Basic Study

Norcantharidin combined with ABT-737 for hepatocellular carcinoma: Therapeutic effects and molecular mechanisms

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Abstract

AIM: To study the therapeutic effect of norcantharidin (NCTD) combined with ABT-737 on hepatocellular carcinoma cells and the molecular mechanism.

METHODS: Two hepatocellular carcinoma (HCC) cell lines, HepG2 and SMMC-7721, were selected. ABT-737 and NCTD were allocated into groups to be used alone or in combination. HepG2 and SMMC-7721 cells were cultured in vitro. Liver cancer cells in the logarithmic phase of growth were vaccinated and cultured to the cell wall stage; these cells were treated for 48 h with different concentrations of NCTD, or ABT-737, or NCTD combined with ABT-737. The cell proliferation inhibition rate was detected by methyl thiazolyl tetrazolium. The expression of Mcl in HCC cells was detected by Western Blotting, and the cells in each group after treatment had apoptosis detected by flow cytometry. The proliferation inhibition rate, the expression of Mcl-1 in cells and the apoptosis inducing effect of treatment were observed in each group, and the effect of NCTD on ABT-737 in the treatment of HCC and its mechanism of action were analyzed.

RESULTS: As the concentration of NCTD increased, the cell proliferation inhibition rate gradually decreased; and the treatment effect of ABT-737 1-3 μm combined with NCTD on cell proliferation inhibition was stronger than that of ABT-737 alone. The difference was statistically significant (P < 0.05). In observing the expression of Mcl-1 in cells after the treatment of different concentrations of NCTD, this was partially
inhibited after treatment with NCTD 15 μm, and the expression of Mcl-1 was almost undetectable after treatment with NCTD 30 μm and 60 μm. The effect on inducing apoptosis with the treatment of ABT-737 or NCTD alone for 48 h was lower than that of the control group. The difference was not statistically significant ($P > 0.05$). The effect on inducing apoptosis in HepG2 and SMMC-7721 cells with the treatment of ABT-737 combined with NCTD for 48 h was greater than that of ABT-737 or NCTD alone. The difference was statistically significant ($P < 0.05$).

CONCLUSION: NCTD combined with ABT-737 has a positive role in the treatment of HCC, and it has great value in clinical research.

Key words: Norcantharidin; Hepatocellular carcinoma cell; Mcl-1

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Core tip: The effects of ABT-737 and norcantharidin (NCTD) alone or in combination on HepG2 and SMMC-7721 cells were tested by methyl thiazolyl tetrazolium, Western blot and flow cytometry. We found that as the concentration of NCTD increased, the cell proliferation inhibition rate gradually decreased; and the treatment effect of ABT-737 1-3 μm combined with NCTD on cell proliferation inhibition was stronger than that of ABT-737 alone ($P < 0.05$). The effect on inducing apoptosis in HepG2 and SMMC-7721 cells with the treatment of ABT-737 combined with NCTD for 48 h was greater than that of ABT-737 or NCTD alone ($P < 0.05$). NCTD combined with ABT-737 has a positive role in the treatment of HCC.

Table 1  List of the main reagents and instruments in the experiments

| Primary reagents          | Source                                      |
|---------------------------|---------------------------------------------|
| ABT-737                   | Biochempartner                              |
| NCTD                      | Nanjing Zelang Medical Technology Co., Ltd.  |
| DMSO                      | Hyclone                                     |
| 96-well and 6-well cell culture | Costar, United States                      |
| Methyl thiazolyl tetrazolium (MTT)  | Sigma, United States                      |
| Trypsin                   | Hangzhou Gino Biomedical Technology Co., Ltd. |
| CO2 Incubator             | Thermo Scientific, United States           |
| Multiskan MK3 microplate reader | Thermo Scientific, United States           |
| Flow cytometer            | BD Biosciences                              |
| TUNEL Assay Kit for Apoptosis Detection | Nanjing KeyGEN BioTECH                     |

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INTRODUCTION

Myeloid cell leukemin-1 (Mcl-1) is a special B-cell lymphoma 2 (Bcl-2) family protein. It can not only control cell survival and death, but also plays an important role in regulating apoptosis signaling[1-3]. Several studies have shown that Mcl-1 generally has a high expression in hepatocellular carcinoma (HCC) and other malignant tumors[4-6], and this has become a cancer research focus of molecular targeted therapy. ABT-737 is a novel cancer therapeutic agent that has good prospects for clinical application[7]. However, ABT-737-mediated apoptosis is limited when there is high expression of Mcl-1 in liver cancer and other solid tumors[8-10], and this has become a major obstacle point in clinical application. Research has shown the treatment sensitivity of tumor cells to ABT-737 can be enhanced by its combination with other chemotherapy drugs[11-14].

Norcantharidin (NCTD) is a derivative of the Chinese medicine cantharidin, which has good anti-tumor effects[15-17]. Studies have reported that the anti-tumor effect of NCTD may be related to the role of Bcl-2 family members[18], which can inhibit Mcl-1 expression in HCC cells[19]. Therefore, this study aims to investigate the therapeutic effects of NCTD combined with ABT-737 on HCC cells, and to preliminarily analyze its mechanism of action for the future development of anticancer drugs, aiming to provide theoretical guidance for clinical applications.

MATERIALS AND METHODS

Materials

HCC cell lines: HepG2, SMMC-7721 (purchased from Cell Bank of Beijing Concord Technical Institute). Reagents and equipment details are displayed in Table 1.

Experimental methods

Cultured cell lines: Hepatoma cell lines HepG2 and SMMC-7721 were cultured in vitro, placed in RPMI-1640 medium containing 10% fetal bovine serum, and placed in an incubator with 5% CO2 at 37℃.

Cell proliferation inhibition detection by methyl thiazolyl tetrazolium assay: HepG2 and SMMC-7721 hepatoma cells in the logarithmic growth phase were seeded into 96-well plates and cultured. Cells were divided into the following groups when they attached to the wall: ABT-737 monotherapy group, NCTD monotherapy group, ABT-737 combined with NCTD group, control group, and apoptosis group; and each group had 3 parallel wells. After treatment,
culture was continued for 48 h. Then, 20 μL of methyl thiazolyl tetrazolium (MTT) solution was added into each well, and incubated for 4 h in an incubator. The supernatant was discarded, 150 μL of DMSO was added into each well, and they were placed in the incubator for 10 min. Optical density (OD) value was measured with an enzyme mark instrument. Measurements were repeated three times, and the average value was obtained. Proliferation inhibitory rates of the drug-treated groups were calculated as follows: inhibition rate (%) = [1 - (average OD value of drug-treated groups - average OD value of the apoptosis group)/(average OD value of the control group - average OD value of the apoptosis group)] × 100%.

**Western blot detection of Mcl expression in hepatoma cells:** Before initiation of the experiment, 4 × 10^5 hepatoma cells were seeded in 6-well plates. After cell adhesion, they were treated with NCTD alone, ABT-737 alone, and NCTD combined with ABT-737, and placed in an incubator for 24 h. After drug-treated cells were trypsinized, cells were collected by centrifugation, total protein was extracted, then protein was quantified by Bradford assay. Western blot detection was carried out as follows: (1) loading volume: the sample injection volume per well was 20 μg, boiled in water for 5 min, centrifuged 5 min, and the supernatant sample was obtained; (2) SDS-PAGE electrophoresis, electrophoresis procedure: 100 V for 15 min and 180 V for 45 min; (3) electricity facing: 45 V for 35 min, 100 V for 10 min and blocked, then the membrane was washed for 5 min twice; (4) the primary antibody was added, incubated at 4 °C overnight and membrane was washed for 5 min three times, the secondary antibody was added, the membrane was washed twice; and (5) development and fixing: the membrane was fixed, chemiluminescent was added, wrapped in plastic wrap after drying, washed after exposure for 1-3 min, then scanned and protein bands analyzed.

**Apoptosis detection by flow cytometry:** After treatment, cells in the NCTD group, ABT-737 group, and NCTD combined with ABT-737 group were trypsinized, collected, centrifuged, washed, and resuspended. Then, flow cytometry detection and analysis were performed according to the TUNEL apoptosis kit manufacturer's instructions.

**RESULTS**

**Cell proliferation inhibition rate in each group**

After treatment of HepG2 and SMMC-7721 cells with different concentrations of NCTD for 48 h, cell proliferation inhibition rates detected by MTT were as follows: when concentrations of NCTD were increased, the cell proliferation inhibition rate became smaller (Figure 1A and B); meanwhile, the effect of ABT-737 1-3 μm and NCTD combined treatment on cell proliferation inhibition was stronger than ABT-737 alone. The difference was statistically significant (P < 0.05) (Figure 2A and B).

**Mcl-1 expression in cells after different concentrations of NCTD treatment**

After HepG2 and SMMC-7721 cells were treated with NCTD 15 μm, the expression of Mcl-1 was partially inhibited; and when the concentration of NCTD was 30 and 60 μm, the expression of Mcl-1 was almost undetectable (Figure 3).

**Effect of NCTD combined with ABT-737 on cytochrome C**

Results showed that the expression of cytochrome C was not detected in cells in the control group or the ABT-737 monotherapy group, and that a low expression of cytochrome C was detected in cells in the NCTD monotherapy group. Cytochrome C was highly expressed in cells in the ABT-737 combined with NCTD group (Figure 4A and B).

**Apoptosis detection by flow cytometry**

In the control group, ABT-737 3 μm monotherapy group, NCTD 30 μm monotherapy group, or ABT-737 combined with NCTD group, after the 48 h treatment of HepG2 and SMMC-7721 cells, cell apoptosis detection by flow cytometry showed the following: in the monotherapy groups, cells showed a small increase in apoptosis induction compared with the control group, and the difference was not statistically significant (P > 0.05); while after the combination treatment for 48 h, HepG2 and SMMC-7721 cells had a greater amount of apoptosis compared with ABT-737 and NCTD monotherapy, and the difference was statistically significant (P < 0.05) (Figure 5A and B).

**DISCUSSION**

ABT-737 is an antagonist of small molecule Bcl-2, and a novel anti-cancer drug that induces tumor cell apoptosis without causing damage to normal cells; it has broad prospects for development. However, ABT-737 is inhibited in the induction process of apoptosis in hepatocellular carcinoma and some solid tumors that have high expression of Mcl-1. Therefore, determining how to reduce the expression of Mcl-1 in cells to increase the efficiency of the therapeutic effect of ABT-737 for liver cancer would be a breakthrough. Studies have reported norcantharidin treatment for cancer can inhibit the expression of Mcl-1. Therefore, norcantharidin combined with ABT-737 was used in this study to analyze its effect in the treatment of liver cancer, and to explore its mechanism.

The results are as follows: when HepG2 and...
Figure 1 Change in cell proliferation inhibition rate after 48 h of treatment with different concentrations of norcantharidin. A: The change of cell proliferation inhibition rate in HepG2 cells; B: The change of cell proliferation inhibition rate in SMMC-7721 cells.

Figure 2 Change in cell proliferation inhibition rate after 48 h of treatment with different concentrations of ABT-737 or with ABT-737 combined with different concentrations of norcantharidin (15 μm, 30 μm, 60 μm). A: The change of cell proliferation inhibition rate in HepG2 cells; B: The change of cell proliferation inhibition rate in SMMC-7721 cells.

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SMMC-7721 cells are treated for 48 h with different concentrations of NCTD, it is apparent that NCTD has a good inhibitory effect on cell proliferation; and comparing 1-3 μm of ABT-737 alone with ABT-737 combined with NCTD, results show that the ABT-737 combined with NCTD treatment has a stronger inhibition of cell proliferation compared with ABT-737 alone\[29,30\]. In order to verify this, we further detected its apoptotic effect by flow cytometry. The results showed that ABT-737 combined with NCTD treatment for 48 h applied to HepG2 and SMMC-7721 cells had a stronger apoptosis-inducing effect than ABT-737 and NCTD monotherapy; and thus, we confirm the rationality of these results. These results also show that NCTD enhances ABT-737 in inhibiting cell proliferation by inducing apoptosis.

Regarding detection of Mcl-1 expression in cells, results showed that after treatment of HepG2 cells and SMMC-7721 cells with NCTD 15 μm, the expression of Mcl-1 was partially inhibited, while the expression of Mcl-1 was almost undetectable when NCTD concentration was 30 and 60 μm. As expected for NCTD, there were better inhibitory effects on Mcl-1 expression in cells at higher doses. To study its mechanism, cytochrome C was further detected in the cytoplasm and the mitochondrial membrane.

### Figure 3: Expression of Mcl-1 in cells after treatment with different concentrations of norcantharidin.

|          | HepG2 | SMMC-7721 |
|----------|-------|-----------|
| ABT-737  | -     | +         |
| NCTD     | -     | +         |

### Figure 4: Expression of cytochrome C in HepG2 cells and SMMC-7721 cells after treatment with ABT-737 and norcantharidin detected by Western blotting.

A: The expression of cytochrome C in HepG2 cells; B: The expression of cytochrome C in SMMC-7721 cells.

### Figure 5: Apoptosis of HepG2 and SMMC-7721 cells detected by flow cytometry after treatment for 48 h. A: The apoptosis of HepG2 cells after treatment for 48 h; B: The apoptosis of SMMC-7721 cells after treatment for 48 h.
In comparing HepG2 cells and SMMC-7721 cells treated in the control group, ABT-737 monotherapy group, NCTD monotherapy group, and ABT-737 combined with NCTD group, the following expressions of cytochrome C were found: in the control group and ABT-737 monotherapy group, the expression of cytochrome C in cells was not detected, while a low expression of cytochrome C was detected in cells in the NCTD monotherapy group; and cytochrome C showed a high expression in cells in the ABT-737 combined with NCTD group. This result prompts us to conclude that this two-drug combination can enhance the expression of cytochrome C, and it also proves that NCTD enhances the release of cytochrome C induced by ABT-737. These results are due to cytochrome C release from the mitochondria into the cytosol in cells, and this is an important symbol of the Bcl-2 family proteins in the regulation of apoptosis. Therefore, we can speculate that NCTD inhibits Mcl-1 enabling ABT-737 to release cytochrome C in cells.

Studies for ABT-737 drugs are promising. Although we have a number of significant results, there are still many issues that need to be explored in in-depth studies, such as: the inhibition by NCTD of the expression of Mcl-1 to enhance ABT-737 in the treatment of hepatocellular carcinoma drug resistance; the release of cytochrome C induced by ABT-737, and whether there is an impact on other factors; and to determine whether ABT-737 combined with other anticancer chemotherapy drugs will show improvements. In our study, these problems are not investigated, and the role of its mechanism needs to be further explored through in-depth studies.

In summary, NCTD and ABT-737 combined can solve the ABT-737 drug resistance problem for the treatment of liver cancer. NCTD can inhibit the expression of Mcl-1 to enhance the release of cytochrome C induced by ABT-737. NCTD has a role of inducing apoptosis to enhance ABT-737 in its inhibition of cell proliferation; thus, enhancing ABT-737 induces hepatocellular carcinoma cell apoptosis. Therefore, NCTD combined with ABT-737 has a positive impact on the treatment of hepatocellular carcinoma cells; clinical research in this field has great value, and it deserves further investigation.

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