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The anti-cancer effects of cisplatin on hepatic cancer are associated with modulation of miRNA-21 and miRNA-122 expression

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Abstract. – OBJECTIVE: Cisplatin is an effective chemotherapeutic drug to treat hepatic cancer, but its efficacy is marred by extensive adverse effects. Micro (mi) RNAs are small regulatory RNAs that may be used as molecular targets to better fine-tune chemotherapy in hepatic cancer. In this study, we examined to what extent the anti-cancer effects of cisplatin are associated with expressions of miRNA (miR)-21 and miR-122.

MATERIALS AND METHODS: The growth-inhibiting effects of cisplatin on the human hepatic cell line HepG2 were assessed by MTT assay, while cell apoptosis was documented using DAPI staining. Also, we tested the effects of cisplatin on tumour growth in a mouse tumour xenograft model. Finally, we quantified expression levels of miR-21 and miR-122 in cisplatin-treated HepG2 cells.

RESULTS: We observed that cisplatin significantly decreased the growth of HepG2 cells ($p < 0.05$ vs control cells) at all tested concentration (5-80 μg/ml) after 24 or 48 hours of treatment. Microscopic studies demonstrated apoptotic signs in cisplatin-treated cells. In the mouse tumour xenograft model, tumour weights and volumes were significantly ($p < 0.05$ untreated animals) lower after treatment with cisplatin. Also, treatment of HepG2 cells for 48 hours with 20 μg/ml cisplatin was associated with significant decreases in miR-21 expression levels and up-regulation of miR-122.

CONCLUSIONS: The anti-cancer effects of cisplatin are associated with down-regulation of miR-21 expression and up-regulation of miR-122.

Key Words:
Cisplatin, Hepatic cancer, miR-21, miR-122, Drug target, Mechanisms, Anti-cancer effect, Mouse xenograft model.

Introduction

Primary hepatic cancer is a malignant cancer of intrahepatic biliary epithelial cells and hepatic cells. This cancer is associated with high mortality[1-4]. In most patients, hepatic cancer is diagnosed at an advanced stage, when the surgical treatment is not feasible due to advanced tumour size, and/or intrahepatic or distant metastases[5,6]. Therefore, non-surgical treatment, and especially chemotherapy remain the principal therapeutic method[7-10].

Cisplatin is a chemotherapeutic drug whose principal mechanism of action is DNA damage. Cisplatin is widely used to treat solid cancer cells[8,11-13]. While effective, chemotherapy causes substantial adverse effects. This could be because of insufficient selectivity of cisplatin.

Newest research indicates the involvement of microRNAs (miRNA) in cancer biology, including hepatic cancer. miRNA are short RNAs (length of ~20-24 nucleotides); they post-transcriptionally regulate protein expression. Studies demonstrate that miRNA regulate biological processes critical to cancerogenesis, such as proliferation and survival. Specifically, there have been reports on the role of miR-21 and -122 in hepatic cancer[14,15]. In this work, we examined to what extent the anti-cancer effects of cisplatin are associated with miR-21 and miR-122. We conducted studies in both cell culture and mouse tumour xenograft model. Our experimental findings demonstrate that inhibition of proliferation of hepatic cells by cisplatin is associated with down-regulation of miR-21 and up-regulation of miR-122 expression levels. This confirms the suitability of both observed miRs as therapeutic targets in hepatic cancer.

Materials and Methods

Cells and Reagents

The HepG2 human hepatic cancer and H22 mouse hepatic cancer cell lines were obtained from the Third Military Medical University (Chongqing, China). Cisplatin was provided by

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Yunnan Gejiu Biological Pharmaceutical Co., Ltd (Yunnan, China). The clean grade male healthy Kunming mice, weighting 18 to 22 g, were purchased from the Kunming Institute of Zoology (Kunming, China). Plasticware (25- and 50-ml culture bottles, 96- and 6-well plates) were from Coster Company (Beijing, China). The utilized equipment included automatic microplate reader Elx-800 (Omega Bio-Tek Inc., Hercules, CA, USA), CO_2 constant temperature incubator (Thermo Fisher Scientific Inc., Shanghai, China), centrifuge (Changsha Xiangyi Centrifuge Co., Ltd, Changsha, China), and Olympus optical microscope (Olympus Corporation, Beijing, China). Cell culture supplies (DMEM culture medium, trypsin, and antibiotics) were provided by Thermo Fisher Scientific Inc., while foetal calf serum, methyl thiazolyl tetrazolium (MTT), and DMSO were from Sigma-Aldrich (Beijing, China). Haematoxylin-eosin staining solution was from Beijing Zhongshanjinqiao Biotechnology Co., Ltd. (Beijing, China). Primers for miR-21, miR-122, and the reference U6 were provided by Beijing Keyingmei Technology Co., Ltd (Beijing, China).

Proliferation Assay

Logarithmically growing HepG2 cells were lifted by trypsinization, counted, and seeded at the concentration of 6×10^3 cells/well cells onto 96-well culture plates. After 24 hours, the culture medium was replaced with culture medium containing cisplatin. Cisplatin was administered at concentrations of 0 (=basal), 5, 10, 20, 40, and 80 μg/ml, in the final volume of 200 μl. All experimental conditions were done in triplicate. Afterward, the cells were cultured for 24 or 48 hours. Then, the culture medium was discarded, and 50 μl/well of MTT solution combined with 150 μl of culture medium were added to each well. The plates were incubated for 4 hours, following which culture supernatants were discarded, and cells were lysed with 150 μl of dimethyl sulfoxide (DMSO) (12 min with mild shaking). Optical densities were measured at the wavelength of 490 nm. The growth inhibition rate (%) was calculated as follows:

Apoptosis Assay

The HepG2 cells were seeded onto sterile glass coverslips placed at the bottom of a 6-well plate. The cells were incubated overnight and then exposed to 10 or 20 μg/ml of cisplatin in a final volume of 2 ml. After incubation for 48 hours, cells were washed three times with cold phosphate-buffered saline (PBS) and then treated for 30 min with 4% triformol as the fixative agent. Afterward, 2 ml of 1 μg/mL DAPI staining solution was added to cells for 15 min to stain nuclei. Coverslips were mounted onto slides. Cell fluorescence was observed with the excitation wavelength of 350 nm and an emission wavelength of 460 nm.

Cisplatin Effects in a Mouse Tumour Xenograft Model

Mice were divided into 5 groups (6 animals per group). Control animals did not receive inoculation with H22 liver cancer cells. Other mice were inoculated with H22 liver cancer cells. Then, ascitic fluid from the abdominal cavity of these animals was collected and diluted with PBS to adjust cell suspension to the concentration of 5×10^6/ml. Afterward, 0.2 ml of H22 cell suspension was inoculated in subcutaneous armpits of left forelegs. Inoculation was complete within 90 min. Following this, the tumour volume was measured. When the volume reached 100 mm^3, mice received gavages. The control group animals (no injection of cancer cells) received gavage of 0.2 ml/10 g of 0.5% sodium carboxymethyl cellulose every day. Mice in the "xenographic model, untreated" group received gavages with 0.2 ml/10 g 0.5% of sodium carboxymethyl cellulose every day. The animals in the "low-dose treated", "medium-dose treated", and "high-dose treated" groups received cisplatin gavages (respectively, 5, 10 or 20 mg/kg cisplatin) every day.

After cisplatin treatment, the shortest and the longest diameter of the tumour were measured to calculate the tumour volume. The tumour volume (mm^3) was calculated as follows: where "a" = the longest diameter in mm, b = the shortest diameter in mm.

The mice were euthanized on day 10. Six mice from each group provided solid tumours. The tumours were washed with normal saline. The tumour inhibition rate (%) was calculated as follows:

Haematoxylin-eosin Staining

Tumours were washed with cold normal saline and fixed with 4% triformol. After fixation, tumours were washed again with saline and kept overnight. Following this, tumours were dehydrated by sequential immersing in 70%, 80%, 90% and 100% ethanol for several hours. After-

ward, tumours were immersed in Xylene I and II (30 min each), and then in paraffin I, II, and III (30 min each). Finally, tumours were embedded in paraffin and sliced into 5 um paraffin sections. The following procedures were applied: dewaxing, staining with haematoxylin, washing with running water, staining with eosin, repeated washing, and sealing. Afterward, the slides were observed under the microscope.

Expressions of miR-21 and miR-122

Logarithmical growing HepG2 cells were lifted by trypsin, counted, and seeded at the concentration of 3×10^5/well onto 6-well culture plates. After 24 hours, the culture medium was replaced with the culture medium containing cisplatin. Cisplatin was administered at 20 μg/ml in the final volume of 2 ml. All experimental conditions were done in triplicate. After 48 hours, the culture medium was discarded, and 1 ml of MZ lysis buffer was added to cells. Cells were transferred into tubes and incubated at room temperature for 5 min. Then, 0.2 ml chloroform was added to the tubes, and the tubes were shaken for 20 sec. Afterward, the tubes were centrifuged for 15 min at 12,000 rpm. This led to the formation of 3 layers. The top aqueous phase layer, containing RNA, was carefully aspirated and mixed with 0.5 volumes of absolute ethanol. Then, the mixture was transferred into minispin columns and centrifuged for 30 sec at 12,000 rpm. The effluents were collected and mixed with 0.75 volumes of absolute ethanol. The mixtures were transferred into miRelute columns, and centrifuged for 30 sec at 12,000 rpm. The effluents were discarded, and 0.5 ml of solution maximum recovery diluent (MRD) was added and incubated for 100 sec. The samples were subsequently centrifuged for 30 sec (12,000 rpm), and the effluents were discarded. The samples were washed with solution RW. Finally, the samples were centrifuged for 1 min at 12,000 rpm, the supernatants were discarded, and miRelute columns were eluted with 20 μl RNase-free H_2O. The samples were reverse transcribed. U6 served as a control for miR expression.

Statistical Analysis

The data were analyzed using SPSS22.0 statistical package (SPSS Inc, Armonk, New York, NY, USA). Outcomes were compared using the *t* and one-way ANOVA tests. The *p*-value of < 0.05 was considered as statistically significant.

Results

Anti-Cancer Effects of Cisplatin on HepG2 liver Cancer Cells

At both studied time points (24 and 48 hours), cisplatin significantly ($p < 0.05$ *vs.* untreated cells) inhibited the growth of HepG2 cells (Table I; Figure 1). The inhibitory effects were more pronounced at 48 hours of treatment (Table I; Figure 1).

Furthermore, the cisplatin-treated cells exhibited shrinkage of cell volume (Figure 2). In addition, DAPI staining revealed a pronounced karyopyknosis and breakage (Figure 2). Also, the cells showed signs of apoptosis with increasing drug concentrations (Figure 2).

The effects of Cisplatin in A Mouse Xenograft Model

Untreated mice in the xenograft model showed tumours with the average weight and volume of, respectively, 1.47 ± 0.13 g and 713.37 ± 72.61 mm^3 (Table II). As expected, cisplatin demonstrated marked inhibition of tumour growth in the mouse xenograft model. Specifically, both the weight and volume of xenograft tumours were diminished by more than 30% ($p < 0.05$; Table II). Unlike cell proliferation (Table I), we did not observe dose-dependent inhibition of tumour growth (Table II). Therefore, the magnitudes of decreases of weights and volumes of xenograft tumours were comparable between different doses of cisplatin (Table II).

These findings were corroborated by haematoxylin-eosin staining. Untreated mice that showed the biggest tumour volume (Table II) demonstrated the most pronounced tissue morphology changes (bigger cytoplasm and more karyokinesis; Figure 3). In contrast, tumours

Table I. Inhibition of growth of HepG2 cells by cisplatin.

Experimental conditions	24 hours %)	48 hours (%)
Untreated cells	0	0
5 μg/ml cisplatin	12.0 ± 1.3*	19.4 ± 1.8*,#
10 μg/ml cisplatin	15.2 ± 1.8*	25.1 ± 2.6*,#
20 μg/ml cisplatin	21.3 ± 1.9*	32.0 ± 3.1*,#
40 μg/ml cisplatin	26.8 ± 2.3*	37.0 ± 3.5*,#
80 μg/ml cisplatin	28.0 ± 2.8*	39.0 ± 3.6*,#

Footnote: Cell growth was assessed by MTT test. Data are presented as mean ± SD. *$p < 0.05$ vs untreated cells; #$p < 0.05$ *vs.* 24 hours.

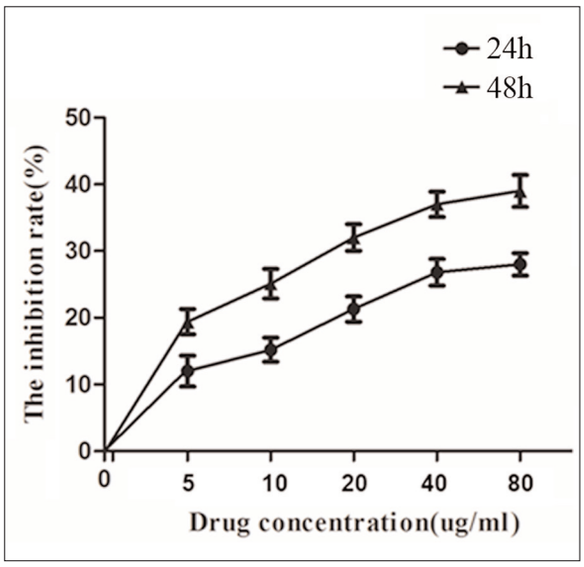

Figure 1. Effects of cisplatin on growth of HepG2 cells. Cell growth was assessed using MTT test at 24 and 48 hours.

from cisplatin-treated mice showed more necrotic areas of the tissue, smaller cells, and darker chromatin and less pronounced karyokinesis (Figure 3).

Expressions of miR-21 and miR-122

We next tested how expressions of miR-21 and miR-122 were modulated by cisplatin (Table III). HepG2 cells were treated with cisplatin (20 μg/ml) for 48 hours. We observed that the expression level of miR-21 decreased, whereas that of miR-122 increased following the treatment with cisplatin.

Discussion

Cisplatin is the first generation platinum drug that belongs to alkylating anti-tumour drugs[12,13,16]. Cisplatin is inactive in high chlorine environment[17]. It is believed that cisplatin enters the cells using the copper transport protein. It has been reported that cisplatin concentration correlates with copper concentration and that absorption and transportation of cisplatin are defined by copper pharmacokinetics[18,19]. A transport protein CTR1 has high affinity to platinum and copper. Deficiency and mutation of the gene that encodes this protein increase cancer resistance to cisplatin[20-22]. Cisplatin causes cross-linking nucleoprotein and DNA strains by adding bivalent

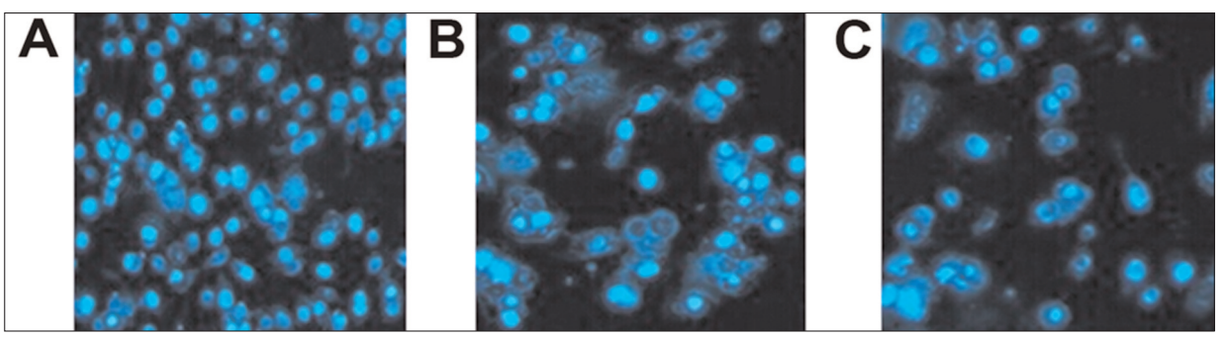

Figure 2. ***A,*** Cell morphology after cisplatin treatment. The left, centre and right images represent HepG2 cells respectively treated for 48 hours with 0.1% DMSO as vehicle control, or 10 or 20 μg/ml cisplatin. DAPI staining; magnification: ×400.

Table II. Effects of cisplatin on tumour weight and volume in a mouse xenograph model.

Groups	Number of mice	Average tumour weight (g)	Inhibitory rate (%)	Average tumour volume (mm^3)	Inhibitory rate (%)
Xenograph model, untreated	6	1.47 ± 0.13	0	713.37 ± 72.61	0
Xenograph model, low-dose cisplatin	6	0.48 ± 0.05*	57.36	143.63 ± 15.32*	68.53
Xenograph model, medium-dose cisplatin	6	0.52 ± 0.06*	55.63	172.93 ± 18.61*	66.46
Xenograph model, high-dose cisplatin	6	0.50 ± 0.05*	56.43	149.25 ± 16.42*	67.32

Footnote: Tumour weights and volumes are presented as mean ± SD. Low-, medium-, and high-doses of cisplatin are 5, 10 or 20 mg/kg. *$p < 0.05$ *vs*. untreated animals.

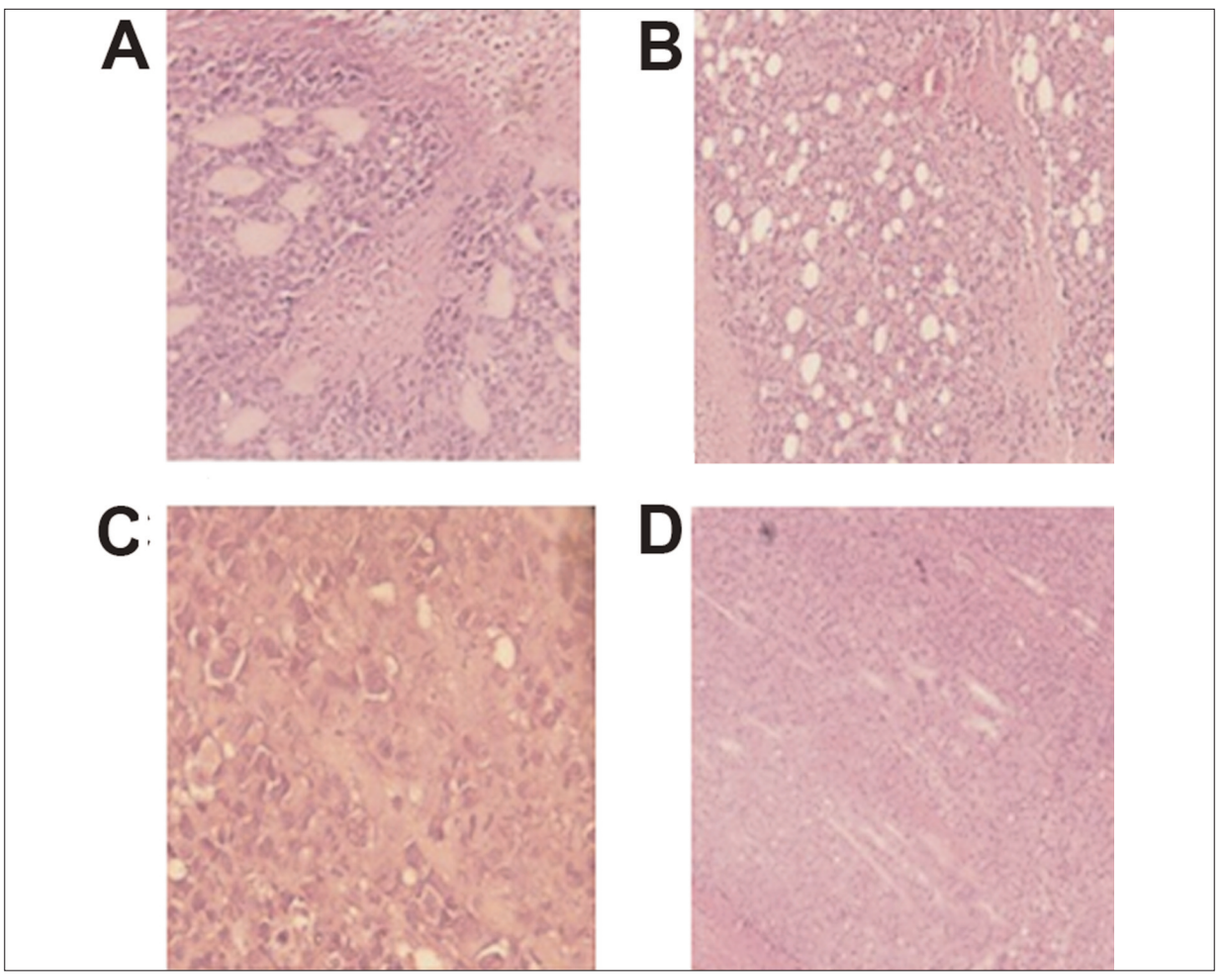

Figure 3. Tumour morphology. ***A,*** Untreated group. ***B,*** Low-dose cisplatin group. ***C,*** Medium-dose cisplatin group. ***D,*** High-dose cisplatin group. Haematoxylin-eosin staining; magnification: ×400.

platinum[23-26]. In cell culture studies, cisplatin was shown to inhibit proliferation of HepG2 human liver cancer cells[27]. Our study showed similar findings. We further observed that cisplatin caused karyopyknosis and breakage, and cell apoptosis in HepG2 cells. In a mouse tumour xenograft model, cisplatin demonstrated inhibition of tumour growth. We also tested whether these effects are associated with modulation of miRNAs. miRNAs are a single strain, small RNAs with short sequence (20 to 24 nucleotides). They exert regulatory function by post-transcriptionally controlling protein expression. Hepatic cancer tissue exhibits abnormal expression of many miRNAs, including miR-224, miR-93, miR-21, and miR-122. In our study, expression of miR-21 was down-regulated by cisplatin. miR-21 is likely to function as a cancer-promoting factor in HepG2 cancer cells. It is plausible that cisplatin exerts its cancer-inhibiting effects by down-regulating miR-21. In contrast, miR-122 was up-regulated by cisplatin treatment. miR-122 inhibits cell proliferation and promotes cell apoptosis in cancer cells.

Table III. Expression levels of miR-21 and miR-122 in HepG2 cells.

Groups	miR-21, expression level	miR-122, expression level
Untreated cells	1.01 ± 0.04	1.02 ± 0.02
Cisplatin treatment (20 μg/ml)	0.79 ± 0.01*	1.31 ± 0.04*

Footnote: Cell growth was assessed by MTT test. Data are presented as mean ± SD. *$p < 0.05$ vs untreated cells; #$p < 0.05$ *vs.* 24 hours.

Conclusions

Cisplatin exerts a pronounced time- and concentration-dependent inhibitory effect on HepG2 human liver cancer cells. In the mouse xenograft model, cisplatin significantly inhibits tumour growth and promotes cell apoptosis. These beneficial effects are associated with down-regulation of miR-21 and up-regulation of miR-122 expression.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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