ORIGINAL ARTICLE

Zinc protoporphyrin IX enhances chemotherapeutic response of hepatoma cells to cisplatin

Yang-Sui Liu, Huan-Song Li, Dun-Feng Qi, Jun Zhang, Xin-Chun Jiang, Kui Shi, Xiao-Jun Zhang, Xin-Hui Zhang

Yang-Sui Liu, Huan-Song Li, Dun-Feng Qi, Jun Zhang, Xin-Chun Jiang, Kui Shi, Xiao-Jun Zhang, Xin-Hui Zhang, The Central Hospital of Xuzhou City, Xuzhou Hospital Affiliated to Medical College of Southeast University, Xuzhou 221009, Jiangsu Province, China
Correspondence to: Xin-Hui Zhang, MD, The Central Hospital of Xuzhou City, Xuzhou Hospital Affiliated to Medical College of Southeast University, Jiefang Rd, Xuzhou 221009, Jiangsu Province, China. 88liuyin888@163.com
Telephone: +86-516-83956131 Fax: +86-516-83956131

Abstract

AIM: To investigate the effect of zinc protoporphyrin IX on the response of hepatoma cells to cisplatin and the possible mechanism involved.

METHODS: Cytotoxicity was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Apoptosis was determined by a flow cytometric assay. Western blotting was used to measure protein expression. Heme oxygenase (HO)-1 activity was measured by determining the level of bilirubin generated in isolated microsomes. Reactive oxygen species (ROS) production was monitored by flow cytometry. Caspase-3 activity was measured with a colorimetric assay kit. Mice were inoculated with 1×10^7 tumor cells subcutaneously into the right flanks. All mice were sacrificed 6 wk after the first treatment and tumors were weighed and measured.

RESULTS: Overexpression of HO-1 in HepG2 cell line was associated with increased chemoresistance to cis-diaminedichloroplatinum (cisplatin; CDDP) compared to other cell lines *in vitro*. Inhibition of HO-1 expression or activity by zinc protoporphyrin IX (ZnPP IX) markedly augmented CDDP-mediated cytotoxicity towards all liver cancer cell lines *in vitro* and *in vivo*. In contrast, induction of HO-1 with hemin increased resistance of tumor cells to CDDP-mediated cytotoxicity *in vitro* and *in vivo*. Furthermore, cells treated with ZnPP IX plus CDDP exhibited marked production of intracellular ROS and caspase-3 activity, which paralleled the incidence of cell apoptosis, whereas hemin decreased cellular ROS and caspase-3 activity induced by CDDP.

CONCLUSION: ZnPP IX increases cellular sensitivity and susceptibility of liver cancer cell lines to CDDP and this may represent a mechanism of increasing ROS.

Core tip: Overexpression of heme oxygenase (HO)-1 in HepG2 cell line was associated with increased chemoresistance to cis-diaminedichloroplatinum (cisplatin; CDDP) compared to other cell lines. Inhibition of HO-1 expression by zinc protoporphyrin IX (ZnPP IX) markedly augmented CDDP-mediated cytotoxicity towards other hepatoma cells. Induction of HO-1 with hemin increased resistance of tumor cells to CDDP-mediated cytotoxicity. Furthermore, cells treated with ZnPP IX plus CDDP exhibited marked production of intracellular reactive oxygen species (ROS) and caspase-3 activity, whereas hemin decreased cellular ROS and caspase-3 activity induced by CDDP. Therefore, administration

of HO-1 inhibitors may evolve into a new liver cancer treatment strategy.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fourth most common cancer and the third leading cause of cancer-related death worldwide[1,2]. Surgical resection is the best treatment to reduce the growth of HCC and improve life expectancy of HCC patients. However, more than 80% of HCC patients are diagnosed with an advanced-stage or unresectable disease. In patients who undergo resection, the prognosis is poor with a recurrence rate as high as 50% at two years[3,4]. Current chemotherapy is also not effective and the development of chemoresistance is the major limitation. Therefore, a new understanding of the molecular mechanisms of drug resistance is urgently needed for treatment of this deadly disease.

Heme oxygenase (HO) is a microsomal initial and rate-limiting enzyme that catalyzes the degradation of heme to produce equimolar quantities of biliverdin, CO and free iron, which play crucial roles in the defense against oxidative and cellular stress[5]. To date, three distinct mammalian HO isoforms have been identified: HO-1, HO-2 and HO-3. HO-2 and HO-3 are constitutively expressed, whereas HO-1 is known to be highly induced by a vast array of stress-inducing stimuli such as H_2O_2, UV irradiation, hypoxia, and extracellular acidosis[6-9]. Induction of the HO-1 protein represents a cytoprotective defense mechanism in the adaptive response to cellular stress[10]. Moreover, new observations indicate that HO-1 and its products also exert anti-inflammatory effects and influence the growth and proliferation of tumor cells. Elevated expression and increased activity of HO-1 have been found in various malignant tumors such as human renal cell carcinoma, pancreatic cancer, hepatoma, prostate cancer, and Kaposi sarcoma[11-15]. Furthermore, overexpression of HO-1 in tumor cells can be further elevated by chemotherapy, radiotherapy or photodynamic therapy[16]. Its overexpression in human cancers may give cancer cells a growth advantage and enhance resistance to chemotherapy and other stressors[16-18]. Inhibition of HO-1 expression or activity suppresses cellular proliferation and increases responsiveness of tumor cells to some anticancer treatments *in vitro* and *in vivo*[18-20]. In contrast, induction of HO-1 expression or activity decreases cell sensitivity to antitumor drugs. Accumulating evidence suggests that HO-1 can be a therapeutic target for antitumor treatment.

In this study, we investigated whether constitutively overexpressed HO-1 in liver cancer cells was associated with resistance to apoptosis induction by cis-diaminedichloroplatinum (cisplatin; CDDP), and then explored the role of HO-1 in protecting tumor cells against chemotherapeutic agents *in vitro* and *in vivo*.

MATERIALS AND METHODS

Reagents

CDDP, zinc protoporphyrin IX (ZnPP IX), hemin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 2,7-dichlorodihydrofluo-recin diacetate (DCFH-DA) were from Sigma (St. Louis, MO, United States). Dulbecco's Modified Eagle's Medium, fetal bovine serum (FBS), and other cell culture reagents were obtained from Gibco BRL Life Technologies (Grand Island, NY, United States). Rabbit anti-human HO-1 monoclonal antibodies were from Cell Signaling Technology (Danvers, MA, United States). Goat anti-glyceralde-hyde-3-phosphate dehydrogenase (GAPDH) polyclonal antibody and horseradish peroxidase-conjugated goat anti-mouse antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, United States).

Cell lines and cell culture

The human liver cancer cell lines HepG2, SMMC7721, and 97H were purchased from the Cell Bank, Chinese Academy of Sciences (Shanghai, China). The three cell lines were cultured in RPMI 1640 containing 100 U/mL penicillin, 0.1 mg/mL streptomycin and 10% heat-inactivated FBS at 37 ℃ in an atmosphere of 5% CO_2 and 95% air. To induce or inhibit the activity of HO-1, hemin or ZnPP IX was added 1 h prior to the addition of CDDP.

Cell viability analysis

In vitro, the cytostatic and/or cytotoxic effects of treatments were determined using the MTT assay. Tumor cells were seeded in a 96-well plate at a concentration of 6×10^3 cells per well and cultured overnight for cell attachment. The following day, cells were treated with the investigational agents for 48 h. After an appropriate time, 20 μL MTT [5 mg/mL in phosphate-buffered saline (PBS)] was added to each well for 4 h. The supernatant was discarded and 150 μL DMSO was added to each well. The plates were shaken until the crystals had dissolved. Absorbance was measured at a wavelength of 570 nm using an enzyme-linked immunosorbent assay reader with background subtraction at 690 nm. Cell viability was expressed as percentage of untreated controls. Experiments were completely randomized in design and repeated six times.

In vitro apoptosis assay

Induction of apoptosis *in vitro* was determined by a flow cytometric assay with an annexin V and propidium iodide apoptosis kit according to the manufacturer's instructions

(Invitrogen, United States). Cells were plated in six-well plates at 1×10^5 cells/well and treated for 48 h. After treatment, the cells were harvested from the plate using trypsin and washed twice with PBS, and then incubated with annexin V-fluorescein isothiocyanate and propidium iodide for 15 min. The number of apoptotic cells was analyzed by flow cytometry using a FACScan Analyzer. Experiments were completely randomized in design and repeated six times.

Protein preparation and Western blotting
Western blotting was used to measure protein expression as follows. Cells were harvested after treatment, and washed twice with PBS. The suspension was resuspended in a buffer containing 1% Triton X-100 with PBS and Halt Protease Inhibitor Cocktail for 30 min on ice and then centrifuged at $14000 \times g$ for 20 min. Protein concentration was measured with the bicinchoninic acid protein assay reagent according to the manufacturer's instructions (Thermo Scientific, United States). Equivalent amounts of total proteins (80 mg) from each sample were separated by 10% gradient SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes. After blocking with 10% milk, the membranes were incubated with the primary antibody for 3 h at room temperature. The dilutions of the primary antibodies were as follows: 1:1000 for anti-hHO-1 antibody and 1:2000 for anti-GAPDH antibody. The membranes were washed four times with 0.1% Tween 20 in Tris-buffered saline and then incubated with a secondary antibody for 1 h. The membranes were washed extensively again and the protein bands were visualized with the ECL-Plus chemiluminescence system according to the manufacturer's instructions (Applygen Technologies, Beijing, China). The relative optical density of each Western blotting band was measured using the Quantity One Quantification Software according to the manufacturer's guidelines (Bio-Rad Laboratories).

HO-1 activity
HO-1 activity was measured by determining the level of bilirubin generated in isolated microsomes. After treatment, cells were collected and homogenized in a homogenization buffer [20 mmol/L potassium phosphate buffer (pH 7.4), 250 mmol/L sucrose, 2 mmol/L EDTA, 2 mmol/L phenylmethyl sulfonyl fluoride (PMSF) and 10 μg/mL leupeptin]. Homogenates were centrifuged at $10000 \times g$ for 30 min at 4 ℃. The resulting supernatants were centrifuged at $100000 \times g$ for 1 h at 4 ℃. The pellet was suspended in phosphate buffer (pH 7.0) and designated the microsome fraction. An aliquot of the microsomal fraction was then added to a reaction mixture containing cytosol of the cells (2 mg cytosolic protein), hemin (20 μmol/L), glucose-6-phosphate (2 mmol/L), glucose-6-phosphate-dehydrogenase (0.2 units), and NADPH (0.8 mmol/L). The reaction mixture was incubated for 60 min at 37 ℃ in the dark and terminated by the addition of 1 mL chloroform. The bilirubin con-

centration was calculated by measuring the difference in absorbance between 465 and 530 nm using a Shimadzu UV-160A spectrophotometer with a molar extinction coefficient of 40/mmol/L/cm. Experiments were completely randomized in design and repeated six times.

Measurement of oxidative stress
ROS production in each sample was monitored by flow cytometry using the DCFH-DA fluorescent probe. DCFH-DA is a stable compound that rapidly diffuses into cells and is activated by intracellular esterases to DCFH, which is converted by H_2O_2 and peroxidases to the DFC fluorescent derivate. Thus, the fluorescence intensity is proportional to the amount of peroxide produced by cells. After treatment, the cells were incubated with 10 μmol/L DCFH-DA for 30 min at 37 ℃ in the dark. Cells were then washed twice with PBS and resuspended again. The intracellular ROS was quantitated as a function of fluorescence intensity measured by flow cytometry.

Measurement of caspase-3 activity
Caspase-3 activity was measured with a Colorimetric Assay Kit according to the manufacturer's instructions (Roche). After treatment cells were collected and lysed with chilled lysis buffer [50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 20 mmol/L EDTA, 0.2% Triton X-100, 1 mmol/L PMSF, 10 μg/mL aprotinin, and 5 mmol/L dithiothreitol] for 10 min on ice. The supernatant containing 100 μg protein was incubated with 0.2 mmol/L AcDEVD-pNA, a specific substrate for caspase-3. Caspase-3 activity was measured at 405 nm with background subtraction at 570 nm and expressed in Ac-pNA cleavage or released absorbance.

Liver cancer xenografts in mice
Female athymic nude mice (6 wk old) were purchased from the Central Animal Laboratory, Nanjing University, Nanjing, China. Mice were maintained according to Nanjing University of Traditional Chinese Medicine institutional policies. Mice were inoculated subcutaneously into the right flanks with 1×10^7 tumor cells, resuspended in 0.2 mL PBS. When tumors reached 100-300 mm^3 in volume, mice were randomly divided into 6 groups of 8 mice each: control (drug vehicle), hemin (10 mg/kg), ZnPP IX (5 mg/kg), CDDP (5 mg/kg), combination of hemin (10 mg/kg) plus CDDP (5 mg/kg), and combination of ZnPP IX (5 mg/kg) plus CDDP (5 mg/kg). All the drugs were injected weekly into their peritoneal cavities four times. The control group was injected with PBS. All mice were sacrificed 6 wk after the first treatment. Tumors were weighed as previously described[21].

Statistical analysis
All data are expressed as mean ± standard deviation (SE). Statistical analysis was performed using one-way analysis of variance and independent-sample t test for each paired experiment with Windows version 15.0. In all assays, $P < 0.05$ was considered statistically significant.
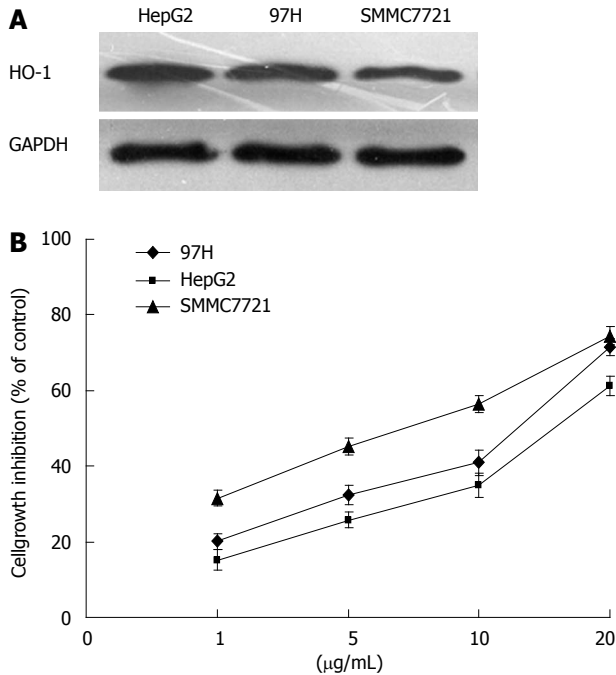
Figure 1 Liver cancer cell lines showed divergent heme oxygenase-1 expression levels, which were associated with variable susceptibility to chemotherapy. In Western blotting, high levels of heme oxygenase (HO)-1 protein were detected in HepG2 cell line, whereas other cell lines revealed lower expression (A) HepG2 > 97H > SMMC7721. Cell viability was assessed 48 h after application of cis-diaminedichloroplatinum (cisplatin; CDDP) *via* MTT assay. All cell lines showed dose-dependent growth inhibition upon treatment with CDDP. The HepG2 cell line, with the highest HO-1 expression, was significantly more chemoresistant to CDDP than the other cell lines with lower HO-1 expression (B).

Figure 2 Heme oxygenase-1 could be induced by cis-diaminedichloroplatinum in liver cancer cell lines. Western blotting showed that heme oxygenase (HO)-1 protein expression was significantly increased after treatment with cis-diaminedichloroplatinum (cisplatin; CDDP) for 24 h and achieved a plateau at a concentration of 10 μg/mL in all liver cancer cells.

RESULTS

HO-1 expression in liver cancer cells is associated with susceptibility to chemotherapy

Liver cancer cell lines showed different expression levels of HO-1 (Figure 1A). Native HO-1 expression was higher in HepG2 than in other cells, without either hemin or antitumor drug treatment. Furthermore, we compared CDDP-induced growth inhibition in three kinds of human liver cancer cells. HepG2 cells with high native HO-1 expression showed significantly greater *in vitro* chemoresistance to CDDP than the other cell lines with low native HO-1 expression (Figure 1B).

HO-1 could be induced by CDDP in liver cancer cell lines

The level of HO-1 was significantly increased in all liver cancer cell lines when cells were treated with CDDP (Figure 2). The highest level of HO-1 expression in all cell lines was achieved with CDDP at concentrations of 5-10 μg/mL. The expression of HO-1 achieved a plateau when CDDP was used at a concentration of 20 μg/mL in all liver cancer cells.

Targeted inhibition of HO-1 activity by ZnPP IX in liver cancer cell lines

As shown in Figure 3, HO-1 activity was significantly elevated in all liver cancer cell lines after treatment with
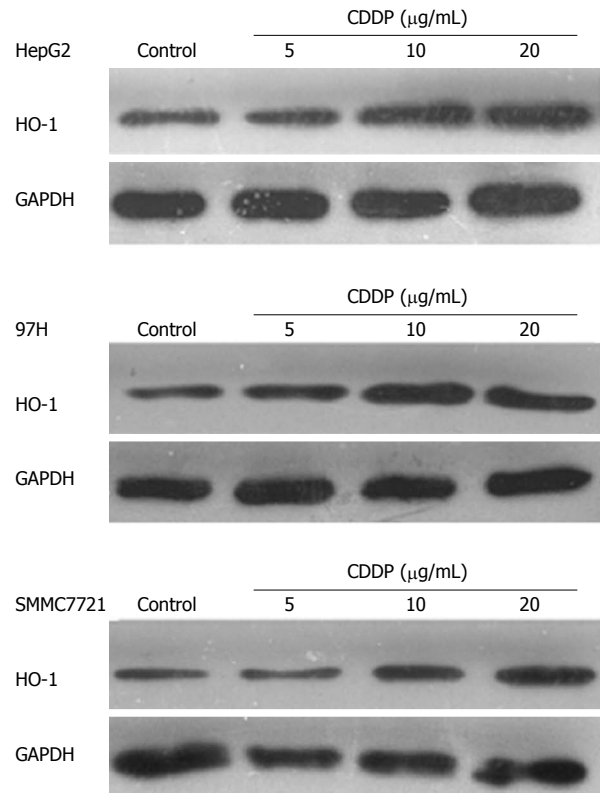
CDDP compared with untreated cells *in vitro* ($P < 0.05$). ZnPP IX decreased HO-1 activity as well as CDDP-induced HO-1 activity ($P < 0.05$). In contrast, administration of CDDP in combination with hemin significantly increased HO-1 activity in all liver cancer cell lines compared to CDDP treatment alone ($P < 0.05$).

Inhibition of HO-1 activity increases susceptibility to chemotherapy in vitro

To demonstrate further the cytoprotective properties of HO-1, the HO-1 inductor hemin and ZnPP IX were added to tumor cells 1 h prior to the application of CDDP. The cytotoxic effect induced by CDDP was largely increased by addition of ZnPP IX, which suggests that inhibition of HO-1 activity by ZnPP IX boosted the anticancer effects of CDDP (Figure 4). In addition, hemin treatment significantly decreased apoptosis induced by CDDP. These findings suggest that augmentation of HO-1 activity induced by hemin is associated with reduced CDDP-induced apoptosis in all liver cancer cell lines. However, it seemed that apoptosis induced by CDDP plus ZnPP IX in the HepG2 cell line was higher than that in the other cell lines.

Cytotoxicity of ZnPP IX is related to increased oxidative stress

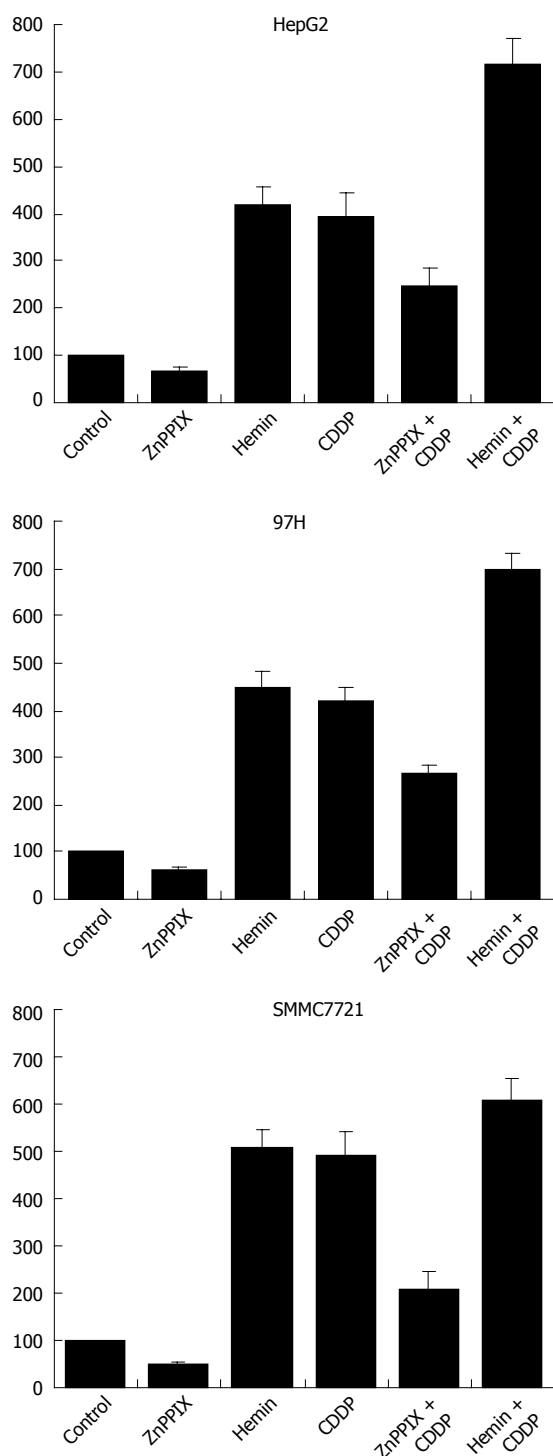To investigate whether apoptosis induced by the addition

Figure 3 Targeted inhibition of heme oxygenase-1 activity by zinc protoporphyrin IX in liver cancer cell lines. Heme oxygenase (HO)-1 activity was measured by determining the level of bilirubin generated in isolated microsomes in cells treated with 10 μmol/L zinc protoporphyrin (ZnPP) IX, 10 μmol/L hemin, and/or 10 μg/mL cis-diaminedichloroplatinum (cisplatin; CDDP) for 24 h. ZnPP IX significantly inhibited HO-1 activity and decreased cis-diaminedichloroplatinum (cisplatin; CDDP)-induced HO-1 activity in all liver cancer cell lines compared with controls or cells treated with cisplatin alone ($P < 0.05$). In contrast, hemin increased CDDP-induced HO-1 activity in all liver cancer cell lines ($P < 0.05$).

of ZnPP IX was related to the increase in intracellular production of ROS in liver cancer cells, flow cytometry

was performed using the oxidant-sensitive fluorescence probe DCDHF. As shown in Figure 5, ZnPP IX (10 μmol/L) increased the fluorescence intensity and CDDP (10 μg/mL)-induced ROS in liver cancer cell lines ($P < 0.05$). In contrast, hemin (10 μmol/L) decreased CDDP-induced ROS in all liver cancer cell lines ($P < 0.05$). These findings suggest that the ZnPP IX-induced increase in apoptosis after exposure to CDDP is related to increased intracellular ROS.

Caspase-3 activity in treated and untreated liver cancer cells

Caspase-3 activity of the cells paralleled cellular apoptotic vulnerability. Caspase-3 activity in cells was not significantly affected by hemin at the concentration used (10 μmol/L) compared with untreated cells (Figure 6). However, caspase-3 activity was significantly increased in all liver cancer cells after treatment with ZnPP IX (10 μmol/L) or CDDP (10 μg/mL) ($P < 0.05$). Furthermore, after treatment with CDDP, caspase-3 activity was re-elevated by the addition of ZnPP IX ($P < 0.05$). Caspase-3 activity induced by CDDP was markedly reduced in the presence of hemin (10 μmol/L) ($P < 0.05$).
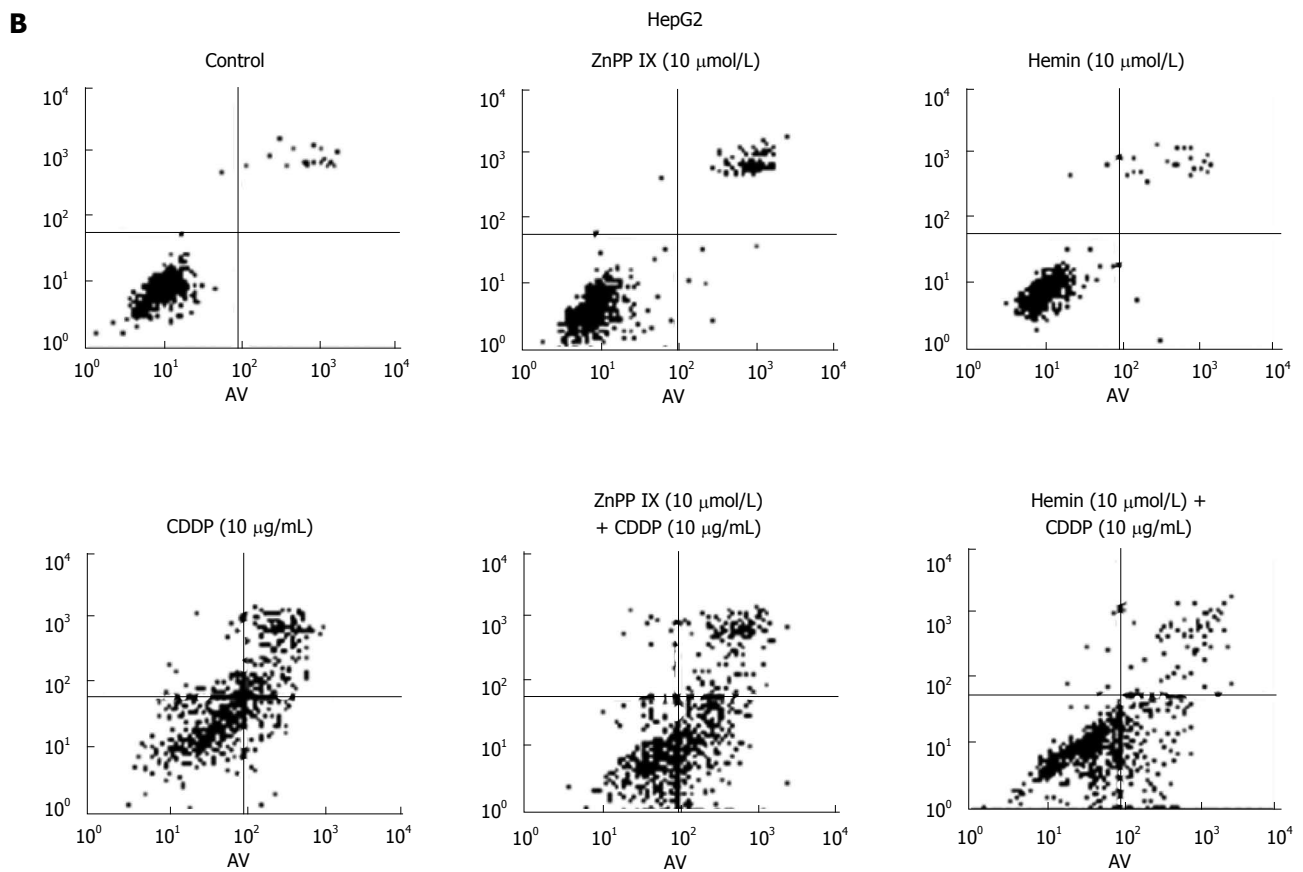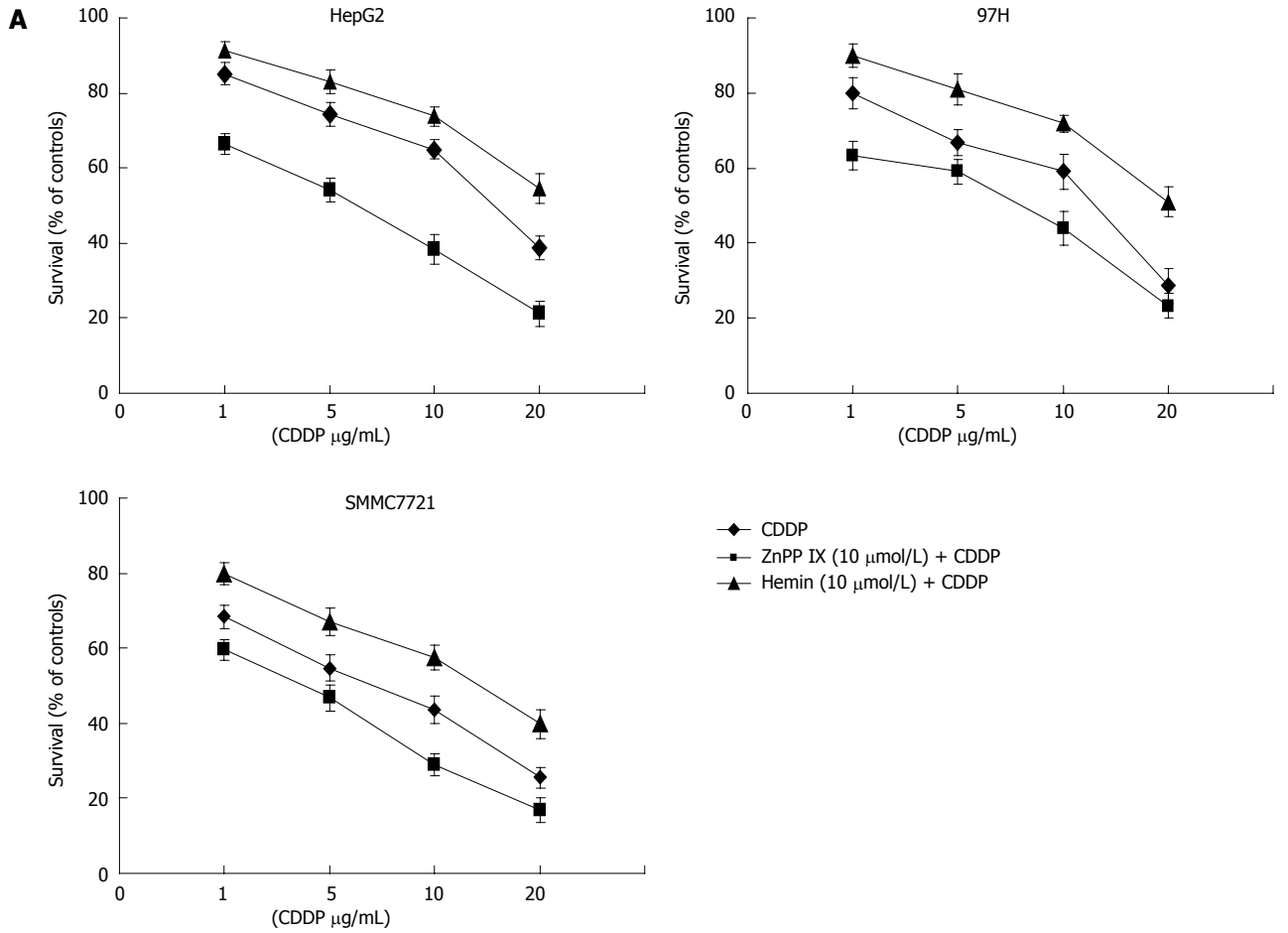
Inhibition of HO-1 expression increases susceptibility to chemotherapy in vivo

Mice treated with ZnPP IX (5 mg/kg) or CDDP (5 mg/kg) showed significantly reduced tumor growth in comparison with untreated control mice (Figure 7). Inhibition of HO-1 activity by ZnPP IX (5 mg/kg) boosted the anticancer effects of CDDP (5 mg/kg), resulting in a significant reduction of tumor growth in CDDP-treated mice, compared to tumors treated with CDDP alone. In contrast, administration of hemin (10 mg/kg) in combination with CDDP tended to increase tumor weight compared to tumors treated with CDDP alone.

DISCUSSION

Accumulating evidence indicates that overexpression of HO-1 increases the proliferation of cancer cells or renders them resistant to apoptosis induced by chemicals, radiotherapy, photodynamic therapy, and other stressors[12,13,17]. Downregulation of HO-1 expression or activity can suppress cell proliferation or increase cellular sensitivity to some therapeutic regimens. For example, inhibition of HO-1 activity could suppress the proliferation of lung cancer, pancreatic cancer and leukemia cells, and sensitize cells to antitumor drugs *in vitro*[22-25]. Moreover, inhibition of HO-1 activity could also lead to increased pancreatic cancer cell susceptibility to chemotherapy *in vivo*[26]. However, little is known about the chemoresistant and poor prognostic function of HO-1 in liver tumors.

In this study, we found that constitutive overexpression of HO-1 was specific to HepG2 cells, and this overexpression of HO-1 was associated with higher resistance to CDDP *in vitro* (Figure 1). We also found that CDDP increased HO-1 activity in liver cancer cells, and
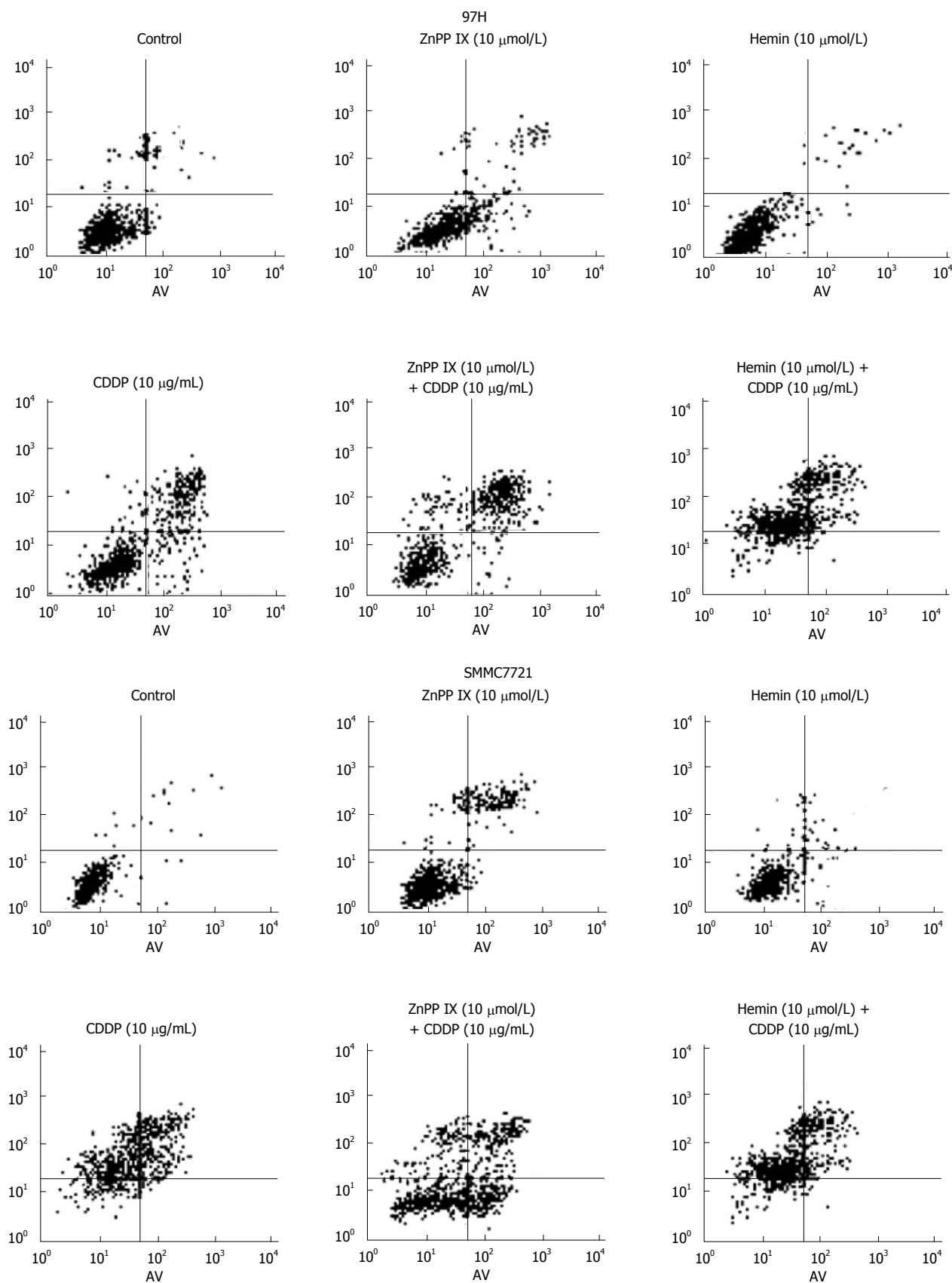
Figure 4 Inhibition of heme oxygenase-1 activity led to increased susceptibility to chemotherapy *in vitro*. Cell viability was assessed *via* MTT assay. zinc protoporphyrin (ZnPP) IX (10 μmol/L) significantly increased apoptosis in cis-diaminedichloroplatinum (cisplatin; CDDP)-treated cells, compared with those treated with CDDP alone (A) (*P* < 0.05). In contrast, hemin decreased apoptosis induced by CDDP in all liver cancer cell lines. FACS analysis with propidium iodide staining also showed that downregulation of heme oxygenase (HO)-1 by ZnPP IX increased apoptosis after exposure to CDDP in comparison with the control group (*P* < 0.05), whereas increased expression of HO-1 by hemin resulted in decreased sensitivity to CDDP (B).
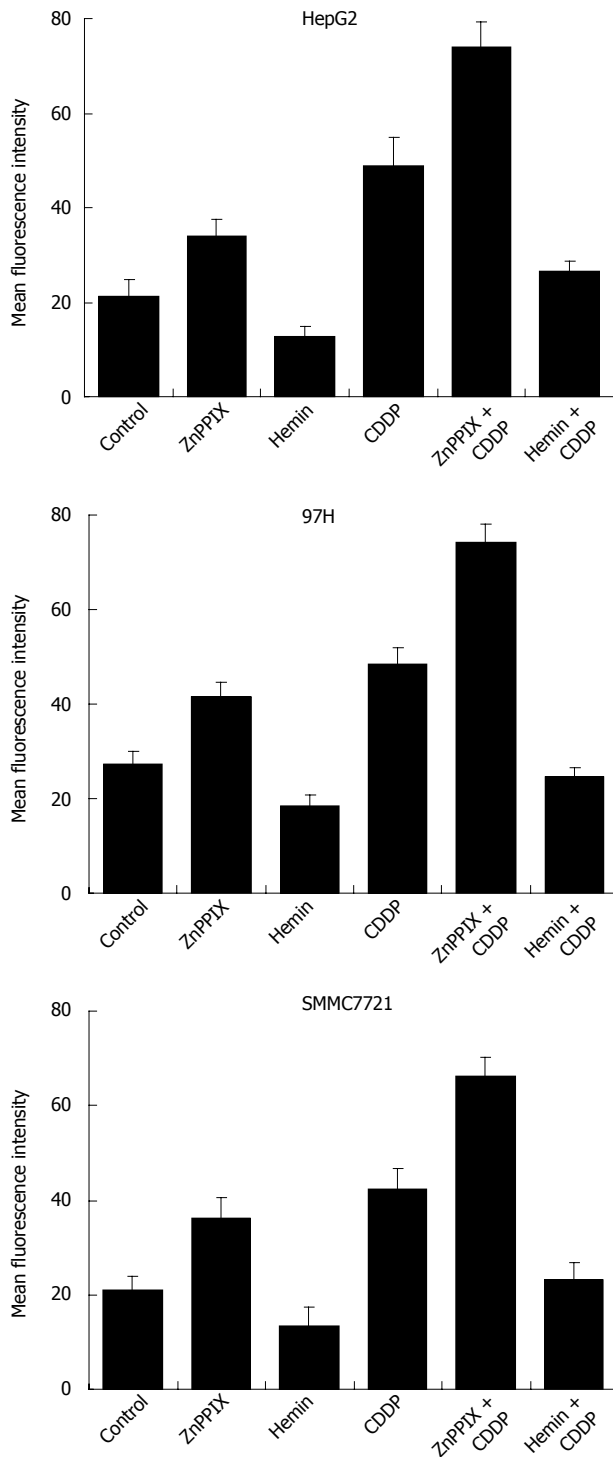
Figure 5 Cytotoxicity of zinc protoporphyrin IX is related to increased oxidative stress. Induction of intracellular ROS was evaluated by flow cytometry by measuring CM-H2DCFDA fluorescence. Each cell line was treated with 10 μmol/L zinc protoporphyrin (ZnPP) IX, 10 μmol/L hemin, and/or 10 μg/mL cis-diaminedichloroplatinum (cisplatin; CDDP) for 24 h. Mean fluorescence intensity was quantified for these treatments. ZnPP IX increased the fluorescence intensity of the cells and drug-induced ROS in liver cancer cell lines compared with controls or cells treated with CDDP alone. In contrast, hemin decreased CDDP-induced ROS in all liver cancer cell lines.

Figure 6 Caspase-3 activity in treated and untreated liver cancer cells. Inhibition of heme oxygenase (HO)-1 activity increased caspase-3 induced by cis-diaminedichloroplatinum (cisplatin; CDDP) in all liver cancer cell lines. Caspase-3 activity was significantly increased in all cells after treatment with cisplatin (10 μg/mL) and re-elevated by addition of zinc protoporphyrin (ZnPP) IX (10 μmol/L). The caspase-3 activity induced by CDDP was markedly reduced in the presence of hemin (10 μmol/L) in all liver cancer cell lines.

inhibition of HO-1 activity by ZnPP IX boosted the anticancer effects of CDDP. These results suggest that the cellular sensitivity of liver cancer cells to CDDP is
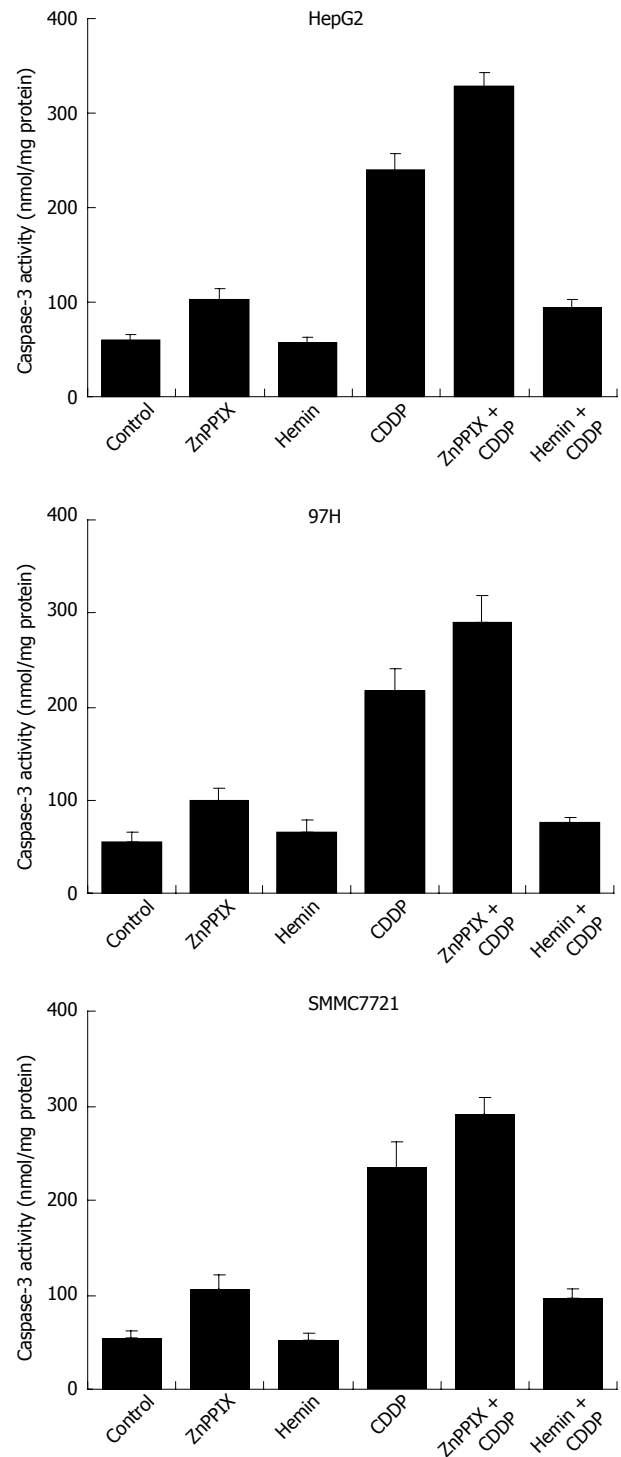
augmented by downregulation of HO-1 activity by ZnPP IX. In support of this conclusion, induction of HO-1 activity by the addition of hemin resulted in a decrease in CDDP-induced apoptosis in all liver cancer cells (Figure 1). These findings suggest that HO-1, as a stress response
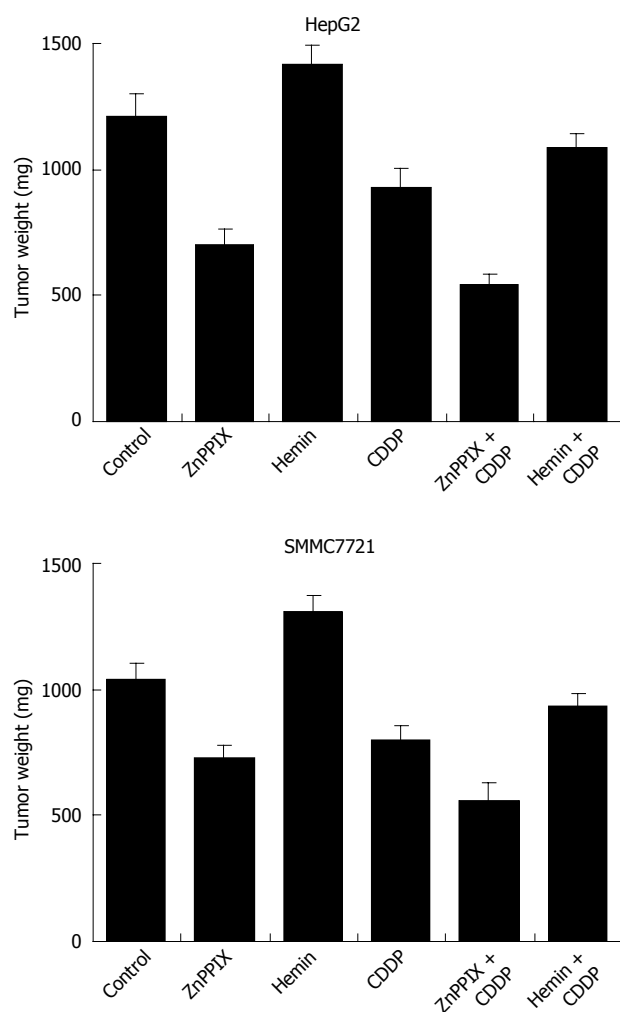
Figure 7 Inhibition of heme oxygenase-1 expression increases susceptibility to chemotherapy *in vivo*. Each nude mouse was treated with 5 mg/kg zinc protoporphyrin (ZnPP) IX, 10 mg/kg hemin, and/or 5 mg/kg *cis*-diamine-dichloroplatinum (cisplatin; CDDP) for 6 wk and tumor weight was assessed after the last treatment. ZnPP IX treatment significantly reduced tumor growth in CDDP-treated mice, compared with those treated with CDDP alone. CDDP in combination with hemin in mice significantly increased tumor weight compared with CDDP treatment alone.

gene, plays an important role in the determination of the sensitivity of liver cancer cells to CDDP. Recent studies in other models support this view: induction of HO-1 expression made colon cancer cells and leukemia resistant to antitumor drugs such as merocyanine and pyrrolidine dithiocarbamate[27,28]. Furthermore, inhibition of HO-1 activity by polyethylene glycol-ZnPP IX augmented the cytotoxic effect of several chemotherapeutic agents in an experimental murine sarcoma model[17]. However, opposite effects were observed in breast cancer and B lymphoblasts, in which HO-1 did not protect the cells from chemotherapy-induced apoptosis[29]. This might be because HO-1 has different biological actions in different cancer cells.

The signaling pathways mediating these effects on cancer are poorly understood, although there is accumulating evidence showing that HO-1 is an antiapoptotic agent in several types of cells. Some previous studies have demon-

strated that apoptosis of human oral and gastric cancer cells could be regulated by Nrf2 and p21[30,31]. Overexpression of HO-1 resulted in a significant increase in Nrf2 and p21 levels and decreased cellular sensitivity to chemotherapy. In contrast, inhibition of HO-1 expression or activity leads to the downregulation of Nrf2 and p21 and decreases cellular sensitivity to chemotherapy. However, we have also shown that the mechanism of resistance to apoptosis by upregulation of HO-1 may be related to increased ROS level and caspase-3 activity. The destructive mechanisms of chemotherapy on cancer cells are mainly based on the generation of oxidative stress and/or induction of apoptosis[32]. The accumulation of ROS results in the subsequent loss of mitochondrial membrane potential and activation of the caspase-9/3 pathway. The present study showed that the activity of caspase-3 and ROS level induced by CDDP were significantly inhibited by elevated hemin (Figures 5 and 6). In contrast, ROS level and caspase-3 activity were significantly re-elevated by the addition of ZnPP IX in liver cancer cells after treatment with CDDP. Therefore, our study suggests that the resistance to apoptosis by HO-1 may be through a pathway with elevated ROS level and caspase-3 activity in human liver cancer cells. In addition, the products of HO-1 enzymatic activity directly influence several other cell signaling pathways, such as the Ras-Raf-ERK pathway, which have crucial roles in human renal cancer cells[33]. However, the mechanisms involved in increased chemosensitivity need additional investigation.

A more important finding, consistent with *in vitro* results, is that inhibition of HO-1 activity by ZnPP IX boosts anti-cancer effects of CDDP, resulting in a significant reduction of tumor growth in mice, compared to those treated with CDDP alone (Figure 7). In contrast, administration of hemin in combination with CDDP tends to increase tumor weight. These findings suggest that inhibition of HO-1 expression leads to increased sensitivity of liver cancer cells to CDDP *in vivo*.

In conclusion, the findings in this study suggest that high HO-1 levels in HepG2 cells are, at least partially, responsible for resistance to chemotherapy and environmental stress. Inhibition of HO-1 activity by ZnPP IX results in sensitizing tumor cells to chemotherapy such as CDDP by increasing the cellular ROS level and caspase-3 activity *in vitro* and *in vivo*. The combined treatment with HO-1 inhibitors may intensify the antitumor activity of chemotherapeutics and may open up new perspectives in the treatment of liver cancer.

COMMENTS

Background
Inhibition of heme oxygenase (HO)-1 expression or activity was shown to suppress cellular proliferation and increase responsiveness of tumor cells to some anti-cancer treatments. In contrast, induction of HO-1 expression or activity decreases cell sensitivity to anti-tumor drugs. Accumulating evidence suggests that HO-1 could be a therapeutic target for anti-tumor treatment.

Research frontiers
The cytoprotective enzyme HO-1 is significantly overexpressed in many tumors and seems to play an important role in cellular resistance to chemotherapy and

radiotherapy. In this study, the authors examined whether constitutively overexpressed HO-1 in liver cancer cells was associated with resistance to apoptosis induction by cis-diaminedichloroplatinum (cisplatin; CDDP), and explored the role of HO-1 in protecting tumor cells against chemotherapeutic agents *in vitro* and *in vivo*.

Innovations and breakthroughs

The results suggested that overexpression of HO-1 in HepG2 cell lines was associated with increased chemoresistance to CDDP compared with other cell lines *in vitro*. The inhibition of HO-1 by zinc protoporphyrin IX (ZnPP IX) was associated with increased cellular sensitivity and susceptibility of liver cancer cell lines to CDDP *in vivo* or *in vitro*. Reactive oxygen species (ROS) and caspase-3 seem to be involved in HO-1-mediated resistance to anti-cancer treatment. Therefore, administration of HO-1 inhibitors may evolve into a new liver cancer treatment strategy.

Applications

The findings in this study suggest that high HO-1 levels in HepG2 cells may, at least partially, be responsible for their resistance to chemotherapy and environmental stress. The combined treatment with HO-1 inhibitors may intensify the anti-tumor activity of chemotherapeutic agents and open up new perspectives in the treatment of liver cancer.

Terminology

High HO-1 levels in HepG2 cells may be responsible for their resistance to chemotherapy and environmental stress. Inhibition of HO-1 activity by ZnPP IX sensitizes tumor cells to chemotherapy (*e.g.*, with CDDP) by increasing the cellular ROS level and caspase-3 activity *in vitro* and *in vivo*.

Peer review

This manuscript reports the results of a study on the effect of ZnPP IX, an HO-1 inhibitor, on the cellular sensitivity and susceptibility of liver cancer cell lines to CDDP *in vitro* and *in vivo*. The results are interesting and may open up new perspectives in the treatment of liver cancer.

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