# Suppression of DRP1-mediated mitophagy increases the apoptosis of hepatocellular carcinoma cells in the setting of chemotherapy

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**Abstract.** The efficacy of chemotherapy for hepatocellular carcinoma (HCC) remains unsatisfactory, primarily due to inherent self-defense mechanisms (e.g., mitophagy and autophagy). In the present study, we aimed to explore the pro-apoptotic effects of targeting mitophagy to potentiate the efficacy of chemotherapy for HCC. HCC cells were subjected to cisplatin, after which cisplatin-induced mitophagy was quantified by immunofluorescence. Mdivi-1, a specific dynamin-related protein 1 (DRP1) inhibitor, was used to study the role of DRP1 in cisplatin-induced HCC mitophagy. The synergistic effect of cisplatin and the DRP1 inhibitor on HCC was assessed *in vitro* and *in vivo*. Accordingly, cisplatin induced mitophagy in surviving HCC cells by activating DRP1. The DRP1 inhibitor (Mdivi-1) increased the apoptosis of cisplatin-treated HCC cells by targeting mitophagy. Mechanistically, Mdivi-1 upregulated Bax and downregulated Bcl-xL, leading to an increase in mitochondrial membrane permeability and subsequent release of cytochrome *c* from mitochondria into the cytosol, thereby aggravating cisplatin-induced apoptosis in HCC cells. Moreover, Mdivi-1 acted synergistically with cisplatin to suppress HCC xenograft growth *in vivo*. Our results indicate that targeting cisplatin-mediated mitophagy increases HCC apoptosis via DRP1 inhibition, providing preclinical proof of concept for combination therapy targeting mitophagy to potentiate the efficacy of chemotherapy.

## Introduction

Hepatocellular carcinoma (HCC) is the fifth most prevalent cancer worldwide, with a vast majority of patients having intermediate to advanced stage disease upon diagnosis. Accordingly, the recommended therapy for such patients includes transarterial chemoembolization, molecular-targeted therapy, or checkpoint immunotherapy (1). However, systemic chemotherapies have demonstrated limited efficacy in the treatment of advanced HCC (2). Indeed, one study showed that FOLFOX4 (fluorouracil, leucovorin and oxaliplatin) chemotherapy for patients with advanced HCC exhibited marginal benefits (3). A plethora of mechanisms have explained the chemoresistance of HCC, such as P-glycoprotein (4), cancer cell stemness (5), DNA repair (6), evasion of apoptosis (7) and autophagy (8).

Mitophagy is a selective form of autophagy that removes malfunctioning or damaged mitochondria and thus maintains cellular homeostasis. Mitophagy is associated with many neurodegenerative disorders and cancer (9). For instance, the dysfunction caused by mutations in phosphatase and tensin homolog-induced putative kinase 1 (PINK1) can impair mitophagy, while accumulation of dysregulated mitochondria results in neuron apoptosis, which may account for Parkinson's disease (10). On the other hand, mitophagy can facilitate cancer cell survival by instantly clearing damaged mitochondria (11).

Mitophagy involves three main stages (9): Mitochondrial fission, autophagosome assembly, and fusion with lysosomes to degrade damaged mitochondria. The first step involves the scission of mitochondria controlled mainly by dynamin-related protein 1 (DRP1) (12), which functions to fragment large mitochondria into smaller ones (13). Given that DRP1-dependent mitochondrial fission is critical for mitophagy, we hypothesized that targeting DRP1 may influence cancer cell resistance to treatment.

Here, we showed that i) cisplatin induced mitophagy in surviving HCC cells by activating DRP1; ii) DRP1 inhibitor Mdivi-1 increased the apoptosis of cisplatin-treated HCC cells by targeting mitophagy; iii) Mdivi-1 downregulated Bcl-xL and upregulated Bax, thereby facilitating cytochrome *c* leakage from the damaged mitochondria into the cytosol; iv) Mdivi-1 acted synergistically with cisplatin to augment HCC apoptosis *in vivo*. Therefore, it was demonstrated that targeting DRP1-mediated mitophagy could be a potential approach toward enhancing chemotherapeutic efficacy for HCC, tipping the balance in favor of cancer cell apoptosis.

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## Materials and methods

*Reagents*. Cisplatin and Mdivi-1 were purchased from MedChem Express, while DMSO was obtained from Sigma-Aldrich/Merck KGaA. Primary antibodies against LC3B (cat. no. 3868; dilution 1:1,000; Cell Signaling Technology), phospho-Ser616-DRP1 (cat. no. 3455; dilution 1:1,000; Cell Signaling Technology), HSP60 (cat. no. 12165; dilution 1:1,000; Cell Signaling Technology), cleaved caspase-3 (cat. no. 9661; dilution 1:1,000; Cell Signaling Technology), Bax (cat. no. 5023; dilution 1:1,000; Cell Signaling Technology) and Bcl-xL (cat. no. 2764; dilution 1:1,000; Cell Signaling Technology), phospho-Ser139-histone H2AX (γ-H2AX) (cat. no. ab2893; dilution 1:1,000; Abcam), DRP1 (cat. no. ab184274; dilution 1:1,000; Abcam), cytochrome *c* (cat. no. ab133504; dilution 1:1,000; Abcam) and CoxIV (cat. no. ab202554; dilution 1:1,000; Abcam), TOM20 (cat. no. sc-17764; dilution 1:200; Santa Cruz Biotechnology, Inc.) and β-actin (cat. no. AA132; dilution 1:1,000; Beyotime Biotechnology, China) were used for western blot analysis.

*Cells and cell culture*. HCC cell lines MHCC97H (Liver Cancer Institute, Zhongshan Hospital, Fudan University, Shanghai, China) and Huh7 (obtained from the Japanese Cancer Research Resources Bank, Tokyo, Japan) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (Thermo Fisher Scientific, Inc.). Cells were incubated at 37˚C in a 5% $CO_2$ atmosphere. After reaching 70-80% confluency, the cells were subjected to subsequent experiments.

*Western blotting*. To obtain total proteins, cells were lysed by radioimmunoprecipitation assay (RIPA) buffer (Beyotime Biotechnology) supplemented with protease inhibitor cocktail (Weao Biotechnology). Mitochondrial protein extraction was performed using a mitochondrial isolation kit (Beyotime Biotechnology) according to the manufacturer's protocol. Protein concentration was determined using an enhanced BCA Protein assay kit (Beyotime Biotechnology). Proteins (30 μg per lane) were loaded onto 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and then separated. Thereafter, the gels were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). After being blocked with 5% bovine serum albumin (BSA) for 1 h, the membranes were incubated with the primary antibodies at 2-4˚C overnight. Next, the membranes were incubated with the appropriate secondary antibodies (anti-mouse IgG HRP-linked antibody, dilution 1:4,000; cat. no. 7076, Cell Signaling Technology; anti-biotin D5A7 rabbit monoclonal antibody, HRP conjugate, dilution 1:4,000; cat. no. 5571, Cell Signaling Technology) for about 1 h at room temperature. After being washed three times, the membranes were visualized using High-Signal ECL Substrate (Tanon).

*Immunofluorescence staining*. Cells or tissues were fixed with paraformaldehyde for 20 min and then permeabilized with 0.3% Triton X-200 (Beyotime Biotechnology) for 10 min to enhance specimen permeability. Thereafter, the specimens were blocked with 10% BSA (Roche) and incubated with primary antibodies (LC3B, cat. no. 3868, dilution 1:100, Cell Signaling Technology; TOM20, cat. no. sc-17764, dilution 1:100, Santa Cruz Biotechnology, Inc.) at 2-4˚C for 16 h. Next, samples were incubated with the appropriate fluorescence-conjugated secondary antibodies (FITC-conjugated goat anti-mouse IgG secondary antibody, F-2761, dilution 1:1,000, Thermo Fisher Scientific, Inc; TRITC-conjugated goat anti-rabbit IgG antibody, T-2769, Thermo Fisher Scientific, Inc.). Hoechst (Thermo Fisher Scientific, Inc.) was used to stain the nuclei. Processed cells or tissues were viewed using a fluorescence microscope (Olympus, magnification, x200). All images were analyzed using software Image-Pro Plus 6 (Media Cybernetics, Inc.).

*Flow cytometry to detect apoptosis and mitochondrial reactive oxygen species (ROS)*. Apoptosis rates were measured using the Dead Cell Apoptosis kit (Thermo Fisher Scientific, Inc.). Briefly, cells were harvested and incubated with Annexin V and propidium iodide (PI) based on the protocol outlined in the kit. Thereafter, the cells were washed and then loaded onto a flow cytometer (BD Biosciences) and FlowJo software (Tree Star), according to the instructions.

Mitochondrial ROS levels were measured using Mitosox (Yeasen). Briefly, cells were incubated in a culture medium containing Mitosox (2 μM) for 10 min and then washed using warm PBS. Next, the mitochondrial ROS level was determined using flow cytometry.

*JC-1 to probe mitochondrial membrane potential*. Changes in mitochondrial membrane potential were measured using JC-1 staining. Briefly, cells ($5x10^5$/well) in a 6-well plate were incubated with JC-1 (1 μg/ml) in culture medium at 37˚C for 10 min. After the culture medium containing JC-1 was removed, samples were washed with PBS and measured using a microplate spectrophotometer and a fluorescence microscope (magnification, x200; Olympus Corporation) to detect relative levels of red J-aggregates (intact mitochondria, excitation/emission: 585 nm and 590 nm) and green J-monomers (uncoupled mitochondria, excitation/emission: 514 and 529 nm). Changes in the red/green fluorescence intensity ratio were used to assess mitochondrial depolarization. A decrease in red fluorescence indicated loss of mitochondrial membrane potential.

*Mitophagy detection*. Cells ($5x10^5$/cm$^2$) in a 6-well plate were fixed with 4% paraformaldehyde for 20 min and then permeabilized with 0.2% Triton X-100 for 10 min. Thereafter, the samples were incubated with primary antibody against TOM20 (cat. no. sc-17764; dilution 1:100; Santa Cruz Biotechnology, Inc.) or LC3B (cat. no. 3868; dilution 1:100; Cell Signaling Technology) for 1 h at room temperature. Next, they were incubated with the appropriate secondary antibodies (FITC-conjugated goat anti-mouse IgG secondary antibody, F-2761, dilution ratio: 1:1,000, Thermo Fisher Scientific, Inc; TRITC-conjugated goat anti-rabbit IgG antibody, T-2769, Thermo Fisher Scientific, Inc.) for 1 h at room temperature and counterstained with Hoechst. Specimens were viewed using a fluorescence microscope (Olympus America Inc.; magnification, x200). TOM20 antibody was used to mark

the mitochondrial outer membrane, while LC3B antibody was used to label the autophagic vesicles. Mitophagy was quantified through the co-localization of these two molecules as previously described (14).

*Animal experiments*. Animal experiments were approved by the Committee on Animal Research (Zhongshan Hospital, Fudan University, Shanghai, China). All animal experiments were performed following the guidelines formulated by Shanghai Medical Experimental Animal Care Commission.

Twelve BALB/c nude mice (4-6 weeks old, male, body weight 18-20 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd., and housed in animal rooms with a 10-h light/14-h dark cycle and at a constant temperature (22-27˚C) and a relative humidity of 40-60% under specific pathogen-free conditions, and with unlimited water and standard laboratory chow. A suspension of $2x10^7$ MHCC97H cells was subcutaneously injected into the right flank of each BALB/c nude mice (18-20 g, 4-6 weeks old). After the tumor size reached 10 mm in diameter, mice bearing tumors were randomly divided into three groups and received a drug (100 $\mu$l) via peritoneal cavity injections every 3 days: control group (10% DMSO, n=4), cisplatin group (2.5 mg/kg, n=4), and combination treatment group (2.5 mg/kg cisplatin and 50 mg/kg Mdivi-1, n=4). Such treatment was continued for 2 weeks. Mice were sacrificed by cervical dislocation at 48 h after the last treatment, and tumor xenografts were harvested for further experiments.

*The Cancer Genome Atlas (TCGA)*. DRP1 expression levels in HCC and normal liver tissue were compared using the UALCAN website (http://ualcan.path.uab.edu/). Survival analysis based on the target gene in the TCGA database of patients with HCC (n=371) (TCGA data portal, http://cancergenome.nih.gov/) was conducted using Kaplan-Meier analysis at the KMplot website (http://kmplot.com) according to the threshold expression value automatically set by the website.

*Statistical analysis*. Data are expressed as means ± standard deviations from three independent experiments and were analyzed using Graphpad Prism 7 (GraphPad Software, Inc.). Comparisons between two samples or among three groups were performed using unpaired Student's t-test or one-way analysis of variance (ANOVA, Bonferroni post hoc test). A two-sided P-value of $<0.05$ was considered statistically significant.

## Results

*Cisplatin increases mitophagy in surviving HCC cells by activating DRP1*. After treating HCC cells (MHCC97H and Huh7) with cisplatin (at 6 and 4 $\mu$g/ml, respectively) for 24 h, a significant increase in mitophagy was observed, as indicated by increased TOM20 and LC3 co-localization (Fig. 1A).

Mitophagy typically occurs when ROS cause mitochondrial damage (9). Upon severe cisplatin-induced DNA damage (Fig. 1D), an increase in mitochondrial ROS among the surviving HCC cells (Fig. 1C) and a marked increase in DRP1 phosphorylation at Ser616 (p-DRP1, Fig. 1D) were observed. More importantly, DRP1-specific inhibitor Mdivi-1 (50 nM) significantly suppressed cisplatin-induced mitophagy (Fig. 1A). Meanwhile, the expression of TOM20 in mitochondria, which is used to identify mitochondrial turnover, was measured in these cells. Accordingly, a significant decrease in HCC cell TOM20 expression was observed after cisplatin treatment (Fig. 1B), indicating accelerated mitochondrial degradation, including mitophagy degradation, after cisplatin treatment. Moreover, Mdivi-1 reversed the cisplatin-induced decrease in TM20 (Fig. 1B), suggesting that inhibition of cisplatin-induced mitophagy can decrease mitochondrial degradation by targeting DRP1. The aforementioned data demonstrated that cisplatin treatment induces mitophagy while inhibition of DRP1 activity can significantly reduce mitophagy. These results suggest that HCC cells survive chemotherapy by activating DRP1-mediated mitophagy, which may be trigged by chemotherapy-induced DNA damage and an increase in mitochondrial ROS. This indicates that DRP1-mediated mitophagy protects HCC cells against chemotherapy insult.

*Inhibition of mitophagy by DRP1 inhibitor leads to increased apoptosis of cisplatin-treated HCC cells*. To validate whether targeting mitophagy could influence the response of HCC cells to cisplatin treatment, HCC cells were subjected to different treatments. Firstly, Mdivi-1 alone did not induce the apoptosis of HCC cells (Fig. 2A). However, cisplatin+Mdivi-1-treated HCC cells exhibited significantly higher apoptosis compared to those treated with cisplatin alone (Fig. 2A), implicating that targeting DRP1-mediated mitophagy promotes apoptosis in cisplatin-treated HCC cells. Cisplatin-treated Huh7 and MHCC97H cells had an apoptosis rate of 8.5 and 12.9%, respectively. Interestingly, following combination treatment, the apoptosis rates of both cell lines increased markedly to 24.8 and 22.2%, respectively. In addition, HCC cell apoptosis was significantly higher with cisplatin and bafilomycin A1 (10 nM, a lysosomal inhibitor) treatment than with cisplatin treatment alone, implying that blockade of autolysosomes, including the inhibition of the lysosomal degradation of mitochondria during mitophagy, facilitates apoptosis of cisplatin-treated HCC cells. Furthermore, the aforementioned findings were confirmed by western blotting for cleaved caspase-3 (Fig. 2B). Hence, our results indicate that inhibition of DRP1-mediated mitophagy increases the susceptibility of cisplatin-treated HCC cells to apoptosis rather than directly causing apoptosis.

*Disruption in mitophagy decreases the mitochondrial membrane potential, subsequently releasing cytochrome c from the damaged mitochondria*. Combination treatment with cisplatin and Mdivi-1 caused more mitochondrial damage in both MHCC97H and Huh7 cell lines, as indicated by the decrease in mitochondrial membrane potential using JC-1 aggregate/JC-1 monomer fluorescence intensity ratio and JC-1 staining (Fig. 3A). More importantly, cisplatin and Mdivi-1 treatment of HCC cells induced considerable leakage of cytochrome *c* into the cytosol (Fig. 3B). This suggests that inhibition of DRP1-mediated mitophagy using Mdivi-1 could promote cytochrome *c* release from mitochondria into the cytosol by decreasing the mitochondrial membrane potential.

Among the major proteins that maintain mitochondrial membrane potential, Bcl-xL and Bax are considered crucial

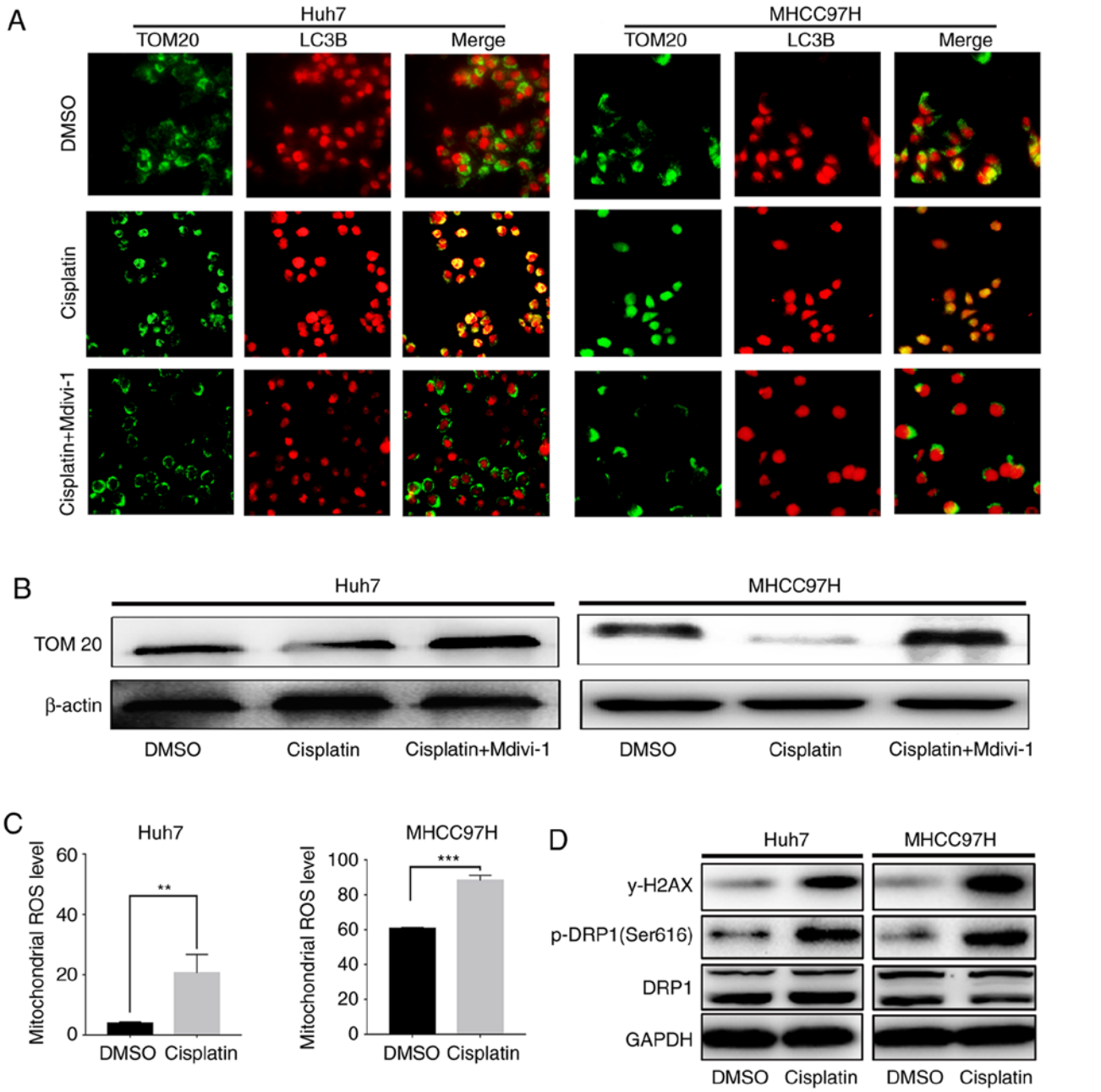


Figure 1. Cisplatin induces mitophagy in surviving HCC cells by activating DRP1. (A) Increased mitophagy was observed in cisplatin-treated HCC cells as indicated by LC3B and TOM20. The red and green signals represent LC3B and TOM20, respectively. Magnification, x200. (B) TOM20 expression in HCC cells receiving different treatments was detected using western blot analysis. (C) Mitochondrial ROS levels were measured using flow cytometry. (D) DNA damage was measured using γ-H2AX. The level of the activated isoform of DRP1 (p-DRP1 Ser616) was detected using Western blotting. $^{**}$P<0.01, $^{***}$P<0.001. HCC, hepatocellular carcinoma; DRP1, dynamin-related protein 1; TOM20, mitochondrial import receptor subunit TOM20 homolog; LC3B, microtubule-associated proteins 1A/1B light chain 3B; ROS, reactive oxygen species.

given that they can counteract each other by competing for voltage-dependent anion channel (VDAC) in mitochondria. More specifically, Bax can increase VDAC activity and helps with the formation of permeability transition pores, whereas Bcl-xL inactivates VDAC (15-17). Although other molecules, such as Bid/Bik, function to maintain integrity, they do not influence the mitochondrial potential (15). Initially, Bax, Bcl-xL, and cytochrome *c* expression was measured at four time points (3, 6, 12, and 24 h). Our initial results showed that the expression of the aforementioned proteins started to change between 12 and 24 h after the treatment (data not shown). Therefore, Bax, Bcl-xL and cytochrome *c* expression in HCC cells was analyzed 24 h after treatment. Notably, mitochondrial Bax expression was markedly higher in HCC cells receiving combination treatment than those receiving cisplatin treatment alone. Accordingly, HCC cells exposed to the combination treatment had much less mitochondrial Bcl-xL. Meanwhile, cytochrome *c* was significantly increased in the cytosol (Fig. 3B).

These results suggest that targeting DRP1-mediated mitophagy using Mdivi-1 downregulates Bcl-xL and upregulates Bax, which decreases the mitochondrial membrane

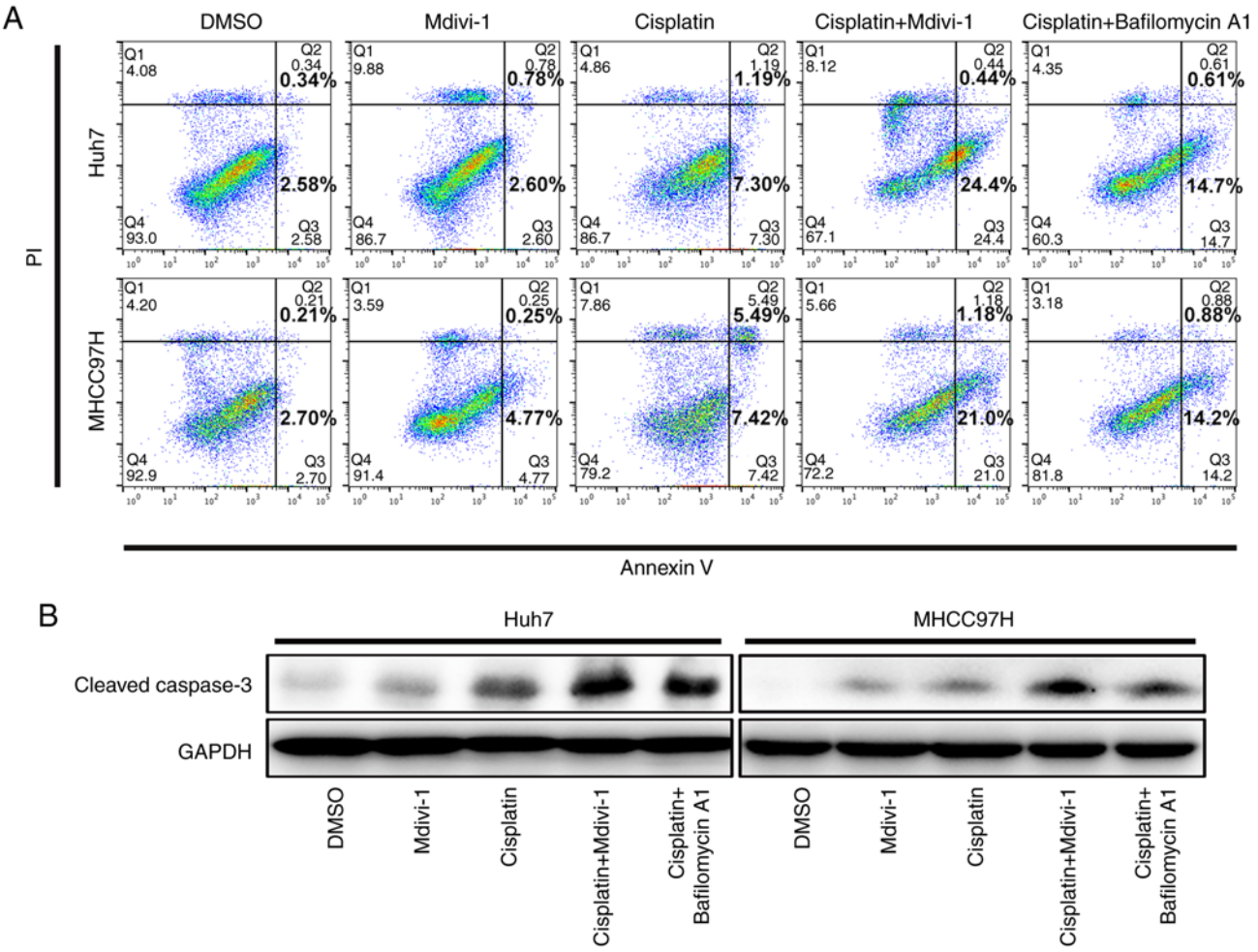


Figure 2. DRP1 inhibitor Mdivi-1 increases the apoptosis of cisplatin-treated HCC cells. (A) Apoptosis of HCC cells receiving different treatments was measured using flow cytometry. (B) Cleaved caspase-3 was detected using western blotting to assess cell apoptosis. DRP1, dynamin-related protein 1; HCC, hepatocellular carcinoma.

potential leading to increased mitochondrial membrane permeability and subsequent release of cytochrome *c* from damaged mitochondria into the cytosol, thereby accelerating cisplatin-induced apoptosis in HCC cells.

*Mdivi-1 exacerbates cisplatin-induced HCC apoptosis in vivo*. We examined whether targeting mitophagy could promote cisplatin-induced HCC apoptosis *in vivo*. After establishing a mouse xenograft model by subcutaneously injecting MHCC97H cells, mice were randomly divided into three groups receiving different treatments: the control group treated with DMSO, the cisplatin group, and the combination treatment group (cisplatin and Mdivi-1). Accordingly, tumor growth was markedly lower in the cisplatin group than that noted in the control group as evidenced by tumor size and weight. Despite the lack of a significant difference in tumor reduction between the cisplatin group and combination treatment group, the addition of Mdivi-1 to cisplatin treatment further inhibited tumor growth (Fig. 4A and B), suggesting a synergistical effect in the combined use of Mdivi-1 and cisplatin. Moreover, cisplatin induced mitophagy in HCC *in vivo*, which was significantly blocked by the DRP1 inhibitor Mdivi-1 (Fig. 4C). Consistent with a markedly inhibitory effect on tumor growth, the expression of apoptotic marker cleaved caspase-3 was significantly increased in the combination treatment group (Fig. 4D). These data suggest that Mdivi-1 acts synergistically with cisplatin to suppress HCC xenograft growth *in vivo* through the blockade of DRP1-mediated mitophagy and an increase in apoptosis.

*Negative correlation between DRP1 expression and survival among patients with HCC*. As shown in Fig. 5A, *DRP1* mRNA expression was markedly increased in the HCC than in the non-tumoral liver tissues. Moreover, patients with HCC having high *DRP1* mRNA expression had worse overall survival compared to those with low *DRP1* mRNA expression (Fig. 5B). Therefore, these results suggest that high *DRP1* expression may serve as an independent predictor for poor HCC prognosis.

## Discussion

The principal finding of the present study was that targeting dynamin-related protein 1 (DRP1)-mediated mitophagy by combining cisplatin with Mdivi-1 (a specific DRP1 inhibitor) could aggravate the cisplatin-induced apoptosis of HCC cells,

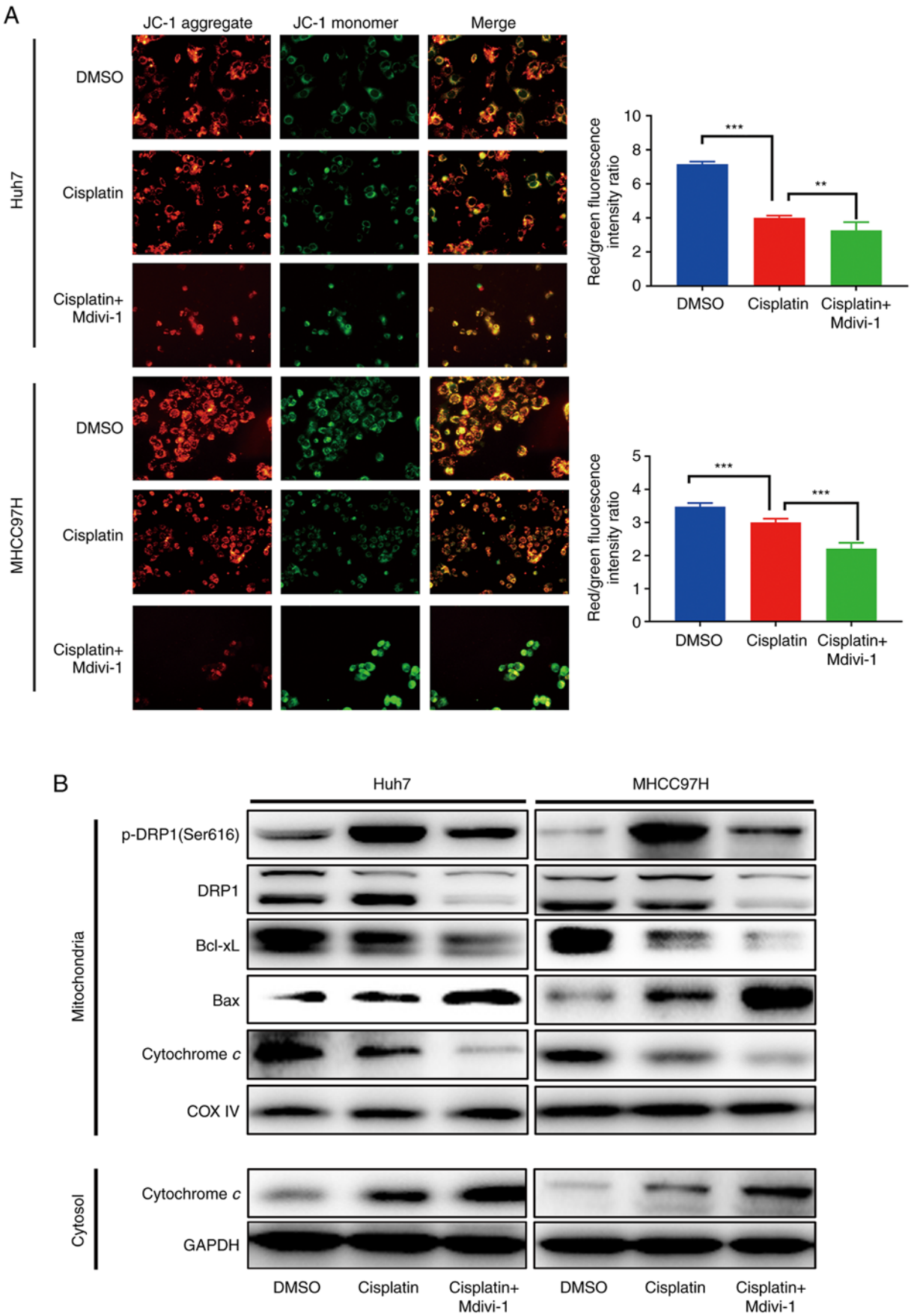


Figure 3. DRP1 inhibitor Mdivi-1 decreases the mitochondrial membrane potential, which promotes the release of cytochrome *c* from the damaged mitochondria. (A) The mitochondrial membrane potential in HCC cells receiving different treatments was measured using JC-1 staining, as indicated by the JC-1 aggregate/JC-1 monomer fluorescence intensity ratio. Magnification, x200. (B) Expression levels of p-DRP1(S616), Bax, Bcl-xL, and cytochrome *c* in mitochondria and expression of cytosolic cytochrome *c* were detected using western blotting. $^{**}$P<0.01, $^{***}$P<0.001. DRP1, dynamin-related protein 1; HCC, hepatocellular carcinoma.

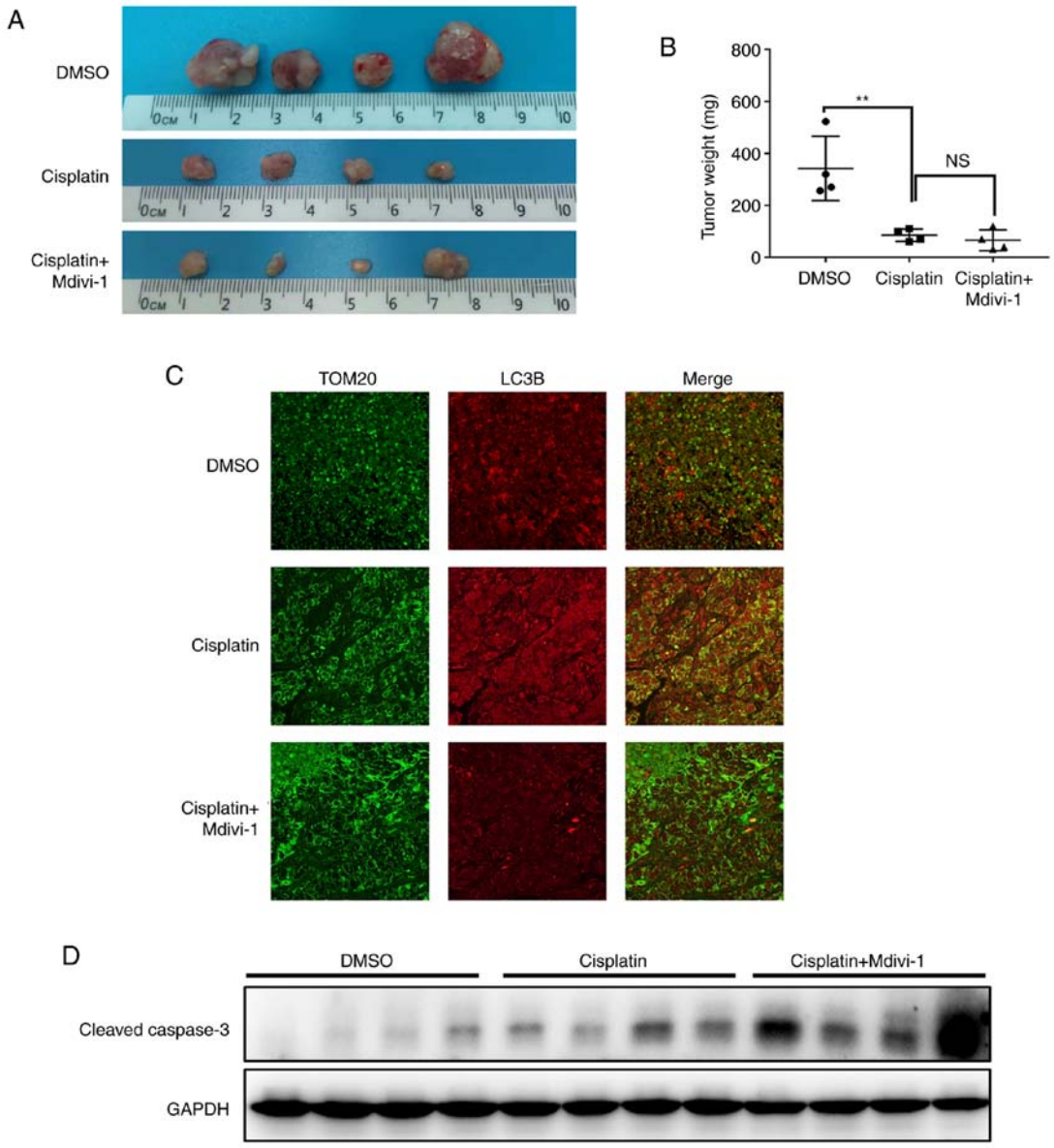


Figure 4. DRP1 inhibitor Mdivi-1 exacerbates cisplatin-induced HCC apoptosis *in vivo*. (A and B) Tumor size and weight in mice receiving three different treatments (DMSO, cisplatin, cisplatin+Mdivi-1) were compared. (C) Mitophagy in tumors was determined using LC3B and TOM20 immunofluorescence staining. Magnification, x200. (D) Expression of cleaved caspase-3 in tumors was detected using western blotting. ns, not significant; $^{**}$P<0.01. HCC, hepatocellular carcinoma; DRP1, dynamin-related protein 1; TOM20, mitochondrial import receptor subunit TOM20 homolog; LC3B, microtubule-associated proteins 1A/1B light chain 3B.

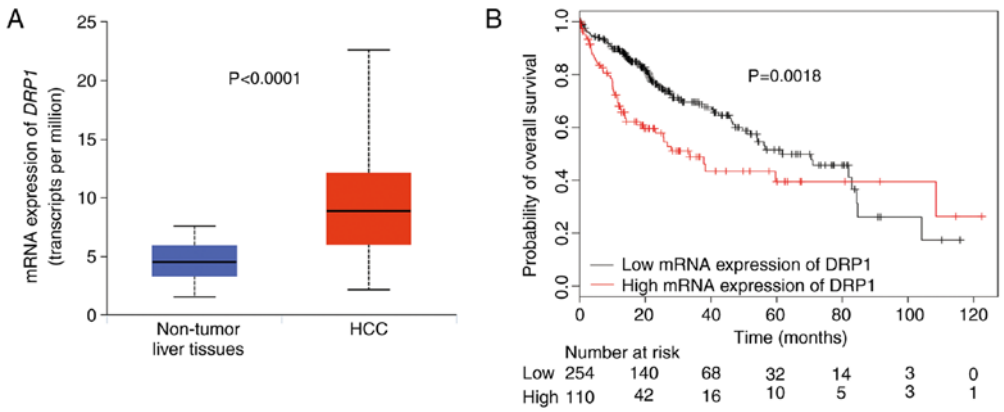


Figure 5. Correlation between *DRP1* mRNA expression and overall survival of patients with HCC. (A) HCC tissues had significantly higher *DRP1* mRNA expression than non-tumor liver tissues (http://ualcan.path.uab.edu/cgi-bin/TCGAExResultNew2.pl?genenam=DNM1L&ctype=LIHC). (B) Compared to patients with low DRP1 expression, those with high expression had poorer survival (https://kmplot.com/analysis/index.php?p=service&start=1). HCC, hepatocellular carcinoma; *DRP1*, dynamin-related protein 1.

providing a novel strategy for boosting the efficacy of hepatocellular carcinoma (HCC) chemotherapy. Furthermore, our results revealed that targeting DRP1-mediated mitophagy could reduce the mitochondrial membrane potential by upregulating Bax and downregulating Bcl-xL, which increased mitochondrial membrane permeability and cytochrome *c* release from damaged mitochondria, thereby augmenting cisplatin-induced apoptosis of HCC cells. Therefore, the clinical implication of our findings is that targeting mitophagy could potentiate the effects of chemotherapy against HCC.

Mitophagy is a vital mechanism for maintaining cellular homeostasis through the selective degradation of damaged or malfunctioning mitochondria. This mechanism promotes cellular survival by preventing the release of apoptotic factors, such as, cytochrome *c* and apoptosis-inducing factors, into the cytosol (18). Mitophagy, however, could be exploited by tumor cells as an adaptive stress response that helps them survive under stressful conditions, such as hypoxia and ischemia, resulting in treatment resistance. Indeed, reports have shown that inhibition of FUNDC1-mediated mitophagy during cardiac ischemia–reperfusion injury promoted cellular apoptosis (19). Anticancer treatment, including chemotherapy, can cause mitochondrial damage (20). Accordingly, damaged mitochondria, if not recycled or cleared through mitophagy, can increase cancer cell susceptibility to death (18). Analogous to the increase in mitophagy among cardiomyocytes suffering from ischemia, we showed that HCC cells survive cisplatin exposure by activating DRP1-mediated mitophagy, suggesting that mitophagy protects HCC cells against chemotherapy.

DRP1, which initiates mitochondrial dynamics, plays a critical role in mitophagy (9). Although other DRP1-independent pathways lead to mitophagy (21), our study showed that DRP1 activation initiates chemotherapy-induced mitophagy. DRP1 is activated under cellular stress, including ROS, ATP deficiency, and calcium overloading (22). The present study showed that DRP1 activation during chemotherapy-induced mitophagy might be triggered by cisplatin-induced DNA damage and mitochondrial ROS. Given that cisplatin can bind not only mitochondrial DNA but also mitochondrial molecules (23,24), it could perhaps directly damage mitochondrial DNA and trigger a burst of mitochondrial ROS, which results in the activation of DRP1 and mitophagy. DRP1 inhibitor Mdivi-1 specifically targets DRP1 GTPase to block DRP1 activity (25). The present study showed that Mdivi-1-induced disruption of mitophagy exacerbated the apoptotic response of HCC cells to cisplatin treatment, suggesting the therapeutic potential of targeting mitophagy in antitumor treatments. Additionally, based on the TCGA database, we found that DRP1 was a promising predictor for outcomes of patients with HCC.

Mechanistically, during the blockade of cisplatin-induced mitophagy by Mdivi-1, we observed more Bax and less Bcl-xL in the mitochondria, which resulted in a decrease in the mitochondrial membrane potential, an increase in mitochondrial permeability and cytochrome *c* release from damaged mitochondria into the cytosol, and the initiation of the apoptotic cascade. Moreover, evidence suggests the interaction between Bcl-xL and DRP1. Accordingly, Li *et al* reported that while Bcl-xL can activate DRP1 and thus mobilize mitochondria to facilitate synapse formation, the depletion of DRP1 may hamper this process (26,27).

This study has some limitations. First, despite highlighting the importance of DRP1 in cisplatin-induced mitophagy, we cannot be certain whether other molecules involved in mitophagy, such as PINK1, Nix, and BNIP3, can hold the same role. Second, only cisplatin was used herein. Apart from cisplatin, doxorubicin has been commonly used in clinical settings. However, there is a possibility that doxorubicin-induced mitophagy may not be dependent on DRP1 activation. Third, we did not assess the changes in DRP-1 and p-DRP1 after Mdivi-1 treatment, thus making it impossible to determine whether Mdivi-1 worked by inhibiting the GTPase activity of p-DRP1 or by altering protein expression. Fourth, Bordt *et al* suggested that Mdivi-1 not only impairs DPR1 GTPase activity but also modulates mitochondrial ROS production (28). Therefore, it is possible that Mdivi-1 could exert its pro-apoptotic effects via other mechanisms. Fifth, considering the small number of mice in each group (n=4), the conclusions presented herein may suffer from low power. Finally, although the apoptosis rate of MHCC97H cells treated with cisplatin alone was not apparent, animal experiments showed that cisplatin can significantly suppress MHCC97H cell growth. This inconsistency may be due to differences in cisplatin concentrations or MHCC97H responses to cisplatin *in vitro* and *in vivo*.

In conclusion, the present study demonstrated that suppression of cisplatin-mediated mitophagy increases cell apoptosis in HCC via DRP1 inhibition, providing a preclinical proof of concept for combination therapy targeting mitophagy to potentiate chemotherapy.

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**Availability of data and materials**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Authors' contributions**

MM and RXC designed the experiments. MM, XHL, HHL and RZ performed the experiments and conducted data collection and analyses. MM and RXC wrote the paper. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

**Ethics approval and consent to participate**

No human patients were enrolled in the present study. The animal experiments were approved by the Committee on Animal Research (Zhongshan Hospital, Fudan University, Shanghai, China). All animal experiments were performed following the guidelines formulated by Shanghai Medical Experimental Animal Care Commission.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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