Antitumor Effects of Dihydromyricetin on the Proliferation, Migration and Apoptosis of Human Hepatocellular Carcinoma Hep3B Cells

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Abstract

Background: Hepatocellular carcinoma (HCC) represents a serious public health problem worldwide and has high morbidity and mortality. Dihydromyricetin (DHM) exhibits anti-tumor effect on a variety of malignancies, but its antitumor function of DHM in HCC has been unclear. The aim of this study was designed to investigate the antitumor effect of DHM on cell apoptosis, proliferation, migration and invasion of hepatoma carcinoma cells.

Methods: Cultured Hep3B cells were treated with different DHM concentrations, follow by cell apoptosis, proliferation, migration and invasion were examined by CCK-8, colony formation assay, wound healing, Transwell and flow cytometry, respectively. The mRNA and protein expression of apoptosis-associated genes and Bcl-2/Caspase-9 signaling pathway were validated by RT-PCR and western blot.

Results: DHM markedly suppressed proliferation, migration, invasion and facilitated apoptosis in Hep3B cells. Mechanistically, DHM significantly downregulated the Bcl-2 expression, and upregulated the mRNA and protein levels of Cleaved-Caspase 3, Cleaved- Caspase 9, Bak, Bax and Bad. Furthermore, in the nude mice tumorigenic model, DHM treatment greatly decreased the weight of the HCC tumors compared to the weights in control and NDP group.

Conclusions: DHM could suppress cell proliferation, migration, invasion, and facilitated apoptosis in Hep3B cells. These findings could provide novel insights to develop potential therapeutic strategy for the clinical treatment of HCC.

Introduction

Hepatocellular carcinoma (HCC) is the most common type of primary hepatocellular carcinoma and is a growing public health problem worldwide. Recently, the incidence, recurrence and mortality of HCC are continuously increasing over years in the majority of countries [1]. The increase in mortality mainly due to a lack of effective therapeutic options [2]. However, most treatments may cause serious side effects, such as nephrotoxicity, neurotoxicity and gastrointestinal (GI) toxicity [3]. Recent studies have illustrated that traditional Chinese medicines (TCMs) have benefic effects on the treatment of a variety of cancers, including HCC [4–6]. Therefore, to find a novel and effective TCM for the treatment of HCC, with the aim to improve the overall survival time of patients with HCC.

Dihydromyricetin (DHM), a biologically active flavonoid compound from *Ampelopsis grossedentata* [7], exerts anti-inflammatory, hypoglycemic, antioxidative, antimicrobial, anti-allergic, and anti-acne effects [8]. Remarkably, this flavonoid compound has attracted considerable attention because of its strong inhibitory effect on colorectal cancer [9], ovarian cancer [10], cholangiocarcinoma [11], and lung cancer [12]. It has been documented that DHM inhibited the progression of colorectal cancer and colon cancer in mouse models [9, 13]. Besides, DHM also plays an important role in various biological processes including cell proliferation, apoptosis, and migration [14]. In vitro experiment demonstrated that DHM suppressed cell proliferation, migration, invasion and promoted apoptosis and cell cycle arrest at the

G1/S phase in melanoma SK-MEL-28 cells, HCC [15], ovarian cancer cells [10], lung cancer cells [16], myelomonocytic lymphoma cells [17], and cholangiocarcinoma cells [14, 18].

In the present study, we mainly investigated the effect of DHM in the biological processes of cell growth and metastasis in Hep3B cells. The different concentrations of DHM were used to stimulate Hep3B cells to reveal the function of DHM on cell proliferation, apoptosis, migration and apoptosis. DHM stimulated Hep3B cells to reveal the anti-tumor characteristics both in vivo and in vitro. The apoptosis-associated genes and Bcl-2/Caspase-9 signaling pathway were analyzed. Further, in the nude mice tumorigenic model, DHM treatment significantly reduced the weight of the HCC tumors. These findings might provide a potential therapeutic candidate for the clinical treatment of HCC.

Materials And Methods

Cell culture and treatment

Hep3B cells derived from American Type Culture Collection (ATCC, Rockville, MD, USA) were grown in Dulbecco's modified Eagle medium (DMEM, Life Technologies, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS, Gibco, Australia). The cells were incubated in an incubator containing 95% air and 5% CO₂ at 37 °C. Dihydromyricetin (DHM) obtained from Sigma-Aldrich (St Louis, MO, USA) was dissolved in dimethylsulfoxide (DMSO, Sigma-Aldrich) and then different concentrations of DHM were used to stimulate Hep3B cells. Nedaplatin (NDP, Nichi-Iko Pharmaceutical Co., Ltd.) was solubilized in sterilized H₂O. DMSO served as a control group.

Animals and tumor models

The mice were housed under standard animal room conditions (temperature 22±1°C and humidity 55±5%). Mice were anesthetized with 2% isoflurane (RWD, Shenzhen, China) using a Rodent Anesthesia Machine (VetEquip Inc., Pleasanton, Ca). Animals were sacrificed with 2–3 times the anesthetic dose of isoflurane (via inhalation), followed by cervical dislocation, shaven and sterilized with 75% ethanol. After opening the abdominal cavity, the liver and tumor tissue were collected and weighed. All animals had free access to sterile tap water and food during the experiments. The mice were randomly divided into three groups, including control groups (n=8), DHM (n=8) and NDP (n=8) for 3 weeks. Hep3B cells were transplanted into the mouse via subcutaneous injection of 1×10⁷ cells. One week after transplantation, tumors had grown to a volume of approximately 20 mm³ with a model success rate of 100%. All the animal experiments and surgical procedures were approved by the Institutional Animal Care and Use Committee of Guangdong Medical University (GDY1802018).

Cell Viability Measurement

The cell viability was assayed by adding Cell Counting Kit-8 assays (CCK-8) solution as described (CCK-8, Dojindo Molecular Technologies, Gaithersburg, MD)[19, 20]. The Hep3B cells were seeded in 6-well plates (1×10^5 cells/ well) and were allowed to adhere for 8 h. The medium was replaced with medium containing different concentrations of DHM (0, 10, 20, 30, 40, 50 μ M). DMSO control wells contained 0.1% DMSO. After 12 h, the culture medium of the cells was discarded, 10 μ L of CCK-8 solution was added into each well and cells were incubated at 37 °C for another 2 h. Finally, the absorbance was analyzed at 450 nm using a Microplate Reader (Molecular Devices, San Jose, CA, USA). All the assays were performed for three times independently. Absorbance of cells in the absence of treatment was regarded as 100% of cell survival. Cell survival was calculated as: absorbance/absorbance of control $\times 100\%$.

Colony formation assay

Cell viability was performed using a colony formation assay [21]. Hep3B cells were seeded into a 6-well plate (3×10^2 cells/well) for 8 h, followed by treatment with two different concentrations of DHM and NDP for 24 h. Hep3B cells were cultured with drug-containing medium for ten days. The cells were fixed with methanol-glacial acetic acid stationary solution (3:1) at room temperature for 10 min and stained with 1% crystal violet (Amresco, Solon, OH, USA). The following formula was used to calculate the colony formation inhibition rate: Colony formation inhibition rate = (control group -experimental group)/control group $\times 100\%$.

Cell apoptosis assay

Cell apoptosis was assessed by flow cytometry assay (BD, FranklinLakes, NJ) [22]. In brief, the cells were seeded in 6-well plates (1×10^6 cells/well), followed by 24 h incubation at 37°C. The cells were then treated with different concentrations of DHM and NDP for 24 h. The assay was performed using the Annexin V-FITC/PI cell apoptosis detection kit (BD Pharmingen, USA) according to the manufacturer's protocol. Subsequently, the cells were monitored by flow cytometry (FACSCalibur, Becton Dickinson, USA), and the data were analyzed using FlowJo™ software (version 10, FlowJo LLC).

Cell migration and invasion Assay

Cell migration and invasion were detected by using Transwell assay with 8.0 μ m porous polycarbonate membranes (Millipore, Bedford, Massachusetts, USA) [20]. In brief, cells were treated with different concentration of DHM and NDP and adjusted cell density to 1×10^5 . The lower transwell contained 600 μ L DMEM with 10% FBS. After incubation 24 h at 37 °C, the non-traversed cells in the upper compartment were wiped by a wet cotton swab. Traversed cells on the lower side of the filter were fixed stained with 0.1% crystal violet (Amresco, Solon, OH, USA). Then these cells were stained with 0.5% crystal violet (Merck, Darmstadt, Germany) for 20 min and counted microscopically (Olympus, Tokyo, Japan). The

method of cell invasion was similar with cell migration, except that the inserts were coated with BD Matrigel™ Matrix (BD Biosciences, NY, USA).

Wound healing assay

Cell migratory abilities was tested by a wound healing assay. Hep3B cells were seeded in 12-well dishes (5×10^4 cells/well), and incubated in DMEM with 10% FBS for 24 h at 37 °C. The cells were then exposed in the absence or presence of DHM and NDP. Then the cells were scratched across the surface of the well by pipette tip. After an incubation at 37°C of 24 h, the scratches were observed.

Western blot analysis

The effects of DHM and NDP on the expression levels of Bcl-2, Cleaved-Caspase 3, Cleaved- Caspase 9, Bak, Bax and Bad were analyzed using western blot [20]. Protein samples were obtained from Hep3B cells that were treated with different concentrations of DHM and NDP for 24 h using cell lysis buffer (RIPA, Beyotime Biotechnology, Shanghai, China). The proteins were collected and detected by using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). Subsequently, total protein (20 µg) samples were separated using SDS-PAGE (10% gel) and transferred onto a polyvinylidene fluoride (PVDF) membranes and blocked in 5% skim milk powder for 1 h at room temperature. Following the membranes were incubated with the corresponding antibodies. Primary antibodies of Bcl-2, Cleaved-Caspase 3, Cleaved- Caspase 9, Bak, Bax and Bad were (all from Cell Signaling Technology) were incubated with the membrane at 4 °C overnight. Then, blots were washed three times with TBST and were incubated with secondary antibodies for 1 h at room temperature. The blots were detected using enhanced chemiluminescence (ECL) reagents (Super Signal Dura kit, Pierce, IL, USA) according to the manufacturer's instructions. The blots were quantified by using Image Lab™ Software (Bio-Rad).

Hematoxylin and Eosin staining

Briefly, liver tissues were immersed in 4% paraformaldehyde for 4h and transferred to ethanol (75%, 85%, 95%). Then they were treated with xylene paraffin-embedded according to a previous report [23]. Before immunostaining, 3-µm-thick liver tissue sections were dewaxed in xylene, rehydrated by decreasing concentrations of ethanol (95%, 85%, 75%) and washed in PBS. And then stained with hematoxylin and eosin (H&E). After staining sections were dehydrated through increasing concentrations of ethanol and xylene.

Statements

The study was approved by ARRIVE guidelines (<http://www.nc3rs.org.uk/arrive-guidelines>). Moreover, we confirm that all methods were performed in accordance with the relevant guidelines and regulations.

Statistical analysis

All data are expressed as the mean $\pm$ S.E.M., and at least three independent replicates were used for per group. All statistical procedures were analyzed by SPSS 22.0 (IBM, Manassas, VA, USA), and plots were generated using GraphPad prism 8.0 (GraphPad Software, La Jolla, CA, USA) (<https://www.graphpad.com/scientific-software/prism/>). The SPSS analysis show that our results are normal distribution, and homogeneity of results between each treatment groups are equal. Significant differences between treatment groups were determined by one-way ANOVA (SPSS 22.0, Chicago, IL, USA).

Results

DHM suppressed cell proliferation and viability of Hep3B cells

The Hep3B cells were cultured with different concentrations of DHM (0 μ M, 10 μ M, 20 μ M, 30 μ M, 40 μ M, 50 μ M for 12 h, and then the cell proliferation was measured by CCK-8 assay. The results of the CCK-8 assay revealed that the proliferation of cells in the DHM group differed compared with the control group (Fig. 1A). The inhibitory effect increased prominently with increasing DHM concentration in a dose-dependent manner ($P < 0.001$) (Fig. 1A). In the subsequent experiments, 25 and 50 μ M of DHM were selected to treat Hep3B cells. According to the colony formation assay results (Fig. 1B and C), the cell viability in each group was significantly inhibited compared with the blank control group ($p < 0.001$), and the inhibition rate of 25 and 50 μ M DHM ($57.85\% \pm 3.24\%$; $88.55\% \pm 0.759\%$) were higher compared with the same NDP ($33.64 \pm 2.73\%$; $81.55\% \pm 1.41\%$). These data indicated that DHM inhibited the viability and proliferation of Hep3B cells, and its effect was comparable to that of NDP.

DHM inhibited the cell migration and invasion of Hep3B cells

Cell migration and invasion of Hep3B cells were measured by Transwell assay in this study. The results of Transwell assay (Fig. 2A) illustrated that numerous cells migrated into the membrane of the upper chamber in the control group, whereas different doses of DHM and NDP treatment significantly inhibited the cell migration rate ($P < 0.001$) (Fig. 2A and 2B). Notably, the inhibitory effect on migration increased gradually with increasing DHM and NDP concentration in a dose-dependent manner ($P < 0.001$). The migration rate in the control group was obviously increased from that of the control group to $23.79 \pm 3.97\%$ in the DHM (25 μ M), $60.42 \pm 2.05\%$ in the NDP (25 μ M), $9.78 \pm 2.33\%$ in the DHM (50 μ M) and $40.26 \pm 8.44\%$ in the NDP (50 μ M) treatment groups (Fig. 2B). Furthermore, the wound healing assay revealed that DHM and NDP treatment significantly reduced wound closure rates in Hep3B cells (Fig. 2C and D). In addition, compared with the control group, DHM and NDP treatment reduced the invasive ability of Hep3B cells (Fig. 2C and D). These results suggested that DHM inhibited cell migration and

invasion of Hep3B cells. Notably, the inhibition of migration and invasion in Hep3B cells following DHM treatment was superior to that by NDP treatment.

DHM induced apoptosis in Hep3B cells

As shown in Fig. 3A and B, the Hep3B cells were stained with FITC-Annexin-V and PI, and early and late apoptotic cells were measured by flow cytometry. The apoptosis rate of DHM and NDP group were significantly different from that of the control group ($P < 0.001$; Fig. 3B), and the apoptosis rate of the DHM group was also significantly different from that of the NDP group ($P < 0.001$; Fig. 3A and 3B). The proportion of apoptotic cells increased from $3.73 \pm 1.57\%$ in the control group to $21.7 \pm 3.57\%$ in the DHM (25 μM), $12.03 \pm 1.98\%$ in the NDP (25 μM), $50.67 \pm 4.80\%$ in the DHM (50 μM) and $34.33 \pm 3.81\%$ in the NDP (50 μM) experimental groups following treatment for 12 h (Fig. 3B; $P < 0.001$ versus control or NDP group). At 12 h, apoptosis was higher ($P < 0.001$) in a dose-dependent manner of DHM-exposed Hep3B cells compared to those in NDP group. The percentages of early and late apoptotic cells significantly increased with an increase in drug concentration. Thus, DHM induced apoptosis of the Hep3B cells ($P < 0.001$), and the ability of DHM induce the apoptosis of Hep3B cells was better than NDP. Next, the protein levels of apoptosis-associated factors were measured by western blot. As displayed in Fig. 3C-3F, DHM and NDP significantly promoted cleaved caspase 3, cleaved Caspase 9, Bak, Bax and Bad expressions and inhibited Bcl-2 expression compared with control group ($P < 0.001$). These results suggested that DHM could induce apoptosis in Hep3B cells.

Antitumor effects of DHM on tumor development in vivo

The effect of DHM on the growth of primary tumor xenografts in nude mice was examined. Tumor volumes were recorded every three days. The volumes of the primary tumors in the DHM and NDP groups were greatly reduced compared with the control group, and the effect of the DHM treatment was superior to the effect of NDP (Fig. 4A). The weight of the tumors in the DHM group was only 0.26 g at the end of the experiment compared with the NDP group (0.65g) and control group (1.73 g) (Fig. 4B, $P < 0.001$). These results illustrated that DHM exhibited the inhibition of tumor development. Liver tissues of nude mice were stained with HE (Fig. 4C). Hepatocytes in the DHM group were significantly enlarged, and could confirm the structure of hepatic lobules, which resulted in hepatic cords disordered and hepatic sinuses narrowed (Fig. 4C). However, DHM increased the weight of the mice compared to the NDP group (Fig. 4D), suggesting that DHM exhibited a better curative effect than NDP in suppressing the tumor development.

Discussion

At present, the treatment options of HCC mainly include orthotopic liver transplantation, surgical resection, local destruction, radiotherapy, and chemotherapy. Although there have been advances in the treatment of HCC patients, the worldwide recurrence and mortality rates of HCC and HCC-associated cases are very high. NDP is a broad-spectrum antitumor drug, and it may be used in the treatment of

malignant tumors, such as cervical, nasopharyngeal, esophageal, and lung cancer [24–28]. In recent years, a number of studies have shown that the molecular mechanism of NDP in tumors, and this potentially involved multiple potential mechanisms. However, NDP treatment led to the autophagosome accumulation and increased LC3-II expression in cisplatin-sensitive nasopharyngeal cancer cell lines [29]. Furthermore, it has been demonstrated that high concentration of NDP could cause treatment-related side effects, such as nephrotoxicity, hematological toxicity, ototoxicity.

DHM, a naturally flavonoids of medicinal plants, has demonstrated therapeutic efficacy in the treatment of various cancer, and it has attracted attention as an antitumor agent against lung cancer, gastric cancer, ovarian cancer and liver cancer. DHM may be combined with or replace other chemotherapeutic drugs, such as NDP, in cancer therapy. Studying these molecular targets also provides novel theoretical foundation for understanding the molecular mechanisms of cancer, as well as novel drugs to replace NDP for cancer treatment. The safety of DHM has been studied in cell cultures, animals, healthy individuals and patients, and DHM is generally recognized as a safe extract of Rattan tea. In Hep3B cell culture studies, DHM inhibited cell proliferation and viability, migration, invasion, and promoted apoptosis. Furthermore, DHM treatment inhibited growth of xenotransplanted tumors in mice [30], suggesting the potential therapeutic effects of DHM as an antitumor agent.

The main antitumor mechanisms of DHM that have been described thus far are as follows: Inhibition of cell proliferation; induces apoptosis; inhibition of tumor metastasis. DHM significantly promoted pro-apoptotic protein expressions, such as cleaved caspase 3, cleaved Caspase 9, Bak, Bax and Bad expressions, but inhibited Bcl-2 expression, induce cell apoptosis of Hep3B cells. In addition, activation of the tumor suppressor gene p53 [10], and inhibition of Semaphorin 4D (Sema4D) [31], multidrug resistance protein 2 (MRP2) [32], NF- κ B [33], and Notch1 pathway [15] and angiogenesis, can promote apoptosis and cytoprotective autophagy [33]. However, to the best of our knowledge, the antitumor effect of DHM in Hep3B cells has rarely been reported to date. The purpose of this study was to determine the effects of DHM on the proliferation, migration and apoptosis of Hep3B cells, implying that DHM may serve as a promising bioactive component for HCC treatment.

As is well known, caspases regulate cell proliferation and apoptosis [34]. Caspase family are usually divided into three protein categories: apoptosis initiators (caspase-9), apoptosis executioners (caspase-3 and -7) and inflammation mediators [35]. Previous research showed that caspase-3 and caspase-9, are key apoptosis proteins in the apoptosis pathway [36]. The caspase-9 protein is the apoptosis initiator and the apoptosis executors (caspase-3 and -7) of cell apoptosis in mammals. The apoptosis initiator is first activated by apoptosis signals, followed by activation of apoptosis executioners of the downstream cascade. Ultimately, large amounts of substrates in cells are hydrolyzed for disintegration. Caspase-3 and caspase-9 are situated at vital junctions in apoptotic signaling pathways. Western blot analyses indicated that DHM treatment markedly promoted cleaved caspase 3, cleaved Caspase 9, Bak, Bax and Bad expressions, while inhibited Bcl-2 expression in Hep3B cells, which were consistent with other apoptosis-related experiments in human myelomonocytic lymphoma cells [17]. Notably, DHM treatment matched or even exceeded the effect of NDP treatment on the caspase expression levels in Hep3B cells.

Invasion and metastasis are key biological characteristics of malignant tumors. Adhesion molecules are involved in its malignant progression, invasion and metastasis. Cancer cells can invade stromal tissue the host stromal of the target organ by the blood vessel wall, which subsequently promotes tumor metastasis and invasion [37]. Transwell assays confirmed that DHM inhibited the migration and invasion of Hep3B cells in a dose-dependent manner, which was consistent with the results of Chen et al. reported that DHM reduced human cholangiocarcinoma cells migration and invasion [14]. Moreover, DHM treatment significantly reduced the weight of the HCC tumors. Thus, the data showed that the antitumor effects of DHM was better than that of NDP treatment.

Conclusion

These data demonstrated that DHM inhibited cell proliferation, migration, invasion, and promoted apoptosis of Hep3B cells. DHM may be critical for cell apoptosis and metastasis. The study hinted that DHM exhibited the anti-tumor effect on HCC, and might provide a novel sight into the clinical treatment of HCC. Further studies are still needed to uncover more potential effect of DHM on HCC.

Abbreviations

ANOVA: One-way analysis of variance; CCK-8: Cell Counting Kit-8; DHM: Dihydromyricetin; HCC: Hepatocellular carcinoma; TCMs: Traditional Chinese medicines; DMEM: Dulbecco's modified Eagle medium; DMSO: Dimethylsulfoxide; NDP: Nedaplatin; FBS: Fetal bovine serum

Declarations

Acknowledgements

Not applicable.

Abbreviations

ANOVA: One-way analysis of variance; CCK-8: Cell Counting Kit-8; DHM: Dihydromyricetin; HCC: Hepatocellular carcinoma; TCMs: Traditional Chinese medicines; DMEM: Dulbecco's modified Eagle medium; DMSO: Dimethylsulfoxide; NDP: Nedaplatin; FBS: Fetal bovine serum

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Authors' contributions

Mingyi li and Lianggui Jiang collected the data. Lianggui Jiang and Wen-Chu Ye conducted experiments and wrote the manuscript. Zuobiao Li, Yongguang Yang and Wei Dai contributed to the discussion and reviewed the manuscript. All authors have read and approved the manuscript.

Availability of data and materials

All data and materials used in this current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All the animal experiments and surgical procedures were approved by the Institutional Animal Care and Use Committee of Guangdong Medical University (GDY1802018).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflicts of interest.

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Figures

Figure 1

DHM inhibited cell proliferation and viability of Hep3B cells. (A) Hep3B cells were treated with six different doses of DHM (0 μM, 10 μM, 20 μM, 30 μM, 40 μM, 50 μM) of DHM for 12 h. Cell proliferation was examined by CCK-8 assays. (B) Effects of DHM and NDP on viability of Hep3B cells according to a colony formation assay. (C) Quantification of colonies number in the colony formation assay. Results are

presented as mean $\pm$ standard error of mean ($n \geq 3$). $**P < 0.01$ and $***P < 0.001$ vs. control group or NDP group. NDP, Nedaplatin.

Figure 2

DHM inhibited cell migration and invasion of Hep3B cells. (A) Effect of DHM and NDP on the migratory ability of Hep3B cells in a Transwell assay. (B) Quantification of migration rate in the Transwell assay. Magnification, x 200. $***P < 0.001$. (C) Wound-healing assay. (D) Quantification of wound-healing rate in

the wound healing assay. Results are presented as mean $\pm$ standard error of mean ($n \geq 3$). ** $P < 0.01$ and *** $P < 0.001$ vs. control group or NDP group. NDP, Nedaplatin.

Figure 3

DHM induced apoptosis in Hep3B cells (A) Following treatment with five different group; Control, DHM (25 and 50 μ M) and NDP (25 and 50 μ M) for 12 h, apoptosis rate of Hep3B cells was determined using Annexin V-FITC/PI dual-staining flow cytometry. (B) Quantification of apoptosis rate in Hep3B cells

detected by Annexin V-FITC/PI dual-staining flow cytometry. (C and D) Apoptosis-associated factors (cleaved caspase 3, cleaved Caspase 9, Caspase 9, Bak, Bax, Bad and Bcl-2) were examined by western blot. Results are presented as mean $\pm$ standard error of mean ($n \geq 3$). (E and F) Integrated density data were quantified. All images are representative of three independent experiments. NDP, Nedaplatin; PI, propidium iodide. Data are means $\pm$ SEM of three independent experiments, *** $P < 0.001$ vs. control group or NDP group.

Figure 4

DHM inhibited tumor development in nude mice. (A) After nude mice were sacrificed, they were dissected to obtain tumors and photograph (lower). (B) Excised tumors were weighed separately. (C) Histologic analysis of HE staining of hepatic cords disordered and hepatic sinuses. (D) One week after last treatment, mice were sacrificed and weighed. Values are presented as the mean $\pm$ SEM (n=6). *P<0.05, **P<0.01 and ***P<0.001 compared with control or NDP group.