by incubation for 2 hr and cell viability was determined by a microculture plate reader at 450 nm (Thermo Scientific, Rockfor, IL, USA). Using the formula: proliferative

inhibition rate = (1- experimental group/control group) × 100% calculated proliferative inhibition rate. The 50% inhibitory concentration (IC_{50}) value was calculated by non-linear regression analysis using SPSS 20.0 software.

Synergy determination

The combination index (CI) was determined using isobologram analysis based on the Chou-Talalay method. The derived combination index equation for two drugs: CI = (D)1/(Dx)1+(D)2/(Dx)2. The CI values represent the modes of interaction between the two drugs. The CI < 1 indicates synergism, CI = 1 indicates an additive effect,

and CI > 1 indicates antagonism.

Colony formation assay

600 per well cells were seeded in six-well plates and cultured for two weeks. Then colonies were fixed in 4% paraformaldehyde and measured colonies number by crystal violet staining assay.

Cell cycle analysis

Cell cycle distribution was determined by Cell Cycle Detection Kit (4A Biotech Co., Ltd. Beijing, China). After treating with Cordycepin or DDP alone or in combination for 48 hr, cells were harvested and fixed in 70% ethanol overnight at 4 °C. Then cells were incubated with 100 μL RNase in 37 °C water bath for 30 min followed by staining with 400 μL propidium iodide (PI) for 30 min at 37 °C. At least 50,000 cells were detected cell cycle distribution for each sample by flow cytometer (Becton Dickinson, San Jose, CA).

Cell apoptosis assay

Cells were treated with different concentration of Cordycepin or DDP alone or in combination for 48 hr, and detected cell apoptosis using an Annexin-V-FITC apoptosis detection kit (4A Biotech Co., Ltd. Beijing, China). All samples were analyzed by flow cytometer.

Western blotting

Cells were treated with Cordycepin or DDP for 48 hr and washed with PBS following with lysing in sodium dodecyl sulfate (SDS) lysis buffer at 100 °C for 20 min. Lysates were centrifuged at 4 °C for 15 min, and then the supernatant were collected. Equal lysate were denatured in 10% SDS sample buffer and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidenedifluoride membranes (0.22 μm, Millipore, MA, USA). The polyvinylidenedifluoride membranes were blocked in 5% skim milk for 1 hr and incubated with anti-p-P53, anti-P53, anti-cyclinD1, anti-P27, anti-caspase-3, anti-p-caspase-3, anti-Bcl-2, anti-Bax, anti-AMPK, anti-p-AMPK, anti-mTOR, anti-p-mTOR, anti-S6, anti-p-S6, anti-P70S6K, anti-p-P70S6K, anti-AKT, anti-p-AKT, anti-GAPDH (1:1000) overnight at 4 °C, followed by incubating with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA, USA) at room temperature for 1 hr. Protein bands were visualized using an enhanced chemiluminescence kit (Beyotime, Shanghai, China) and detected with an

electrochemiluminescence system (hermo Fisher Scientific, MA, USA). The densitometry of the protein bands were measured using the Image J (NIH image software) and normalized to their relevant controls.

Potential target recognition based on PharmMapper

PharmMapper (http://lilab.ecust.edu.cn/pharmmapper/index.php) is supported by a large, in-house repertoire of a pharmacophore database extracted from all the targets in TargetBank, DrugBank, Binding DB and PDTD. PharmMapper contains more than 7,000 receptor-based pharmacophore models (covering information related to 1,627 drug targets, 459 of which are human protein targets). First, the SDF of RA was downloaded from PubChem Compound (https://www.ncbi.nlm.nih.gov/pccompound/) and then uploaded to PharmMapper. Following proper parameter setting, target identification was then carried out, and information regarding the top 300 potential protein targets was obtained.

RNA- Seq analysis

Total RNA was extracted using Trizol reagent (Invitrogen, CA, USA) following the manufacturer's procedure. RNA-seq analysis was completed using Illumina Hiseq 4000 (LianChuan Sciences, Hangzhou, China). Gene ontology (GO) terms for functional categorization were carried out according to molecular function, biological process and cellular component ontologies with an E-value threshold of 10^{-5}. The pathway assignments were performed by sequence searches against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database and using the BLASTX algorithm with an E-value threshold of 10^{-5}. Fragments per Kilobase of exon medel per Million mapped reads values were used to measure the expression abundance of each assembled transcript. Between the two samples, a minimum of a two-fold difference expression were considered as expression differences.

Measurement of GSH

Intracellular levels of GSH were determined by using GSH-Gloglutathione Assay Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Mouse xenograft model

2×10^6 A549 or A549DDP cells were suspended in 100 μl of narmal saline and subcutaneously injected into unilateral axillary fossae of BALB/c nude mice (4 week old, n = 5 per group). Forty mice were purchased from Charles River Laboratories (Beijing, China) and randomly divided into eight groups for the construction of a subcutaneous tumorigenesis model. The tumor sizes were measured and the volumes were calculated according the formula: $0.5 \times \text{length} \times \text{width}^2$. When the volume of tumors reached 450–500 mm^3, the mice received an equal volume of Cordycepin (15 mg/kg), DDP (1.5 mg/kg), Cordycepin (10 mg/kg) and DDP (1 mg/kg) via intraperitoneal injections twice a week. At the end point of study, mice were sacrificed by cervival dislocation and tumors were excised. The animal experiments were administered according to the guidelines of Institution Animal Care and Use Committee and all protocols were approved by the First Affiliated Hospital of Sun Yat-sen University (Guangzhou, China).

Statistical analysis

All experiments were repeated at least three times. All values are presented as means ± standard deviation (SD). Statistical analysis was performed by the Student's t test. $P < 0.05$ was considered significant. All statistical analysis was performed using SPSS 22.0 software (Chicago, IL, USA).

Results

Cor reverses cisplatin resistance in NSCLC cells

Previously we established A549DDP cell line with persistent DDP resistance and the resistance index (RI) value was 11.19 ± 0.50 (21). To investigate the effect of Cor on NSCLC cells, we treated A549 and A549DDP cells with various concentrations of cordycepin for 48 hr and measured cell viability by CCK-8 assay. Our data showed that Cor significantly induced concentration –dependent NSCLC cell death, with IC_{50} values of 74.05 μg/mL in A549 cells and 85.26 μg/mL in A549DDP cells (Fig. 1A and 1B). The ability of Cor to inhibit NSCLC cell proliferation was similar between A549 and A549DDP cells. We also found combination treatment with Cor and DDP significantly increased the sensitivity of NSCLC cells to DDP (Fig. 1C and 1D). Moreover, the combination index of Cor and DDP is below 1 in both A549 and A549DDP cells, which indicated the combination of Cor and DDP could exert synergic action in NSCLC cells (Fig. 1E and 1F). These data suggest that Cor has the similar effector on inhibiting tumor cell proliferation in A549 and A549DDP cells, and Cor resensitizes NSCLC cells to DDP.

The combination of Cor and DDP suppresses cell proliferation in NSCLC cells

Colony formation assay and cell cycle arrest were performed to evaluate the effects of combination of Cor and DDP on proliferation in A549 or A549DDP cells. When Cor and DDP were combined, cell colonies formation efficiency of A549 and A549DDP were markedly suppressed with dose-dependent manner as compared to DDP single treatment groups (Fig. 2A, 2B, 2C and 2D). Further, we analyzed whether Cor combined with DDP-induced inhibition of cell proliferation was related to cell-cycle regulation based on DNA content by flow cytometry analysis. With A549 and A549DDP cells treated with DDP individually, cells in the G0/G1 phase were 35.03% and 35.66%, respectively. With combination treatment with Cor and DDP, the cell cycle arrest of A549 and A549DDP were concentration-dependent increased (Fig. 2E, 2F, 2G and 2H). Collectively, our data showed that Cor enhanced DDP treatment effect associated with tumor cell cycle arrest.

As P53, cyclinD1 and P27 are G1-S modulators (22), we examined the expression of these cell-cycle-regulated proteins by western blotting. As indicated in Fig. 2I and 2J, p-P53 and P27 came up to some extent with a decrease in cyclinD1 dese-dependently of the A549 and A549DDP cells treated with Cor plus DDP, whereas it did not alter protein expression of P53. In addition, Cor promotes the phosphorylation of the p53 tumor-suppressor protein to induce NSCLC cells apoptosis. These results are evidence that the synergistic effect of Cor with DDP on cell cycle arrest at G0/G1 mainly through the key G1-phase regulatory molecules P53, cyclinD1 and P27.

Cor enhances apoptotic effect of DDP on NSCLC cells

To further assess the effects of Cor combined with DDP; we incubated cells with DDP alone or with Cor to investigate the two drugs effect on cell apoptosis by AnnexinV-FITC/propidium iodide staining and western blotting. Cell apoptosis induced in a dose-dependent manner when cells were incubated with the combination of Cor and DDP. In comparison with DDP, the combination of Cor (μM) and DDP (μM) had a greater effect on cell apoptosis of A549 and A549DDP, respectively (Fig. 3A, 3B, 3C and 3D). These results document that DDP combined with Cor has the prior in apoptotic effect on NSCLC cells than single treatment with these drugs.

The Bcl-2 family proteins including both pro- and anti-apoptotic proteins are key regulators in the mitochondrial apoptotic pathway. The low expression of Bcl-2, a founder member of the Bcl-2 family of apoptosis regulator protein, leads to apoptosis by activating pro-apoptotic protein Bax oligomerization to releasing cytochrome c into the cytosol, and activates caspases including caspase-3 to cause cascade reactions that cleave essential proteins complement throughout the cell (23). Western blot assay revealed that the amounts of caspase-3 cleaved, caspase-3 and pro-apoptotic protein Bax were markedly increased in both A549 and A549DDP by treatment with the two drugs combined as compared with the DDP alone, while the expression of anti-apoptotic protein Bcl-2 were decreased (Fig. 3E and 3F). These data indicated that the combination of two drugs had a greater effect than DDP treatment individually, which might induce cell apoptosis including upregulation of Bax and downregulation of Bcl-2 to facilitate activation of the caspase cascade.

Potential proteins and signaling pathways involving in NSCLC cells resistance to DDP by GO and KEGG analyses

Based on RNA sequencing (RNA-Seq) screening of differentially expressed genes (DEGs), for further understanding of functions and signaling pathways involved in NSCLC cells resistant to DDP, GO analysis and pathway enrichment analysis were performed. Our findings showed that there were 304 differential genes between A549 and A549DDP cells (Table S1). GO analyses revealed that these DEGs were associated with biological process (BP), cellular component (CC) and molecular function (MF) (Fig. 4A, 4B and 4C). KEGG pathway analysis showed that AMPK and AKT signaling pathways and Glutathione metabolism were mainly involved in NSCLC cells resistant to DDP (Fig. 4D). These results indicated that the major cause for the onset of DDP resistance might be due to intracellular GSH levels as well as AMPK and AKT signaling pathways.

Cor induces a dose-dependent reduction in GSH-mediated DDP resistance in NSCLC cells

We obtained top 241 potential targets of Cor using Pharma Mapper, which indicated that Cor had superb druggability with these potential targets (Table S2). Combined with KEGG pathway information analysis, we identified glutathione metabolism, AMPK and AKT signaling pathways were involved in Cor treatment of NSCLC (Fig. 5A and 5B). We quantified intracellular GSH)level to explore the DDP resistance

mechanism of A549DDP cells. GSH expression was significantly higher in the resistant A549DDP cells than that in A549 cells (Fig. 5C). The resulting increasing intracellular GSH levels may explain the attenuation of DDP-mediated apoptosis effects. This is supported by the demonstration that prominent elevations in GSH levels have been correlated directly with DDP resistance in a panel of tumor models (24). Our results showed that GSH expression was downregulated in A549 cells but not in A549DDP cells by treatment with DDP alone. When an increase in Cor concentration with DDP, the GSH level were significantly reduced leading to mitochondrial-mediated apoptosis. These results indicate that upregulation of GSH for DDP detoxification is an important way for NSCLC cells to resistant. More importantly, Cor might reverse resistance to DDP in NSCLC cells by down-regulating the expression of GSH.

Cor reverses DDP resistance via activation of AMPK and inactivation of AKT signaling pathways in NSCLC cells.

Based on above results, Cor exhibits a significant reverse effect on NSCLC cells resistance to DDP by activating AMPK and inactivating AKT signaling pathways. We further select several target proteins regulating the signaling transduction pathways to investigate in order to verify the reliability of the bioinformatics data by western blotting. After 48 hr of drug treatment, DDP promoted phosphorylation of AMPK but did not significantly affect total amount of proteins including AMPK, AKT, mTOR, P70S6K and S6. Cor, in combination with DDP, not only upregulated AMPK phosphorylation but also reduced p-AKT, p-mTOR, and downstream p-P70S6K, p-S6 in both A549 and A549DDP cells compared with DDP single treatment in a dose-dependent manner (Fig. 6A and 6B). Moreover, the increased sensitivity was seen in both DDP-sensitivity and –resistant cell lines, drawing the rational conclusion that Cor and DDP combination might be related to the AMPK and AKT signaling pathways. Our results suggested Cor treatment activated AMPK and inhibited the activity of AKT, which associates with phosphorylates mTOR, thereby depressing the phosphorylation of S6K to suppress proliferation.

Cor combines with DDP potentiates tumor regression in NSCLC xenograft mice model

To further determine the efficacy of Cor and DDP combination treatment on tumor regression, A549 or A549DDP cells were implanted subcutaneously in nude mice. After tumor attained size of 450 to 500 mm^3, mice were treated with drugs twice a week as mentioned in the methods section (Fig. 7A). Xenograft tumors grew slower in combination of Cor and DDP-treated group compared to that in the control group (Fig. 7C and 7D). While administration of Cor or DDP alone marginally inhibited tumor growth in comparison with the control group, a combination of Cor + DDP administration regressed the tumor volume to a greater extent (Fig. 7E, 7F, 7G and 7H). We also noted that Cor did not affect the health of the mice (Fig. 7B). Our data suggest that Cor combined with DDP can significantly inhibit tumor growth compares with Cor or DDP single treatment. Thus indicates that Cor not only reverses NSCLC resistance to DDP but also alleviates DDP-induced weight loss.

Discussion

DDP is the first member of a class of platinum-containing anticancer drugs, which causes DNA crose-linking to trigger cell apoptosis (25). Although DDP is widely known anticancer agent, a significant risk of drug resistance is the major limitation the clinical usefulness. Thus, it is high priority to explore combined strategies to improve the therapeutic effect of DDP on NSCLC. In the present study, we demonstrated that Cor and DDP were synergistic at inhibiting cell proliferation and inducing apoptosis of NSCLC in the presence or absence of DDP resistance. Moreover, the AMPK and AKT signaling pathways as well as glutathione metabolism were involved in attenuation of DDP resistance in NSCLC cells by Cor and DDP combination treatment.

The mechanisms contributing to DDP resistance including DDP damage/repair proteins, prevention of drug to reach DNA target, and activating signaling pathways to prevent apoptosis (2). Apoptotic inhibitor molecules overexpression directly or indirectly attenuates the activation of caspase cascade contributing to DDP resistance (26). Hayakawa *et al*. reported that HER-2 overexpression involved in DDP resistance is due to inactivation of the pro-apoptotic protein Bad following its phosphorylation by Akt (27). Several measures have been proposed to decrease DDP resistance. Inhibition of miR-196a is revealed to be able to restore sensitivity to DDP in lung cancer via downregulation of drug-resistant proteins expression and inhibition of drug efflux (28). mTOR inhibitor (CCI-779) appears to reverse DDP resistance by increasing the growth inhibition and enhancing the apoptotic effect in DDP resistant cells (29). Rosmarinic acid reverses NSCLC resistant to DDP by activating the MAPK signaling pathway (21). Our results indicated that AMPK and AKT signaling pathways involved in NSCLC cells resistant to DDP by GO and KEGG analysis, which provided potential targets for overcoming DDP resistance.

Cor, as traditional Chinese medicine, is revealed to play an important role in the treatment of cancer including inhibiting proliferation of tumors and reducing toxicity of conventional therapy in recent years (30). It is noteworthy that Cor in reversing drug resistance has made some achievements, especially used in combination. Several combination therapies using Cor with other chemotherapeutic drugs have been explored in different cancers. Cor combined with temozolomide synergistically inhibits glioma cells via AMPK signaling pathway (11). The combination treatment of Cor and DDP shows an enhanced apoptotic effect on OC3 human oral cancer through the JNK/caspase-7 signaling pathway (31). Wei *et al*. confirmed that Cor in combination with gefitinib can be used as a new therapeutic strategy for gefitinib-resistant lung cancer (10). It is reasonable to suggest that there are different mechanisms underlying the pharmacological effect of combination with Cor and other chemotherapeutic drugs in different types of cancers. Therefore, we investigated effects of Cor in combination with DDP on reducing DDP resistance in NSCLC and explored the possible underlying mechanisms. We demonstrated in the present study that the combination between Cor and DDP had synergistic effect on proliferation inhibition and apoptosis induction in A549 and A549DDP cells. These findings indicated that Cor might enhance DDP treatment in both sensitive and insensitive NSCLC cells. Hence, a combination of Cor and DDP provides a novel approach in the treatment of NSCLC especially for DDP resistant patients.

Based on differentially expressed genes (DEGs) between A549 and A549DDP cells in RNA-Seq, we found glutathione metabolism, AMPK and AKT signaling pathways might play major roles in NSCLC cells resistant to DDP. Furthermore, we found Cor in treatment of NSCLC were mediated though reducing glutathione metabolism, activation of AMPK and inhibition of AKT signaling pathways based on Pharma Mapper analysis of 241 potential protein targets for Cor. On the one hand, our results showed that intracellular GSH in A549DDP was significantly increased than that in A549, which is consistent with reports that in a number of cisplatin-resistant tumor models (24). It is generally accepted that the increased conjugation reaction between GSH and cisplatin is a significant factor in resistance (2). Studies have demonstrated that GSH elevated by cisplatin contributes to increase DNA repair or increase the inhibitory effect on apoptosis (24, 32). Our results also showed that GSH as well as Bcl-2 was overexpression in A549DDP cells. It is consistent with reports that higher intracellular GSH levels have correspondingly overproducing the Bcl-2 protein, which may associate with the anti-apoptotic functions of Bcl-2 (33, 34). Conversely, downregulation of GSH levels by Cor potentiated DDP cytotoxicity, which might in fact increase in the Bax: Bcl-2 ratio as well as activation of Caspase cascade contributing to cell apoptosis. Members of the Bcl-2 family localized in the mitochondria are key players involving in regulating apoptosis (35). This understanding is consistent with overexpression *bcl-2* is facilitated with DDP resistance, and this is likely associated with an increase in GSH levels (34). On the other hand, our results showed that the attenuation of DDP resistance was associated with upregulation of p-P53. Studies have proposed several genes transactivated by p53 as a result of Cor and DDP combination exposure are associated with DNA damage, cell cycle arrest and apoptosis (36, 37). When DNA damage overwhelms cellular repair capacity, the well-orchestrated cell apoptosis activates with Cor and DDP-induced Bax translocated from the cytosol to the mitochondria, where cytochrome *C* activates the caspase 3 pathway, and leads to apoptosis (38). Our results indicates that Cor-mediated apoptosis of NSCLC cells is associated with the decreased expression of Bcl-2, accompanying with overexpression of Bax and p53.

It should be noted that many of the studies to define the apoptotic function of p53 is dependent on several DDP-induced upstream signaling pathways that activate the tumor-suppressor protein by upregulating tis phosphorylation (20, 39). AMPK activation is also revealed to cause cell cycle arrest associated with stabilization of p53 and the cyclin-dependent kinase inhibitor p27 (14). It is reported that a lack of p53 phosphorylation due to defective upstream activation of AMPK is the probable mechanism contributing to drug resistance (14). In this regard, the AMPK protein is activated by Cor and DDP, contributes to activate the tumor-suppressor activator p53. Once the AMPK is activated by combination between Cor and DDP, downstream signaling is propagated through either the p53 pathway, which in turn activates the apoptosis-related proteins such as Bax and the cell cycle-related proteins including p27, or the suppresses mTOR/S6K pathway. Basal activity of the AMPK pathway facilitates the induction of Bax and P27 by Cor and DDP in a p53-dependent manner. Meanwhile, AMPK amplification is revealed to reduce expression of the downstream mTOR, which is an essential component of the mTOR/p70S6K1 pathway (40). Cor was found to inhibit NSCLC cells proliferation by decreasing phosphorylation of 70S6K1 and S6, which was greatly associated with mTOR inhibition following AMPK activation. As a

result, Cor reversed resistance of NSCNC cells to DDP through upregulating expression AMPK related to mTOR/p70S6K1 pathway to reduce cell proliferation.

We then unveiled that Cor increased sensitivity in both DDP-sensitive and –resistance cell lines related to inactivate AKT pathway. In many tumors the protein kinase Akt is hyperactivated and p-Akt is the activated form of Akt that has biological function. Akt has been reported to be a negative regulator of AMPK and upstream positive regulator of mTOR (14). Once activated, Akt activation of the mTOR pathway leads to increase cell proliferation and reduced cell apoptosis involved in the pathogenesis of cancer (2). The AKT and mTOR constitutive activation confers drug resistance to many types of cancer, including NSCLC. Activated downstream protein S6 in mTOR signaling often regulates cell proliferation and survival, thus diminishing the inhibitory effect of DDP on NSCLC, with resultant resistance of DDP to the drug. As results above, AMPK and AKT signaling pathways are intimately associated with DDP resistance. On contrast, Cor appears following perturbation of the pathways by dysfunction of Akt and activation of AMPK, which are upstream regulators of mTOR. This perturbation in the two pathways is consistent with the finding that Cor effectively inhibition of cellular growth due to mTOR inhibition via AMPK and AKT signaling pathway (11).

Conclusions

Taken together, we demonstrated that Cor and DDP had synergistic effect on inhibiting proliferation and inducing apoptosis in the DDP-sensistive and -insensitive NSCLC cells. Cor mediates enhanced DDP therapeutic efficacy, but more importantly reverses DDP-resistance of NSCLC associated with upregulation of AMPK and inactivation of AKT signaling pathways (Fig. 8). Based on these findings, it is worthwhile to further explore the potential application of Cor combination with DDP in treatment for NSCLC patients, especially for DDP resistant patients.

Abbreviations

Non-small-cell lung cancer (NSCLC); DDP (cisplatin); glutathione (GSH); Cordycepin (Cor); AMP-activated protein kinase (AMPK); mammalian target of rapamycin (mTOR); fetal bovine serum (FBS); dimethyl sulfoxide (DMSO); 50% inhibitory concentration (IC50); combination index (CI); propidium iodide (PI); Gene ontology (GO); Kyoto Encyclopedia of Genes and Genomes (KEGG); standard deviation (SD); resistance index (RI); RNA sequencing (RNA-Seq); biological process (BP); cellular component (CC); molecular function (MF); differentially expressed genes (DEGs);

Declarations

Ethics approval and consent to participate

The research protocol was approved by the Institutional Ethical Review Boards of the First Affiliated Hospital of Sun Yat-sen University. All animal experiments were performed in accordance with animal

protocols approved by the Institutional Animal Care and Use Ethics Committee of First Affiliated Hospital of Sun Yat-sen University.

Consent for publication

All the authors are consent for publication of the article.

Availability of data and material

The datasets generated for this study are available on request to the corresponding author.

Competing Interests: None.

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Authors' Contibutions

L.Z.L and M.F.H designed the research study; X.Z.L, Y.G and M.Z performed the majority of the experiments and data analysis; L.L.S and L.T.T contributed to specimen preparation; J.H.L, W.G, H.R.C, LY and Z.Z.C assisted with in vivo experiments. X.Z.L and Y.G wrote the manuscript. All authors approved the final version of the manuscript.

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Figures

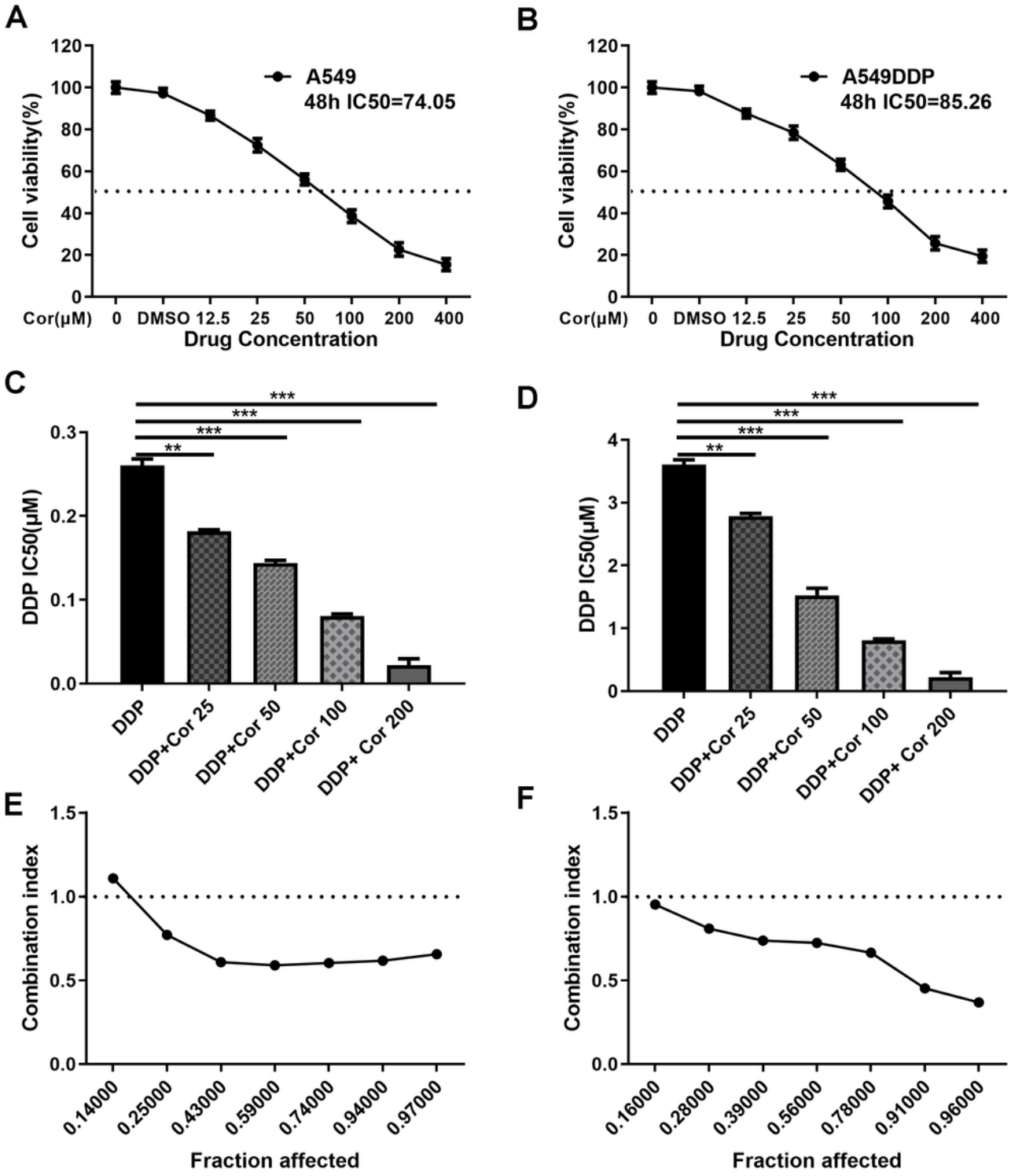

Figure 1

Proliferative inhibitory effect of Cor, DDP and the combination treatment on non-small-cell lung cancer cells (NSCLCs) or DDP-resistant NSCLs. The survival rations of A549 cells (A) or A549DDP (B) with different Cor concentration were detected by CCK-8 assay. IC50 of DDP was detected for A549 cells (C) or A549DDP (D) with DDP and different concentration of Cor in combination. Synergistic effects between Cor and DDP were presented as Fa=CI plots for A549 cells (E) or A549DDP (F).

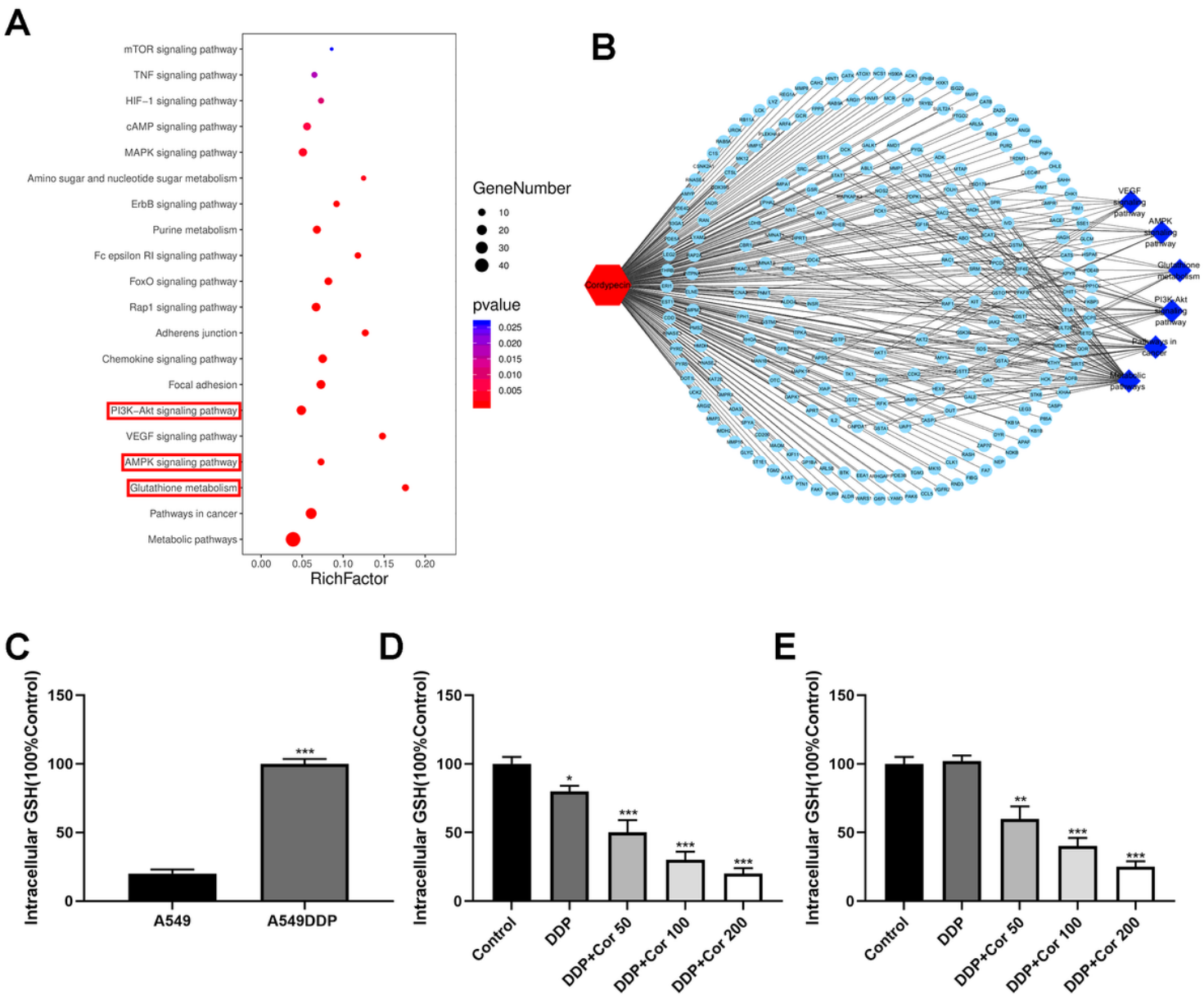

Figure 2

Inhibition of cell proliferation and cell cycle arrest by DDP or combination of Cor and DDP in NSCLC cell lines. Representative images (left) and quantification (right) of A549 cells (upper panel) or A549DDP (lower panel) with 0, 50, 100, 200 μg/mL Cor combined with 1.5 μg/mL or 15 μg/mL DDP for 48 hr for colony formation assay (A)for cell cycle assay (B); for apoptosis assay (C).

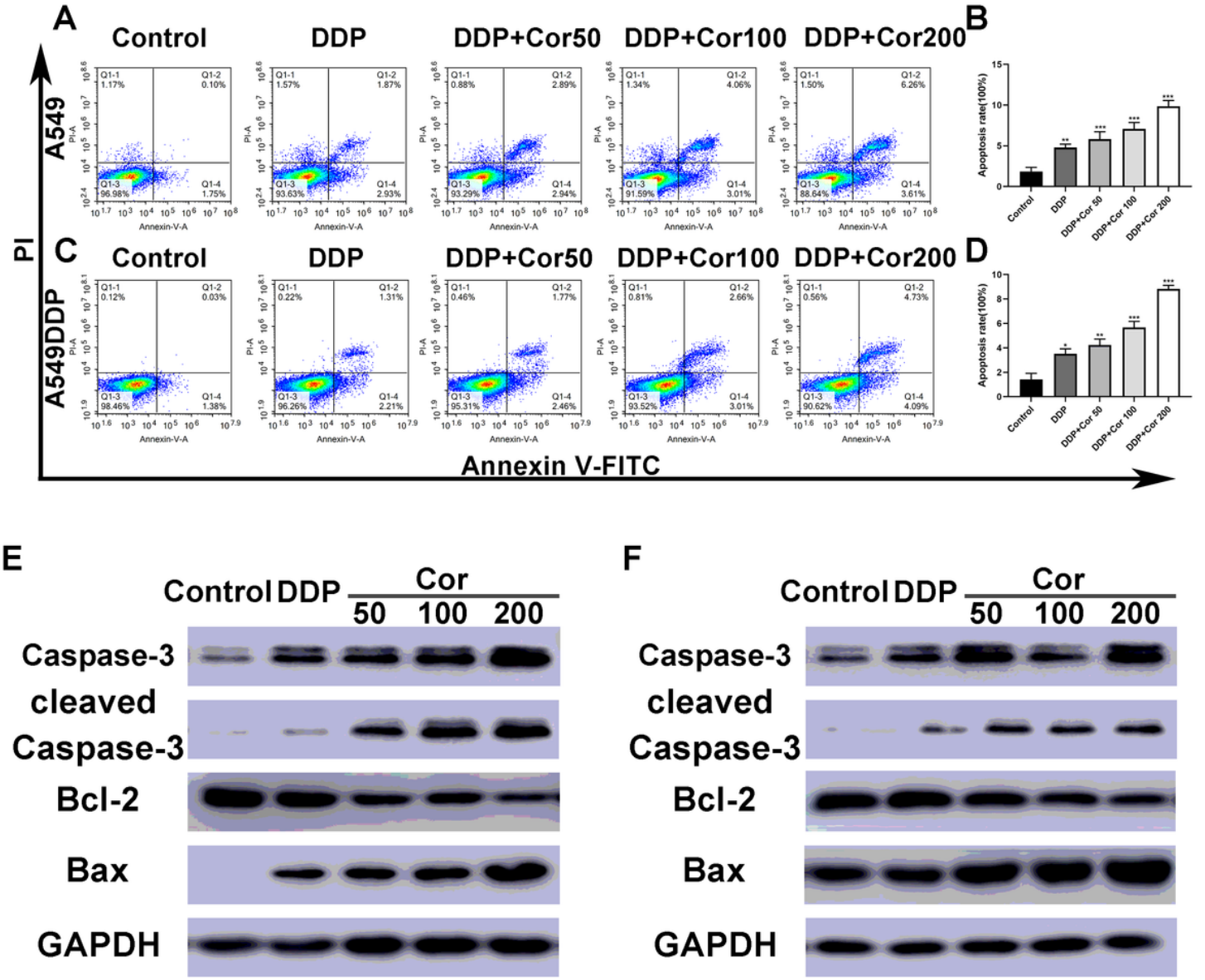

Figure 3

Induction of apoptosis by DDP with or without Cor in NSCLC cell lines. Induction of apoptosis with DDP or combined with different concentration of Cor in both A549 (A and B) and A549DDP (C and D) cell lines using Annexin-V-FITC/PI staining. Western blot analysis of caspase-3, cleaved caspase-3, Bcl-2 and Bax and GAPDH was used as control in A549 (E) or A549DDP cell lines treated with DDP or combination between DDP and different concentration of Cor.

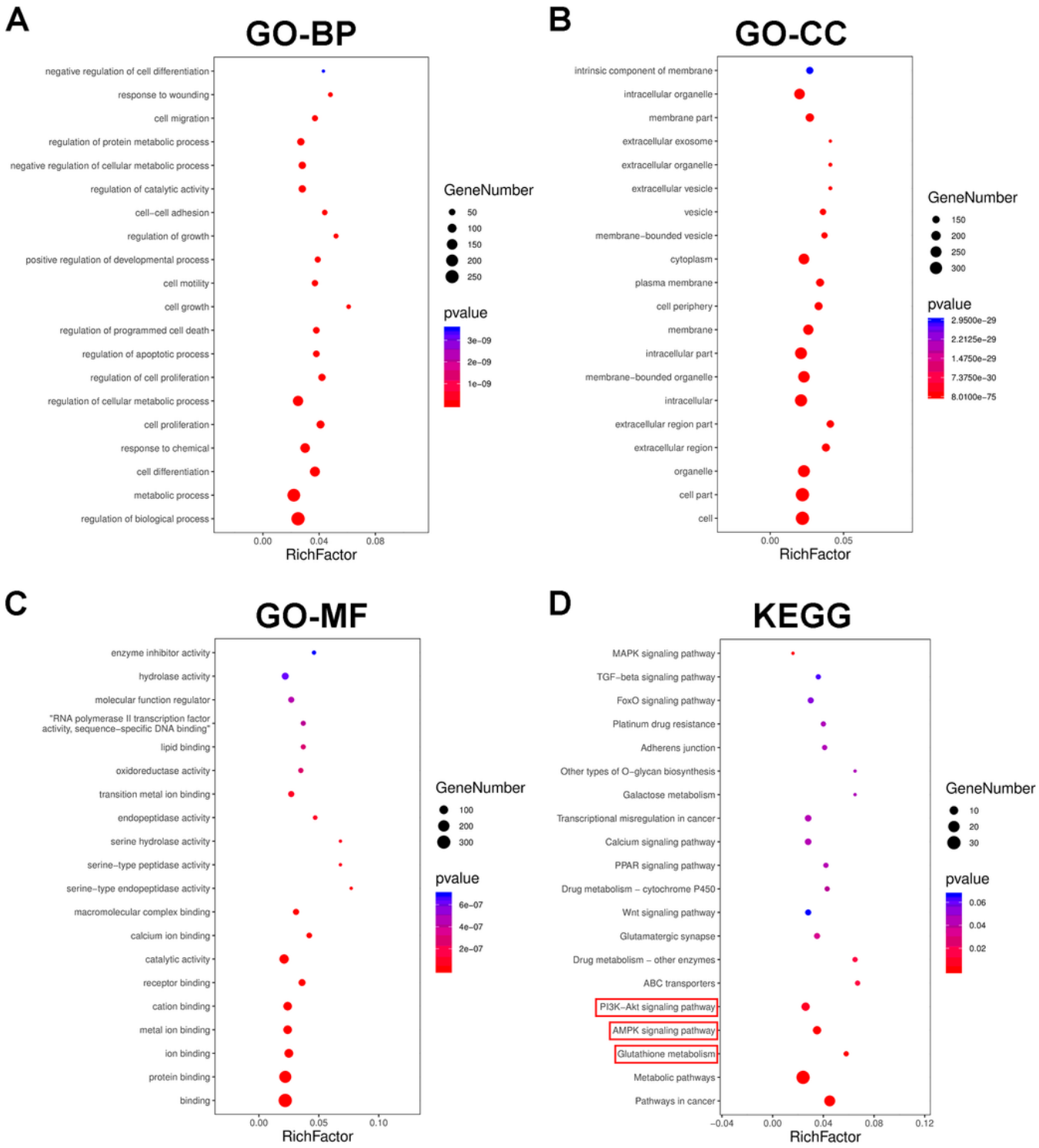

Figure 4

The transcriptional changes between A549 and A549DDP cell lines by GO and KEGG pathway analysis. GO term analysis of differently expression genes (DEGs) in Biological Process (A), Cellular Component (B) and Molecular Function (C). (D) KEGG pathway analysis of signal transduction pathways involved in NSCLC cell lines resistant to DDP.

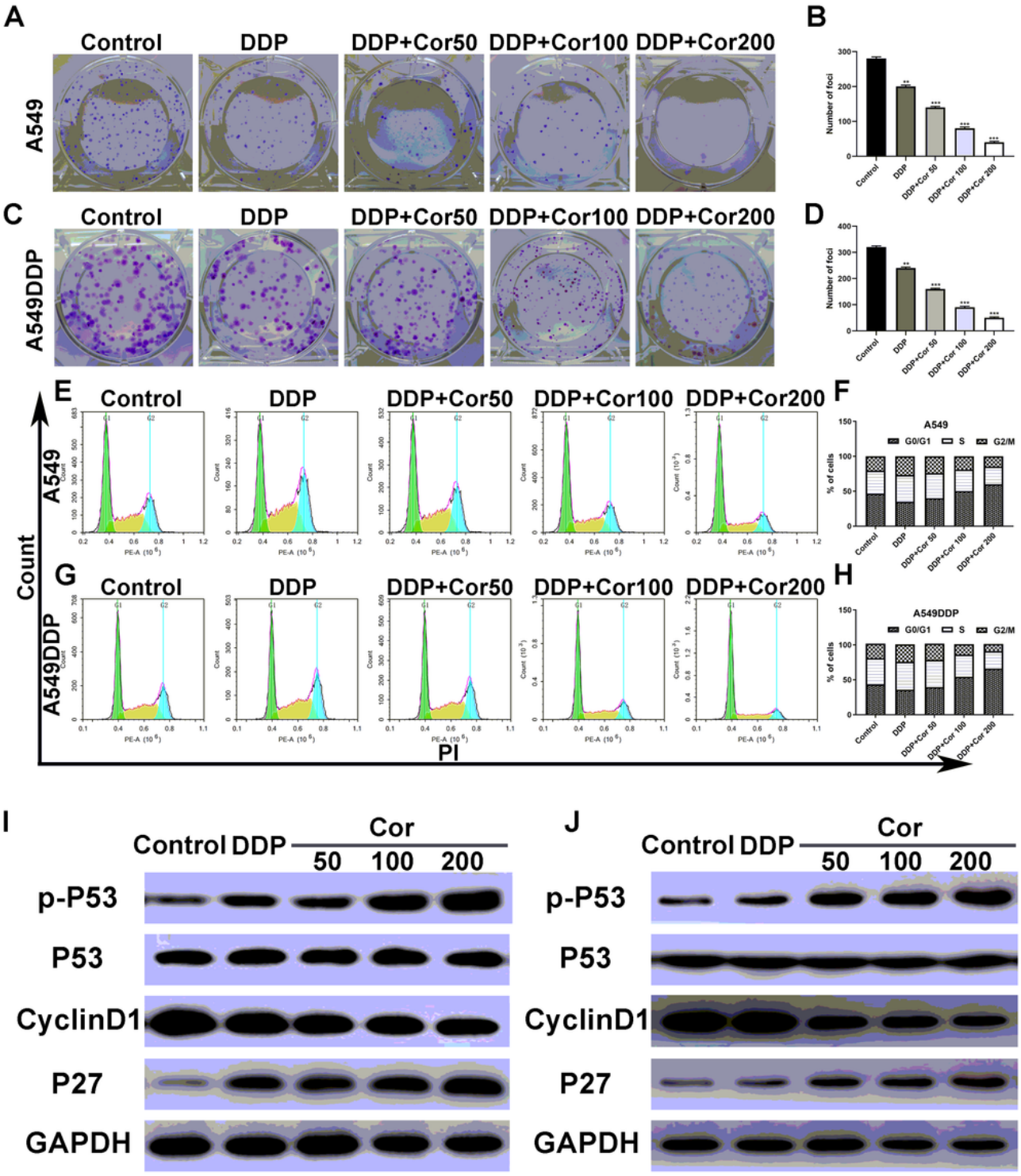

Figure 5

Suppression of intracellular glutathione (GSH) by Cor and DDP in NSCLC cells. (A) KEGG pathway analysis of signal transduction pathways involved in Cor treating NSCLC cells. (B)Potential targets by Cor in NSCLC cells using Pharma Mapper. (C) Measuring the intracellular glutathione in A549 and A549DDP. Intracellular glutathione assay of A549 cells (D) or A549DDP (E) with 0, 50, 100, 200 μg/mL Cor combined with 1.5 μg/mL or 15 μg/mL DDP for 48 hr.

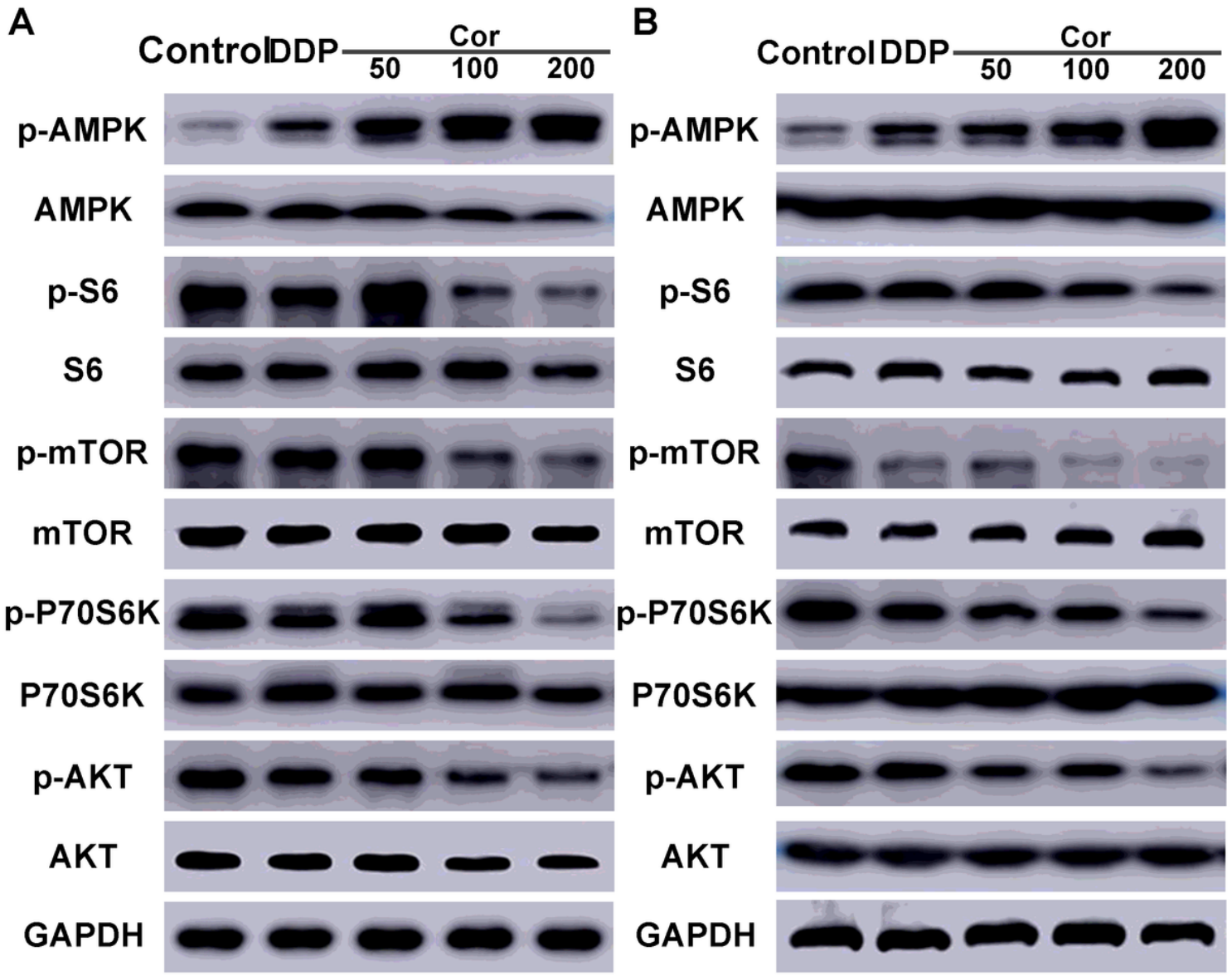

Figure 6

Western blot assay of AMPK, p-AMPK, S6, p-S6, mTOR, p-mTOR, P70S6K, p-P70S6K, AKT, p-AKT in A549 (A) and A549DDP (B) cell lines treated with DDP or combination between DDP and different concentration of Cor.

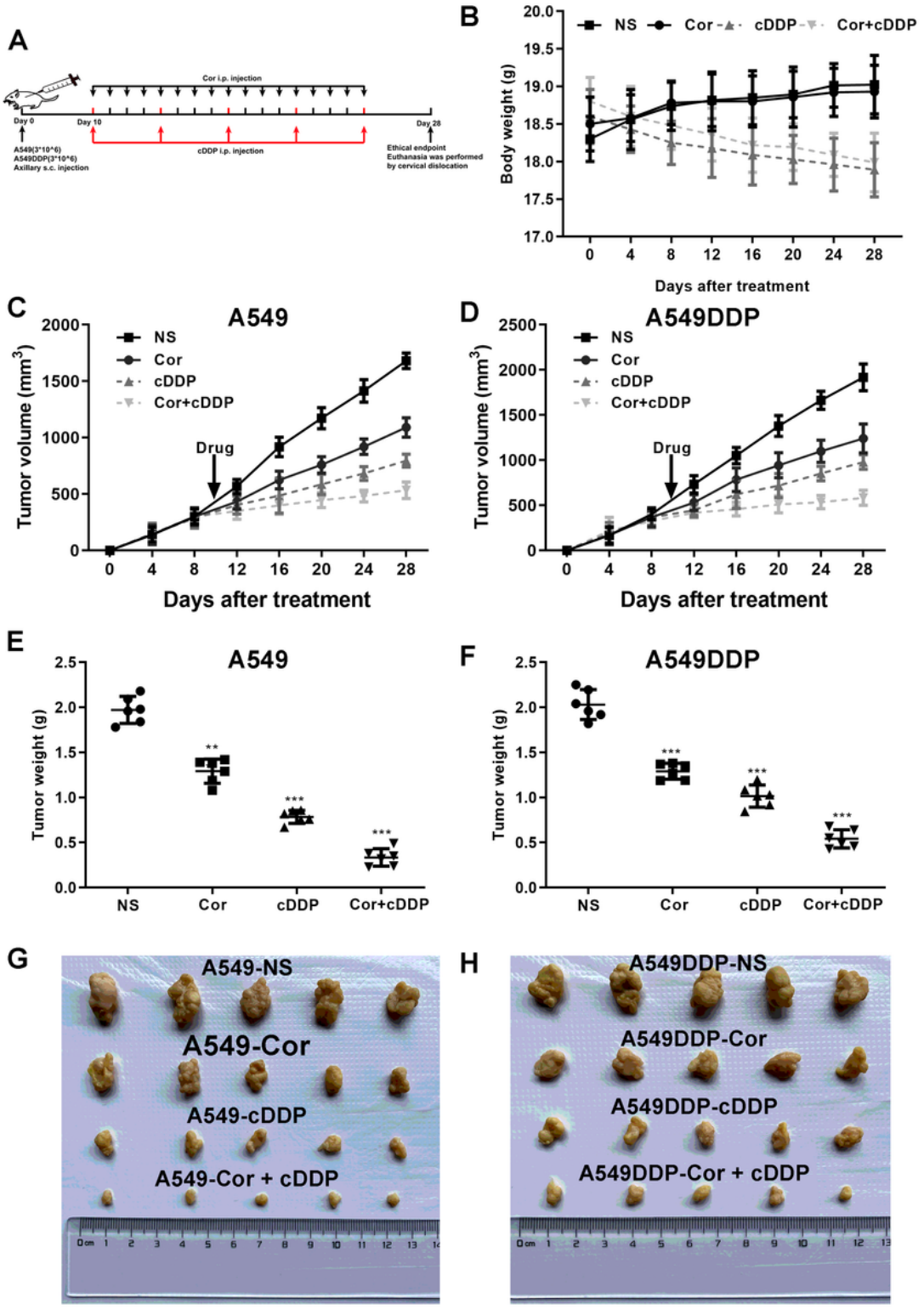

Figure 7

The effect of DDP or Cor alone or combined between DDP and Cor compared with control on A549 or A549DDP cells xenograft tumor growth. (A) The timeline of A549 or A549DDP cells incubation subsequently treated with drugs. (B) Time course of mice body weight. Time course of A549 (C) or A549DDP (D) cells tumor growth. Dots graphs represent the weight of the A549 (E) or A549DDP (F) at the

indicated time point of different treatments. Visual comparison of the A549 (G) or A549DDP (H) dissected tumor tissues.

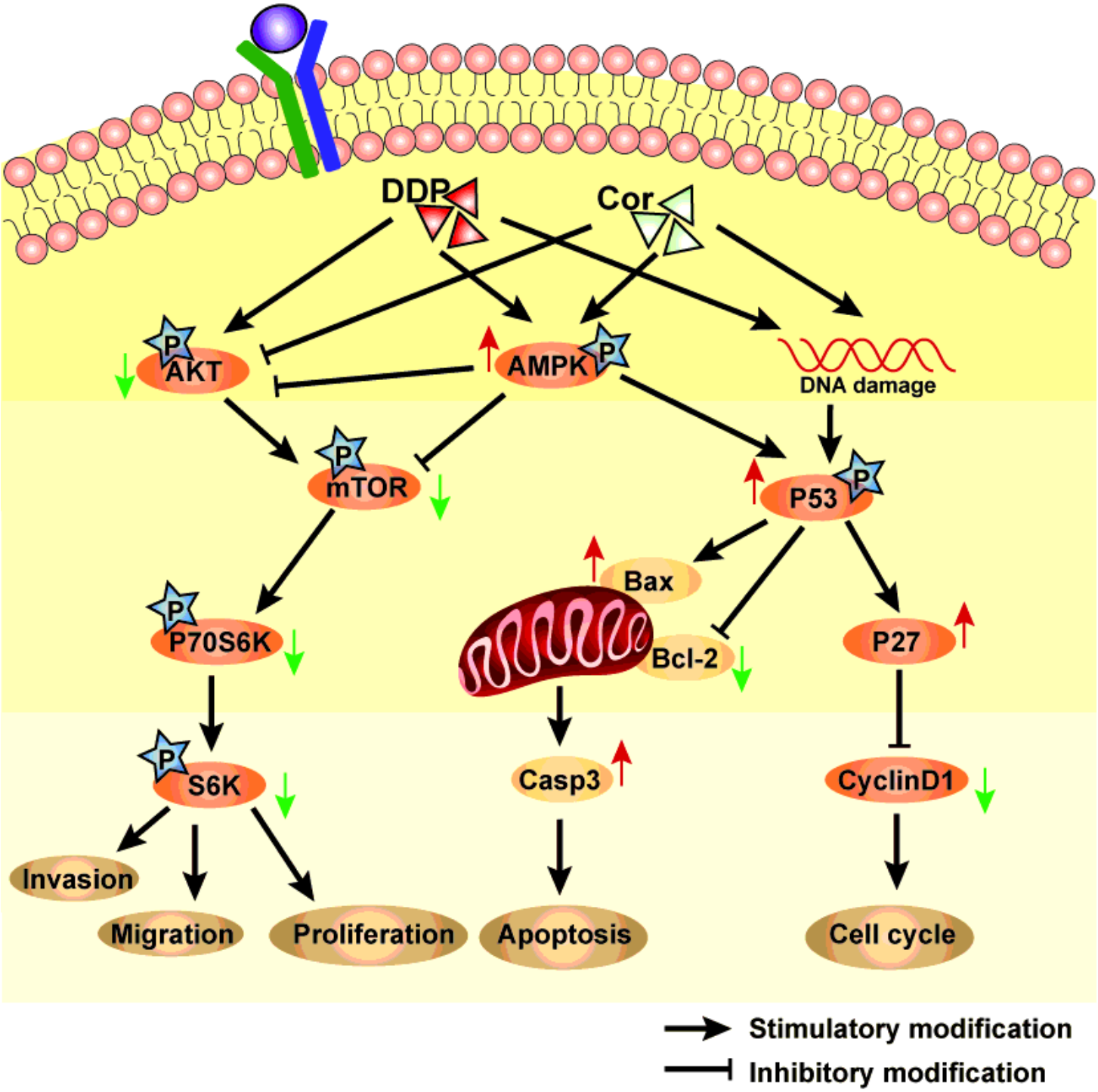

Figure 8

An overview of pathways involved in mediating Cor and DDP-induced cellular effects. Cell proliferation or cell apoptosis will depend on the relative intensity of the signals generated and the crosstalk between the pathways involved.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- table1.docx
- table2.docx