

# Cisplatin induces apoptosis of hepatocellular carcinoma LM3 cells via down-regulation of XIAP

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**Abstract. – OBJECTIVE:** Cisplatin is an important anti-cancer drug. However, the molecular mechanism of cisplatin on inhibition of the proliferation of liver cancer cells is unclear. Thus, we aimed to investigate the regulatory role of cisplatin on the growth and apoptosis of hepatoma LM3 cells.

**MATERIALS AND METHODS:** LM3 cells were treated with cisplatin (2  $\mu\text{mol/L}$ ) for 48 h. MTT assay, flow cytometry, and caspase-3 activity assay were used to detect the growth, proliferation and apoptosis of LM3 cells. Western blot was applied to detect the expression of the inhibitor of apoptosis protein, XIAP. siRNA was used to knockdown the level of XIAP followed by cisplatin (2  $\mu\text{mol/L}$ ) treatment, and then the apoptosis of LM3 cells was measured.

**RESULTS:** The treatment of cisplatin significantly inhibited the growth but induced the apoptosis of LM3 cells. Cisplatin also downregulated the expression of XIAP. The downregulation of XIAP by using siRNA enhanced the apoptosis of LM3 cells induced by cisplatin.

**CONCLUSIONS:** Downregulation of XIAP enhanced the proapoptotic effect of cisplatin on LM3 cells, suggesting that XIAP might be used as a potential target in the treatment of liver cancer.

*Key Words:*

Cisplatin, Inhibitor of apoptosis protein, XIAP, LM3 cell, Apoptosis.

## Introduction

The mortality of liver cancer presents an increasing trend recently<sup>1,2</sup>. The occurrence of liver cancer is closely related with genetic factors, viral infections, and living habits<sup>3,4</sup>. The combination therapy of surgery and radiotherapy is often used for liver cancer treatment. Although the efficacy of combined treatment has improved the survival rate of patients, yet unsatisfied result exists due to deficiencies, such as bleeding

and other side effects<sup>5-7</sup>. The improvement on accuracy and success rate of liver cancer therapy become the focus in both medical and scientific community. With the development of genetic engineering, targeted molecular therapy has been gradually adopted<sup>8-10</sup>. This therapy has drawn extensive attention owing to its little toxicity to normal cells and low side effect. For instance, imatinib mesylate has been used in targeted molecular therapy. It is a selective tyrosine kinase inhibitor that can interact with ATP-binding site of the ABL kinase, thereby preventing the phosphorylation of downstream proteins. Recent evidence has demonstrated its significant effect in the treatment of chronic phase, advanced phase, or blastic phase of chronic myelogenous leukemia (CML)<sup>11,12</sup>.

Cisplatin, an anti-cancer chemotherapy drug widely used in clinical, can inhibit the replication of DNA. DDP cells are most sensitive to cisplatin, which can diffuse through the charged cell membrane and inhibits the RNA and protein synthesis at high concentrations. The main acting sites of DDP are the purine and pyrimidine bases of DNA. So far, it has been demonstrated that cisplatin has a strong broad-spectrum anti-cancer effect and it is used for the treatment of ovarian cancer, prostate cancer, testicular cancer, lung cancer, nasopharyngeal cancer, esophageal cancer, malignant lymphoma, and thyroid cancer<sup>11,12</sup>. XIAP is a kind of the protein kinases which plays an important role in apoptosis<sup>13</sup>, programmed necrosis<sup>14</sup>, autophagy<sup>15</sup>, and NF- $\kappa$ B signaling<sup>16</sup>. Studies have shown that silence of XIAP using siRNA enhanced the inducing effect of cisplatin on apoptosis of LM3 cells<sup>17</sup>. However, the exact mechanism remains unknown. This article aims to investigate the molecular role of cisplatin on the apoptosis of LM3 cells.

## Materials and Methods

### Reagents and Consumables

Mitochondrial membrane potential dye tetramethylrhodamineethyl ester (TMRE) was purchased from Biyuntian Biotech (Beijing, China). Other inorganic reagents were provided from Sigma-Aldrich (St. Louis, MO, USA). Rabbit anti-human XIAP antibody was from Sigma-Aldrich (St. Louis, MO, USA). siRNA of XIAP and control siRNA were synthesized by Shanghai Jima Biotechnology (Shanghai, China) with the following sequences: 5'GCGTTATATGATTGGCGT3' and 5'TGGTTCTAGAGTGATCACAG-3', 5'-GTAACGACGGAACCAGT-3' and 5'AATCATCAATAGGATGGT3'. Liposomal transfection reagent Escort™ was bought from Sigma-Aldrich (St. Louis, MO, USA). Cell culture reagents were collected from Dingguo Biological Technology (Beijing, China).

### Cell Culture

LM3 cells were purchased from ATCC (Manassas, VA, USA). Cells were conventionally cultured in Dulbecco's Modified Eagle Medium (DMEM) medium containing 10% fetal calf serum (FCS). Cisplatin (2  $\mu\text{mol/L}$ ) or solvent control (DMSO) was used to treat the cells.

### Cell Transfection

siRNA of XIAP or control siRNA was transfected into LM3 cells using Escort™ Transfection Reagent according to manufacturer's instruction (Sigma-Aldrich, St. Louis, MO, USA). Cells were then for another 24 h to precede subsequent experiments<sup>7</sup>.

### Flow Cytometry

TMRE (Mitochondrial membrane potential dye) and Annexin-V-FITC (membrane phosphatidylserine specific dye) were used to measure the apoptosis of LM3 cells<sup>18,19</sup>. Briefly, cells were collected and resuspended. 100  $\mu\text{l}$  of reaction buffer and 2  $\mu\text{l}$  of Annexin-V-FITC were added to 500  $\mu\text{l}$  of cell suspension, mixed, and incubated for 20 min in dark for flow cytometry analysis with 488 nm and 625 nm. Cells were also stained with TMRE (final concentration: 10  $\mu\text{mol/L}$ ) for 20 min in dark and then measured by flow cytometry.

### MTT Test

MTT test was performed according to conventional methods<sup>20</sup>. Cells were seeded into 96-well plates at a density of 1000 cells/well. MTT solution was added to each well and incubated for 5

h. Optical density at 420 nm was recorded using a microplate reader to draw a growth curve.

### Caspase-3 Activity Assay

Caspase-3 activity was detected according to kit instructions. Briefly, transfected LM3 cells were collected, lysed, mixed with chromogenic substrate, and incubated on ice for 10 min. Absorbance of each sample was recorded by a microplate reader.

### Western Blot

LM3 cells of each group were collected, lysed, electrophoresed, and blotted<sup>11</sup>, and expression level of XIAP were analyzed.

### Statistical Analysis

SPSS 18.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. All data were expressed as mean  $\pm$  standard error. Differences between groups were analyzed by *t*-test.  $p < 0.05$  was considered statistically significant.

## Results

### Cisplatin Inhibited the Growth of LM3 Cells

The effect of cisplatin on the growth and survival of LM3 cells were measured. As shown in Figure 1, the treatment of cisplatin significantly inhibited the growth of LM3 cells, compared to that of control ( $p < 0.05$ ).

### Cisplatin Decreased Mitochondrial Membrane Potential of LM3 cells

Mitochondrial membrane potential assay showed that of cisplatin treatment significantly re-

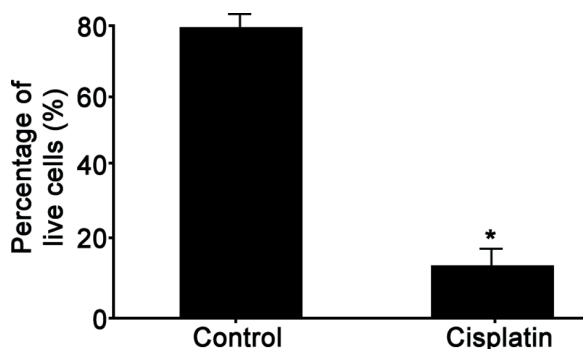
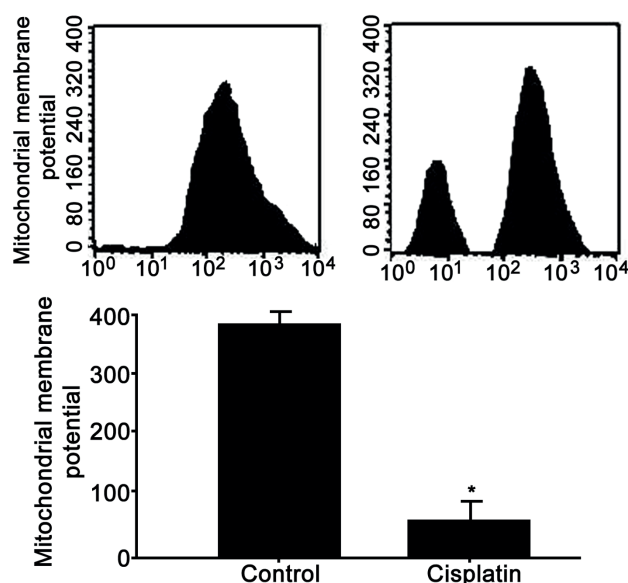


Figure 1. Cisplatin inhibited the growth of LM3 cells. \* $p < 0.05$ , compared with control group.



**Figure 2.** Cisplatin decreased mitochondrial membrane potential of LM3 cells. \* $p < 0.05$ , compared with control group.

duced mitochondrial membrane potential of LM3 cells, compared with that of control, suggesting that cisplatin treatment induced apoptosis of LM3 cells ( $p < 0.05$ ) (Figure 2).

#### **Cisplatin Induced Apoptosis of LM3 Cells**

In order to further evaluate the proapoptotic role of cisplatin, flow cytometry analysis was applied and the result showed that cisplatin significantly increased the eversion of phosphatidylserine in LM3 cells, compared with that of control, suggesting that cisplatin treatment induced apoptosis of LM3 cells ( $p < 0.05$ ) (Figure 3).

#### **Cisplatin Activated Caspase-3 in LM3 Cells**

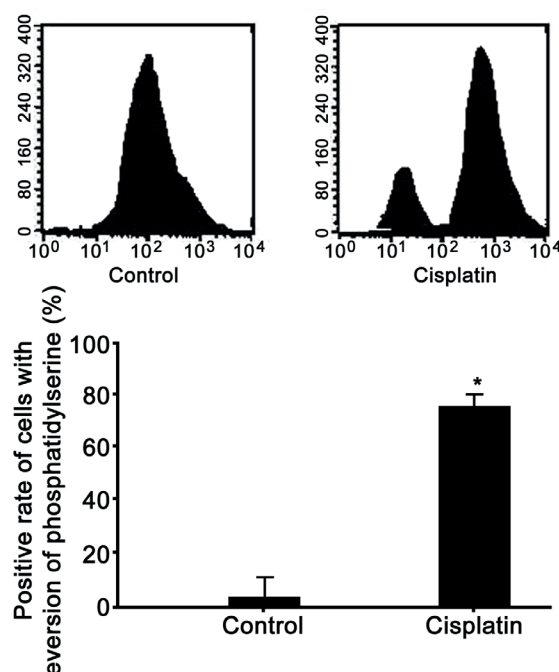
In a similar fashion, caspase-3 activity assay results showed that cisplatin significantly activated caspase-3 in LM3 cells, suggesting that cisplatin induced apoptosis of LM3 cells ( $p < 0.05$ ) (Figure 4).

#### **Cisplatin Down Regulated the Expression of XIAP in LM3 Cells**

Western blot analysis showed that cisplatin treatment remarkably lowered the expression level of XIAP in LM3 cells, compared with that of controls (Figure 5).

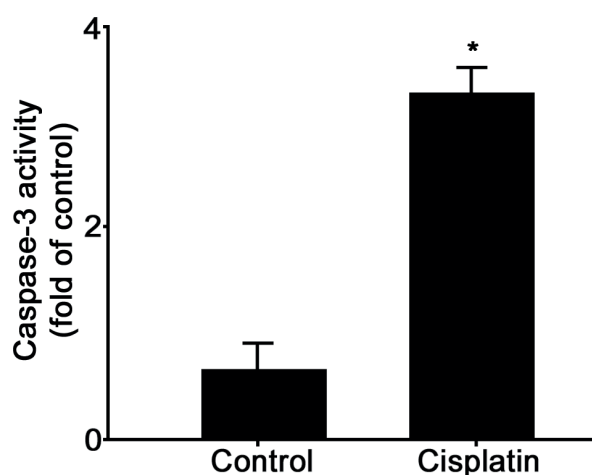
#### **Silence of XIAP Enhanced Cisplatin-induced Apoptosis of LM3 Cells**

We then determined the effect of XIAP on the apoptosis of LM3 cells. The expression



**Figure 3.** Cisplatin induced the eversion of phosphatidylserine in LM3 cells. \* $p < 0.05$ , compared with control group.

of XIAP was significantly decreased by the treatment of siXIAP. The reduction of XIAP increased the level of caspases-3 and the combined treatment of cisplatin and siXIAP-LM3 cells significantly enhanced the activation of caspase-3, compared with that of control group



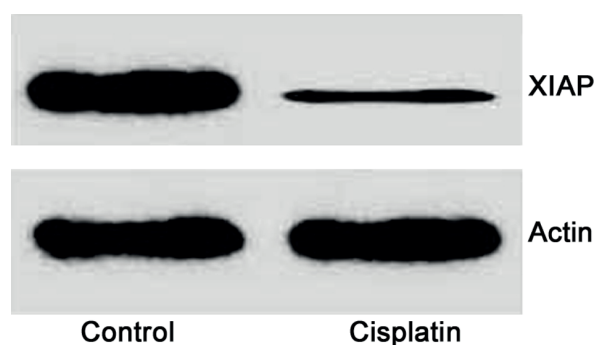
**Figure 4.** Cisplatin activated caspase-3 in LM3 cells. \* $p < 0.05$ , compared with control group.

( $p < 0.05$ ). The results suggested that suppression of XIAP promoted cisplatin-induced apoptosis of LM3 cells (Figure 6).

## Discussion

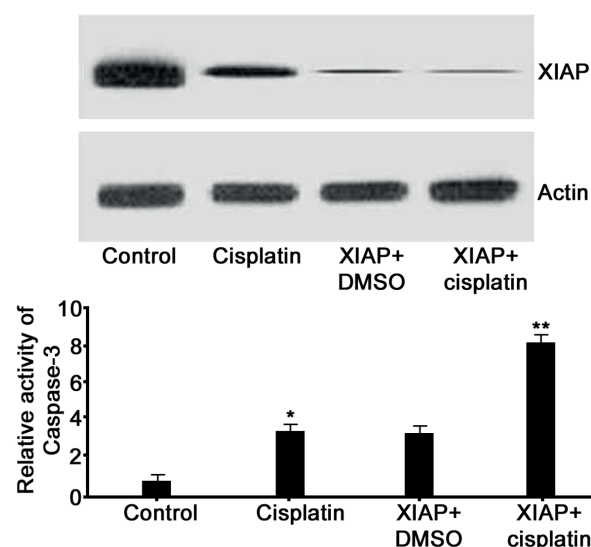
Cisplatin is a type of the clinically important anticancer drugs<sup>21</sup>. Recent evidence also indicated that specific miRNAs induced the apoptosis of cancer cells via the down-regulation of XIAP<sup>22</sup>. Our data presented that cisplatin significantly inhibited the growth of LM3 cell, which is consistent with previous studies. Moreover, our results of flow cytometry showed that cisplatin activated caspase-3 and reduced mitochondrial membrane potential of LM3 cells, suggesting that cisplatin induced apoptosis of LM3 cells, which is in agreement with previous data<sup>23,24</sup>. Notably, studies showed that under the same concentration of cisplatin (2  $\mu\text{mol/L}$ ), LM3 cells were more sensitive to cisplatin than other types of cancer cells, this may be due to the different sensitivity of cells to the same chemical drugs<sup>15-17</sup>.

Accumulative studies showed that signaling pathway was crucial to apoptosis<sup>25,26</sup>. In this study, we thus explored the exact pathway during the regulation of cisplatin on the proliferation of LM3 cells. It is known that caspase proteins regulate apoptosis. Extrinsic pathway activates caspase-8, while intrinsic mitochondrial pathway is mainly mediated by the activation of caspase-3/7. This study suggests that cisplatin caused the apoptosis of LM3 cells on the mediation of mitochondrial intrinsic pathway, which is consistent with



**Figure 5.** Cisplatin down regulated the expression of XIAP in LM3 cells.

previous results<sup>18,20</sup>. It has been shown that XIAP involves in apoptosis<sup>3</sup>, programmed necrosis<sup>4</sup>, autophagy<sup>5</sup>, and NF- $\kappa$ B signaling pathways<sup>6</sup>. The results of this paper demonstrated that silencing of XIAP enhanced cisplatin-induced apoptosis of LM3 cells, suggesting that the down regulation of XIAP might enhance the sensitivity of LM3 cells to cisplatin. However, the limitation in our paper still exists that clinical specimens at different stages of liver cancer need to be further collected for the measurement of apoptosis and level of XIAP. Derivatives of cisplatin will be evaluated for the treatment of LMs. An XIAP knockout model of mice will be established for the study of XIAP in cisplatin-induced apoptosis of liver cancer cells.



**Figure 6.** Silence of XIAP enhanced cisplatin-induced apoptosis of LM3 cells. \* $p < 0.05$ , compared with control group; \*\* $p < 0.01$ , compared with control group.



## Conclusions

Down-regulation of XIAP enhanced the proapoptotic role of cisplatin on LM3 cells.

## Conflict of Interest

The Authors declare that they have no conflict of interest.

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