

Original Article

Megestrol acetate and oxaliplatin fail to enhance the chemosensitivity of hepatocellular carcinoma in vitro and in vivo

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Abstract: Megestrol acetate and oxaliplatin have positive clinical efficacy in the treatment of hepatocellular carcinoma individually, but these drugs have not yet been studied in combination. In this study, we found that megestrol acetate (75 μ M and 112.5 μ M), oxaliplatin (3 mM), and a combination of these drugs all showed significant suppression of cellular activity, but the effect was no better in the combined medicine groups than in the single-drug groups. Cell cycle distribution analysis showed that with megestrol acetate (75 μ M), the number of cells increased in the G1 phase, with a subsequent reduction in the S phase when megestrol acetate was combined with oxaliplatin (3 mM). We also found that megestrol acetate, oxaliplatin, and combined treatment significantly induced cell apoptosis of HepG2, and the combined medicine group showed a remarkable decrease in apoptosis. In a tumor model of nude mice, megestrol acetate (10 mg/kg/day) and oxaliplatin (1.5 mg/kg/day) were effective against tumor growth. In addition to the single-medicine group, we administered megestrol acetate to mice at two different time points. The oxaliplatin group and the combined medicine group pretreated with megestrol acetate showed reductions in tumor mass and volume, but the megestrol acetate group and the combined medicine group simultaneously treated with megestrol acetate showed only inhibition of tumor mass. We also found that oxaliplatin, megestrol acetate, and both combined medicine groups showed reduced mean microvascular density. In conclusion, this study indicates that oxaliplatin, megestrol acetate, and a combination of the two can inhibit tumor progression of hepatocellular carcinoma, but megestrol acetate does not increase the chemotherapeutic sensitivity of oxaliplatin in vitro and in vivo.

Keywords: Megestrol acetate, oxaliplatin, hepatocellular carcinoma, HepG2, apoptosis

Introduction

Hepatocellular carcinoma (HCC) is a highly malignant tumor [1] with an insidious onset, rapid invasion, and high rates of recurrence and fatality. Because most HCCs are discovered at a late stage, there is little opportunity for radical operation upon diagnosis. Because advanced HCC has a poor prognosis, treatment programs for HCC have proliferated, including radical resection or liver transplantation for resectable HCC and ablation, arterially directed therapies, and external-beam radiation therapy for unresectable HCC. Systemic chemotherapy has also been considered as a palliative treatment for patients with advanced HCC, especially in cases with extrahepatic spread [2]. Be-

cause of the short overall survival time, low objective response rate, and obvious side effects, application of the traditional chemotherapeutic agents in advanced HCC is limited. Oxaliplatin is a newer chemotherapeutic agent that is currently used for advanced HCC because of its greater efficiency and better tolerance. Some phase II studies have also shown that oxaliplatin is effective against advanced HCC [3].

Megestrol acetate (MA) is a synthetic progesterone agent with multiple pharmacological actions. MA has been reported to favorably influence the course of advanced malignancy [4], reduce tumor size, improve patient survival rates, and have positive effects on quality of life

in the palliative management of patients with HCC [5, 6]. However, MA has no role in prolonging overall survival in patients with advanced treatment-naïve HCC [7]. However, to our knowledge, oxaliplatin and MA have been studied separately, but detailed and specific research on a combination of the drugs for the treatment of HCC has not yet been reported. Therefore, we report here the results of an investigation of the combined effects of MA and oxaliplatin on human HCC *in vitro* and *in vivo*, which would benefit clinical use and provide crucial experimental support for clinical studies.

Materials and methods

Reagents

MA was purchased from Sigma Chemical Co. (St. Louis, MO). Oxaliplatin was purchased from Sanofi S.A. (Paris, France). A human hepatocellular cell line, HepG2, was obtained from the Shanghai cell bank of the Chinese Academy of Science. Cell Counting Kit-8 (CCK8) was purchased from the Chemical Society of Japan (Tokyo, Japan). Alexa Flour Dyes was obtained from Invitrogen Life Technologies (Gaithersburg, MD). The primary antibodies against anti-p53 (Pab 240) antibody, anti-caspase 3 antibody, anti-XIAP antibody, anti-PARP antibody, and anti-GAPDH antibody were purchased from Abcam (Cambridge, UK). The Matrigel Matrix was obtained from BD Biosciences (Franklin Lakes, NJ).

Cell culture

HepG2 cells were maintained in Dulbecco's modified Eagle medium-high glucose (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum, 2 mM of L-glutamine (Invitrogen), and 100 µg/mL of penicillin-streptomycin (Invitrogen). The cells were cultured in a humidified atmosphere in 5% CO₂ at 37°C.

Cell viability assay

To evaluate the cell viability rate, we plated 5 × 10³ cells per well in 96-well plates with 100 µl of maintenance medium. HepG2 cells were treated with various concentrations of drugs. CCK-8 was used to monitor cell viability at 24 hours, and the number of viable cells was assessed by measuring the absorbance at 450 nm with a microplate reader (Thermo Scientific,

Waltham, MA) [8]. The cell viability was then measured as described above. Three independent experiments were done in quadruplicate wells.

Analysis of cell cycle and apoptosis

HepG2 cells were cultured in each well (2.7 × 10⁵/well) of a 6-well plate. After overnight incubation at 37°C, the culture media were removed and replaced with media containing either diluents control or various concentrations of the drugs (as discussed above). Cell suspensions from both the control cultures and the indicated drugs were prepared by trypsinization (not containing ethylenediaminetetraacetic acid) and washed twice with phosphate-buffered saline solution with 500 µl of annexin-binding buffer (Multisciences, Hangzhou, China). Cells were stained with 10 µl of propidium iodide (Multisciences) and 5 µl of annexin V-fluorescein isothiocyanate (Multisciences) according to the established manufacturer's protocol, and assayed on an Accuri C6 flow cytometer (BD Biosciences). Cell cycle distribution was analyzed by flow cytometry using a Becton Dickinson FACSCalibur, and the DNA histograms were analyzed with Winndi multicycle software.

Animal model

This study was carried out in strict accordance with the recommendations in the Guide for the Institutional Animal Care and Use Committee of the College of Medicine, Shenzhen University. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Shenzhen (Permit Number: SCXK-2012-0002). The surgery was performed under urethane, and the mice were sacrificed by inhalation of CO₂. Male BALB/C nude mice (4 to 6 weeks old) were purchased from SLAC Laboratory Animal Shanghai Co. Ltd. and kept at the Laboratory Animal Center of the College of Medicine, Shenzhen University. The experimental mice were housed in individually ventilated cages and had free access to food and drinking water. A metastatic model of human HCC in mice using HepG2 cells was used for this pilot study [9]. Briefly, HepG2 cells (5 × 10⁶) were given by subcutaneous injection into the right axilla. When the subcutaneous tumor reached approximately 1 cm in length, the mice were randomly distributed into a control group (9

Megestrol acetate and oxaliplatin in hepatocellular carcinoma

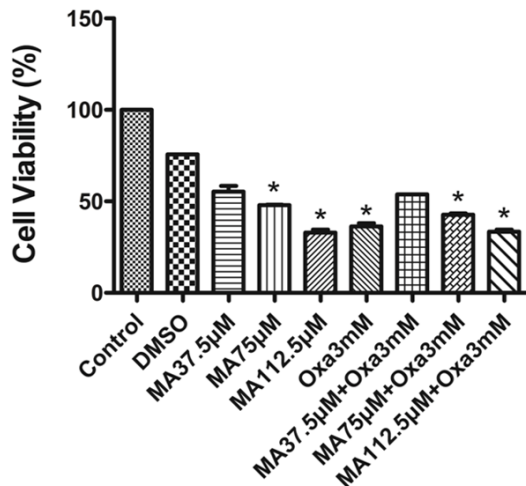


Figure 1. Dose-dependent inhibition of HepG2 cells by megestrol acetate (MA) and oxaliplatin. HepG2 cells were subcultured in each well of 96-well plates and incubated overnight for attachment. For the dose-dependent study, HepG2 cells were treated with 0.1% dimethyl sulfoxide (DMSO; control) or various concentrations of MA and oxaliplatin, and incubation was carried out at 37 °C for 24 h. Cell viability was then determined by CCK-8 assay as described in Materials and Methods. Means \pm standard errors of cell viability percentages from three experiments are plotted. * $P < 0.05$ as compared with control. Oxa, oxaliplatin.

mice), an MA group (9 mice), an oxaliplatin group (9 mice), (MA+Oxa) group I (9 mice), and a (MA+Oxa) group II (10 mice). Based on the literature [9] and the results of our previous studies, a dosage of 10 mg/kg MA and 1.5 mg/kg oxaliplatin once per day, was adopted. The control group received 0.2 mL of 0.9% sodium chloride by intraperitoneal injection 21 days after inoculation. From the 80th day, MA or oxaliplatin was administered by intraperitoneal injection to the mice in the corresponding group for 14 days. From the 80th day, both MA and oxaliplatin were given to the (MA+Oxa) group I mice at the same time for 14 days. The (MA+Oxa) group II mice were pretreated with MA for the first 7 days, and oxaliplatin was added from day 8, and each drug was administered for 14 days. The tumor volume was calculated by the following formula: tumor volume [mm³] = (length [mm]) \times (width [mm])² \times 0.5. At the end of the experiment, the tumors were excised, weighed, and fixed for further study. The body weights were assessed by the following formula: body weight (g) = total weight (g) - tumor volume (mm³, density = 1).

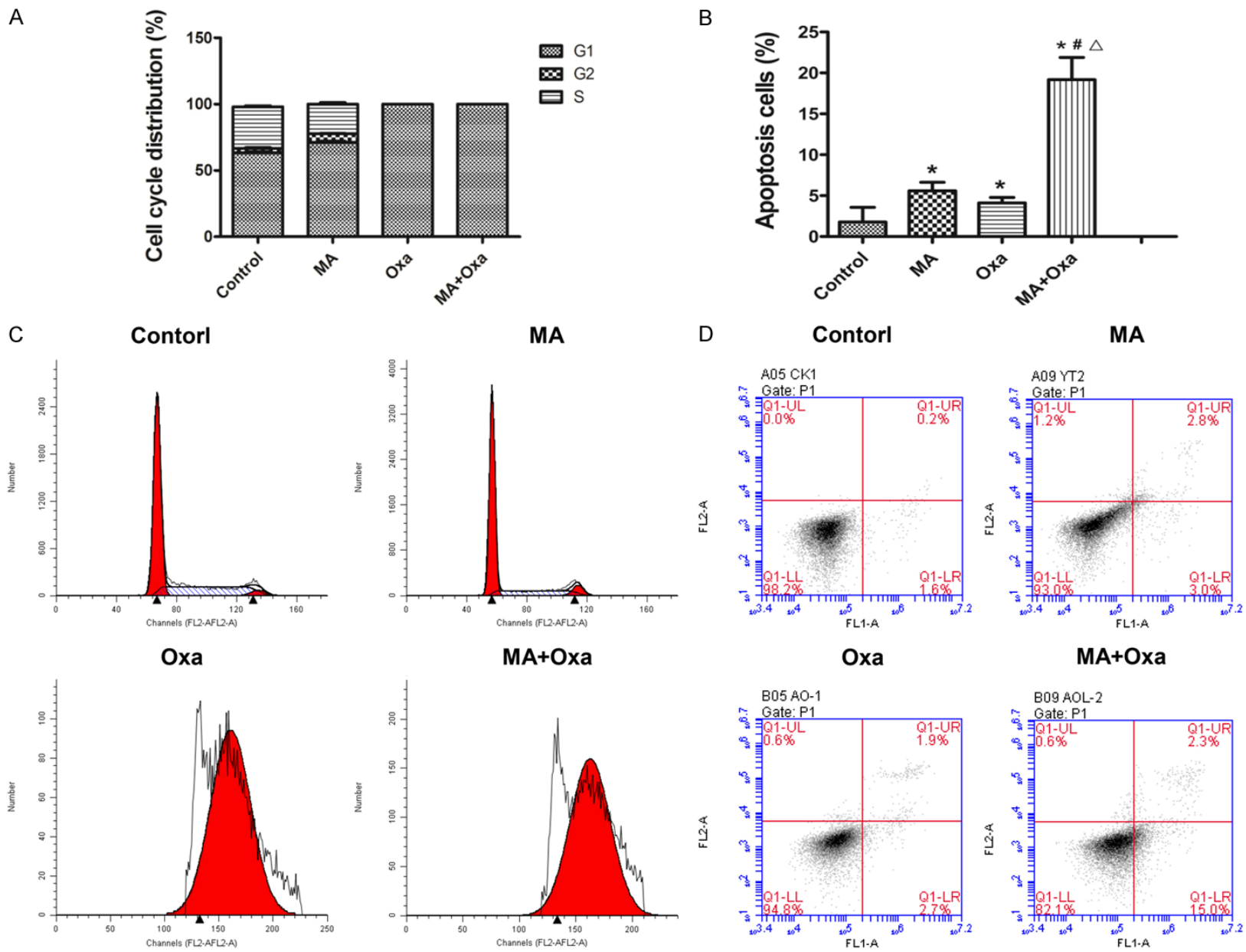
Immunohistochemical analysis

The tumor tissues were embedded in paraffin, cut into 4- μ m-thick sections, and sliced on a microtome. The sections were deparaffinized and dehydrated. Endogenous peroxidase activity was quenched by incubation in 3.0% (v/v) H₂O₂ for 30 minutes at room temperature. Heat-induced epitope retrieval was performed at 120°C for 20 minutes in 0.001 M of citrate buffer. The sections were incubated with blocking serum (Life Technologies) and monoclonal goat anti-CD34 antibody (1:200, Abnova) at 4°C overnight, and the control groups were treated with phosphate-buffered saline solution. After washing three times in phosphate-buffered saline solution (5 minutes \times 3), the sections were incubated with rabbit anti-goat IgG secondary biotinylated antibody (Abcam, Cambridge, UK) at 37°C for 1 h. They were then stained with 3,3'-diaminobenzidine (Maixin Biotech, Fujian, China) under a microscope according to the manufacturer's instructions. The sections were then counterstained with hematoxylin (Sigma) and observed under a microscope (BH2, Olympus, Tokyo, Japan). The vessel density was estimated by calculating the number of blood vessels that stained with the anti-CD34 antibody in three microscopic high-power fields in the most densely vascularized "hotspot" area. All quantitative assessments of immunohistochemical staining were conducted in a double-blinded fashion on the section next to the slide that contained the largest tumor cross-sectional area. For some very small tumors, more than one section had to be included in the analysis to allow analysis of three different high-power fields.

Statistical analysis

Graphs were constructed using GraphPad Prism (Graphpad Software, San Diego, CA). The data are presented as means with standard errors (SE). In vitro cell migration and proliferation assays were analyzed with the Student's *t*-test. One-way analysis of variance was performed to compare results with more than one treatment, and the Student's *t*-test was performed to compare the differences between two groups. Statistical analysis was performed with SPSS 15.0 for Windows (SPSS Inc., Chicago, IL). *P* values of less than 0.05 were considered to indicate statistical significance.

Megestrol acetate and oxaliplatin in hepatocellular carcinoma



Megestrol acetate and oxaliplatin in hepatocellular carcinoma

Figure 2. Flow cytometric analysis of cell cycle and apoptosis of HepG2 cells treated with MA and oxaliplatin. Exponentially growing cells were incubated with MA 75 μ M or oxaliplatin 3 mM for 24 hours. After processing, cells were analyzed by flow cytometry as described in Materials and Methods. A. Bar chart of cell cycle distribution. B. Quantitation of cell apoptosis. C. Results shown by histogram. D. Results presented as means \pm standard errors. * P < 0.05 compared with control; # P < 0.05 compared with MA; ΔP < 0.05 compared with Oxa.

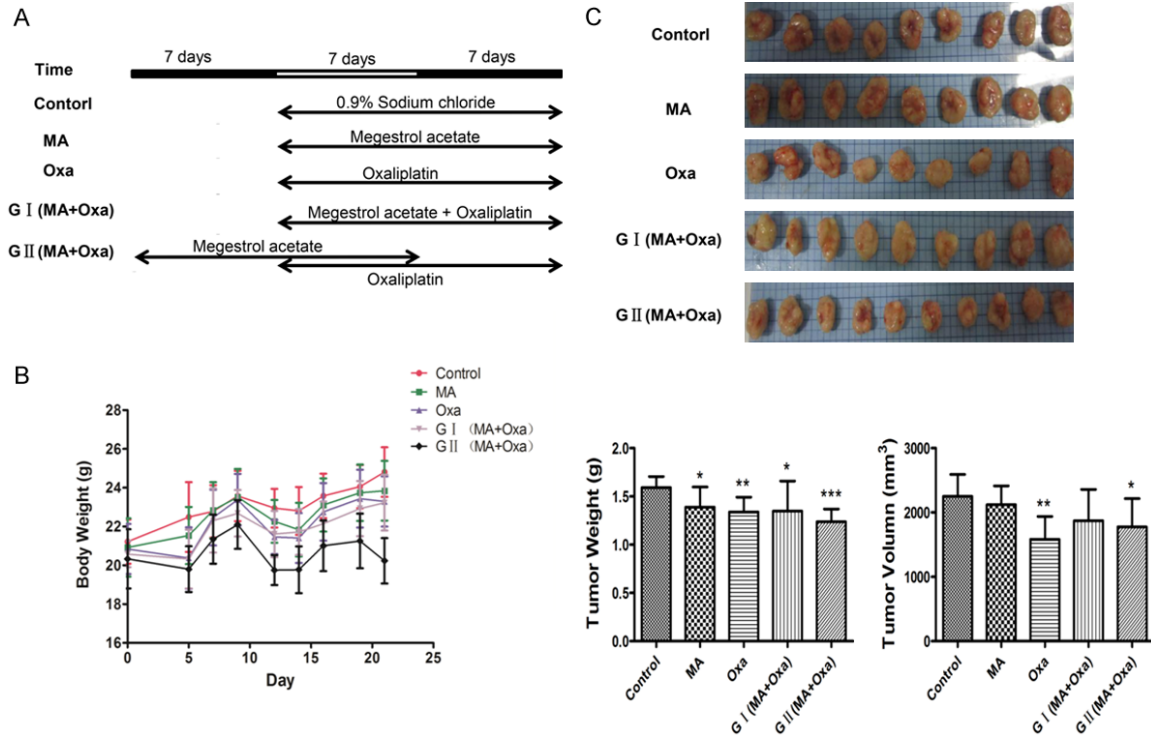


Figure 3. Effect of MA and oxaliplatin treatment on tumor-bearing nude mice. HepG2 cells were transplanted as a solid tumor in nude mice, and tumor volumes were measured as described in Materials and Methods. Tumor-bearing mice received peritoneal injections of MA (10 mg/kg/day), oxaliplatin (1.5 mg/kg/day), or 0.9% sodium chloride (control). A. Sketch of mice grouping and administration. B. Changes in body weight. C. Tumor mass and volume presented as means \pm standard errors ($n \geq 9$). * P < 0.05, ** P < 0.01, *** P < 0.001, compared with control. GI (MA+Oxa), simultaneous treatment with MA and oxaliplatin; GII (MA+Oxa): pretreatment with MA, then combined with oxaliplatin.

Results

HepG2 cell growth inhibition after treatment with megestrol acetate and oxaliplatin in vitro

The effects of MA and oxaliplatin on human HepG2 cells were examined by increasing the concentrations of MA from 37.5 to 112.5 μ M for 24 hours. Cell viability was then measured with an CCK-8 assay (Figure 1). The addition of MA in cultures of HepG2 cells showed dose-dependent inhibition of cell growth. MA 75 μ M and 112.5 μ M, oxaliplatin 3 mM, and the combination of the two drugs all significantly suppressed cellular activity (P < 0.05 compared with controls). We also compared cell viability after HepG2 treatment with MA alone or in

combination with oxaliplatin, but no significant difference was seen.

Effects of megestrol acetate and oxaliplatin on cell cycle progression and apoptosis

Cell growth and inhibition are mediated by cell cycle progression [10]. Based on the above results showing the inhibitory effects of MA and oxaliplatin on HepG2 cells grown in vitro, the effect of the drugs on cell cycle progression was also examined as a sequential step to elucidate the possible mechanisms involved in their inhibitory effects. HepG2 cells were incubated with MA 75 μ M, oxaliplatin 3 mM, and the drug mixture after 24 h. Cell cycle distribution analysis showed that after incubation with

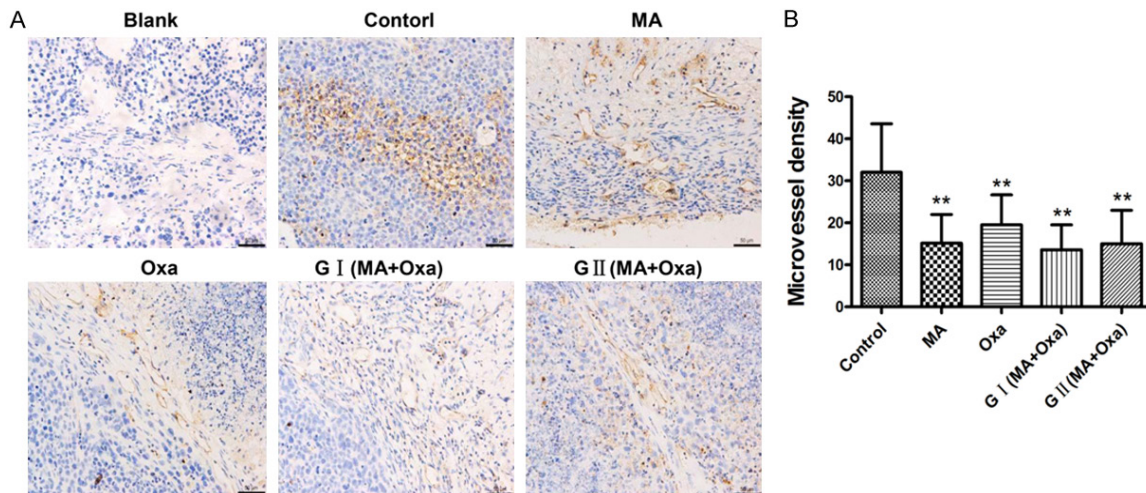


Figure 4. Immunohistochemical staining of CD34 in hepatocellular carcinoma tissues. A. Immunohistochemical staining of CD34 in hepatocellular carcinoma tissues. B. Microvascular density of each group is presented as mean \pm standard error ($n \geq 9$). ** $P < 0.01$ compared with control.

MA 75 μM , the cells were increased in the G1 phase and then reduced in the S phase (**Figure 2A**). Oxaliplatin 3 mM and the drug mixture increased the G1 phase population up to 100% (**Figure 2A**). Furthermore, the annexin V-fluorescein isothiocyanate/propidium iodide apoptosis data showed that after co-culture with MA 75 μM and oxaliplatin 3 mM, the proportion of apoptotic cells increased by 19.18%. We also found that MA 75 μM or oxaliplatin 3 mM can induce significant cell apoptosis at 24 hours ($P < 0.05$ compared with controls, **Figure 2B**). The combined medicine group also differed significantly from the group treated with oxaliplatin alone ($P < 0.05$ compared with control; $P < 0.05$ compared with MA; **Figure 2B**).

Effects of megestrol acetate and oxaliplatin treatment on hosts of transplanted tumors

The grouping and administration of the mice are shown in **Figure 3A**. The mean body weight of the mice treated with MA was higher than that of the other groups. The growth curve of body weight in (MA+Oxa) group I was flatter than that in (MA+Oxa) group II (**Figure 3B**). To evaluate the effects of the drug on HCC tumors, the tumor volumes and masses in the trial and control groups were measured after 21 days. The oxaliplatin group and (MA+Oxa) group II showed significant reductions in tumor volume ($P < 0.01$ and $P < 0.05$, respectively, compared with controls; **Figure 3C**). However, no obvious changes were observed between the tumor volume of (MA+Oxa) group I and that of the control

group. The MA group, the oxaliplatin group, and (MA+Oxa) groups I and II all showed significant reductions in tumor mass ($P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, compared with the controls; **Figure 3C**). No significant differences were found in tumor volume or mass among the other groups.

Effects of megestrol treatment and oxaliplatin on microvascular density

Angiogenesis plays an important role in HCC development [11]. We assessed tissue's mean microvascular density (MVD) using an anti-CD34 antibody in the HCC tissue samples. Compared to the control group, the MA group, the oxaliplatin group, and (MA+Oxa) groups I and II showed reduced MVD ($P < 0.01$; **Figure 4A, 4B**). As shown in **Figure 4B**, the MVD was lower in (MA+Oxa) groups I and II than in either the MA or oxaliplatin groups, but the difference was not statistically significant.

Discussion

MA is a steroidal progestin and progesterone derivative with predominantly progestational and anti-gonadotropic effects [12]. MA has been used in the clinical treatment of malignancies such as endometrial carcinoma, ovarian cancer, breast cancer, prostate cancer, renal cell carcinoma, and malignant melanoma for more than 30 years [6, 13, 14]. HCC has been shown to be sex hormone dependent [15, 16], and MA may be a useful treatment method

for HCC. Oxaliplatin is a platinum-based anti-neoplastic agent used in cancer chemotherapy [17], typically along with folinic acid and 5-fluorouracil in a combination known as FOLFOX. However, many fewer studies have been done on HCC than on other types of malignant tumors with respect to treatment with hormonal agents combined with chemotherapeutic drugs. The results of this study will be useful for consideration of clinical studies of the therapeutic effects of MA combined with oxaliplatin on HCC. Treatment of HCC with MA or oxaliplatin alone provides a definite curative effect, but the effects of combined treatment have not been reported and are worthy of our discussion to provide a basis for clinical treatment.

We have shown that treatment with MA and oxaliplatin led to a decrease in the viability of HepG2 cells in vitro in a dose-dependent manner (**Figure 1**). **Figure 1** shows that cell viability is significantly inhibited by oxaliplatin 3 mM, and this suppression effect is enhanced as the concentration of MA increased (**Figure 1A**). However, our data suggest that the cell activity suppression with the combination of the medicines was no better than that with the medicines individually.

Cell apoptosis and the cell cycle were determined by flow cytometry analysis. **Figure 2** shows that cells treated with MA were arrested mostly in the G1 phase at an incubation time of 24 hours. Incubation of HepG2 cells with oxaliplatin alone or combined with MA was arrested irreversibly in the G1 phase by preventing progression to the S phase, followed by the triggering of DNA fragmentation and morphological changes associated with apoptosis [18]; these findings are consistent with our earlier results (**Figure 2B**). The relationship between cell cycle arrest and apoptosis is best understood in the context of G1 arrest [19, 20]. It appears in this study that oxaliplatin combined with MA arrested the HepG2 cells in the G1 phase and consequently caused these G1-arrested cells to undergo apoptosis instead of entering the S phase. It is worthy of note that the combined medicine group had no significant effect on cell cycle distribution compared to the single drugs, but it did cause more apoptosis among HepG2 cells. We also found that MA did not change the cellular morphology, but oxaliplatin and the combined medicines had a significant influence on the shape of the HepG2 cells (data not

shown, [Supplementary Figure 1](#)). The analysis of p53, caspase-3, PARP, and XIAP expression showed that apoptosis-associated protein is not unprecedented. Our study shows that oxaliplatin and MA alone and a combination of the two medicines can induce the apoptosis of HepG2. However, no significant difference was seen in apoptosis-related protein expression (data not shown, [Supplementary Figure 1](#)). The mechanism by which oxaliplatin and MA induce apoptosis of HepG2 is unclear, and this is a limitation that requires further research. As in all negative studies, we can infer that MA cannot enhance the chemosensitivity of oxaliplatin treatment of HCC in vitro.

However, in vivo study is more complicated. Chemotherapy not only affects tumor cells but also damages blood vessels and inhibits the host's antitumor system, which may contribute to cancer metastasis [21, 22]. In a clinical setting, oxaliplatin is used as an effective chemotherapy drug for all kinds of malignant tumors, but it has severe side effects. In accordance with the results of a clinical trial [3], oxaliplatin led to obvious decreases in tumor volume and mass in mice in our study. One study found that MA significantly decreased tumor growth and improved the survival rate in treated patients compared to a placebo group [4]. The growth of HepG2 was inhibited in a dose- and time-dependent manner and in HepG2 transplanted tumor in vivo [9]. MA improves HCC patients' appetite and body weight and gives them a feeling of well-being with minimal side effects. It also results in a minor reduction in tumor size and prolonged survival [5]. Consistent with our results, MA caused a significant reduction in tumor mass compared to the control group mice. To verify the synergistic effects of MA and oxaliplatin, we observed their effects on tumor-bearing nude mice and found that tumors that were pretreated with MA showed reductions in volume and mass compared to those treated with MA and oxaliplatin at the same time. These studies reveal that the anti-hepatoma functions of the combination of MA and oxaliplatin are related to the administration time and schedule. Further evidence was provided by immunohistologic analysis of CD34 in HCC tissues of tumor-bearing nude mice [23]. Immunohistologic analysis of the mean intratumoral MVD is the most commonly used method to assess angiogenesis, and the MVD apparently decreased in each group compared with con-

trol group. This observation indicates that MA and oxaliplatin may inhibit tumor metastasis, but no statistical difference was seen between the single-drug groups and the combined medicine group. Further investigations are needed to characterize the underlying mechanisms in more detail.

In clinical settings, patients with advanced malignancies are frequently given MA to improve their quality of life and appetite [24, 25]. The curative effects of MA on HCC are controversial, but the effect of oxaliplatin-based chemotherapy is definite [3, 7]. It is unclear whether MA and oxaliplatin had a combined effect on HCC. To conclude, we show that MA cannot increase the chemotherapeutic sensitivity of oxaliplatin. However, only experimental data exist to support this conclusion, and more clinical data are required to obtain more accurate knowledge.

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Disclosure of conflict of interest

None.

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Megestrol acetate and oxaliplatin in hepatocellular carcinoma

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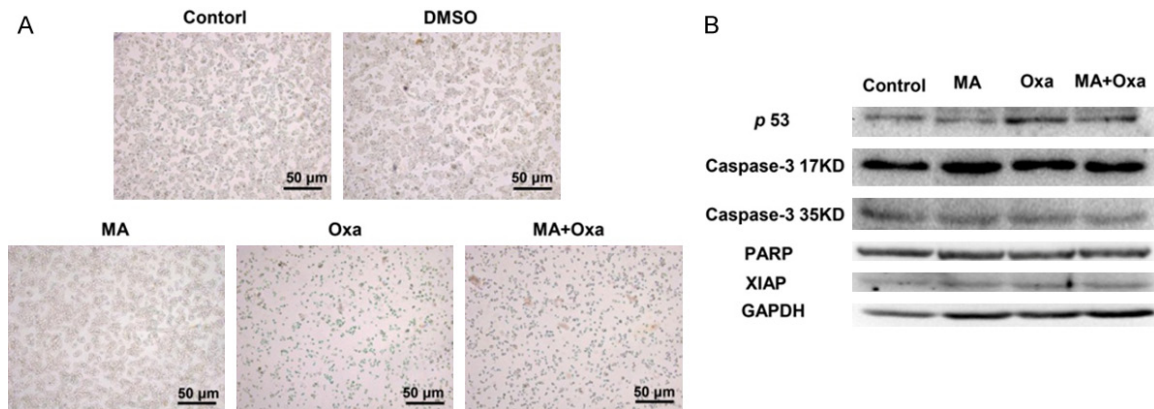
Megestrol acetate and oxaliplatin in hepatocellular carcinoma

Western blot analysis

To further confirm the underlying molecular mechanism, the related proteins were examined using western blot analysis. The HepG2 cells were seeded in 6-well plates using 5×10^5 cells per well and exposed to media containing either diluents (control) or previously mentioned drugs (discussed in the main text) incubated in a CO₂ incubator for 24 h. Proteins were extracted from pretreated cells using RIPA lysis buffer (Beyotime, Shanghai, China) on ice and quantified using a BCA protein assay (Beyotime, Shanghai, China). The same amount of proteins (15 µg) was loaded into each well. The proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to immobilon PVDF membrane (Merck Millipore, Billerica, MA, USA). The membranes were blocked for 1 h with tris-buffered saline (TBS) containing 5% nonfat dry milk and 0.5% tween 20 (tris-buffered saline tween, TBST) (Sinopharm Chemical Reagent, Shanghai, China). After blocking, the PVDF membrane was incubated with primary antibody at 4°C for 24 h. The primary antibodies were shown as follows: anti-p53 (PAb 240) antibody, anti-caspase 3 antibody, anti-XIAP antibody, anti-PARP antibody and GAPDH as a loading control. The PVDF membrane was then washed with TBST and incubated for 1 h with secondary alkaline phosphatase-conjugated anti-rabbit IgG or anti-mouse IgG (Bioworld, Sydney, Australia) antibody at room temperature. The proteins were visualized and semi-quantified with the UVItec system (Uvitec, Cambridge, UK).

Effect of megestrol acetate combined with oxaliplatin on apoptosis-related protein expression in HepG2 cells

For observing the molecular mechanisms of megestrol acetate combined with oxaliplatin in HepG2 cells, the expression of protein related to apoptosis was investigated. As shown in [Supplementary Figure 1A](#), the cell morphology showed significant changes after the cells were co-cultured with oxaliplatin 3 mM or a drug mixture for 24 h, including cell shrinkage, cell size reduction and turnaround and cytoplasmic vacuolar changes ([Supplementary Figure 1A](#)). Each group was then lysed to detect the variation of p53, caspase-3, PARP and XIAP expression. The results showed no statistical difference in the expression of p53, caspase-3, PARP and XIAP between oxaliplatin or combined with megestrol acetate-treated HepG2 cells ([Supplementary Figure 1B](#)).



Supplementary Figure 1. Effects of megestrol acetate and oxaliplatin on HepG2 cells. HepG2 cells were subcultured in each well of six-well plates and incubated overnight for attachment. The cells were treated with 0.1% DMSO (control), megestrol acetate 75 µM or oxaliplatin 3 mM for 24 h. A. Cell histology. B. Changes in P53, caspase-3, PARP and XIAP were analyzed by western blot. The results were taken from a representative of three independent experiments. MA: megestrol acetate; Oxa: oxaliplatin.