Lidocaine Induces Apoptosis and Suppresses Tumor Growth in Human Hepatocellular Carcinoma Cells *In Vitro* and in a Xenograft Model *In Vivo*

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ABSTRACT

Background: Recent epidemiologic studies have focused on the potential beneficial effects of regional anesthetics, and the differences in cancer prognosis may be the result of anesthetics on cancer biologic behavior. However, the function and underlying mechanisms of lidocaine in hepatocellular carcinoma both *in vitro* and *in vivo* have been poorly studied.

Methods: Human HepG2 cells were treated with lidocaine. Cell viability, colony formation, cell cycle, and apoptosis were assessed. The effects of lidocaine on apoptosis-related and mitogen-activated protein kinase protein expression were evaluated by Western blot analysis. The antitumor activity of lidocaine in hepatocellular carcinoma with or without cisplatin was investigated with *in vitro* experiments and also with animal experiments.

Results: Lidocaine inhibited the growth of HepG2 cells in a dose- and time-dependent manner. The authors also found that lidocaine arrested cells in the G0/G1 phase of the cell cycle (63.7 ± 1.7% *vs.* 72.4 ± 3.2%; $P = 0.0143$) and induced apoptosis (1.7 ± 0.3% *vs.* 5.0 ± 0.7%; $P = 0.0009$). Lidocaine may exert these functions by causing an increase in Bax protein and activated caspase-3 and a corresponding decrease in Bcl-2 protein through the extracellular signal-regulated kinase 1/2 and p38 pathways. More importantly, for the first time, xenograft experiments (n = 8 per group) indicated that lidocaine suppressed tumor development ($P < 0.0001$; lidocaine *vs.* control) and enhanced the sensitivity of cisplatin ($P = 0.0008$; lidocaine plus cisplatin *vs.* cisplatin).

Conclusions: The authors' findings suggest that lidocaine may exert potent antitumor activity in hepatocellular carcinoma. Furthermore, combining lidocaine with cisplatin may be a novel treatment option for hepatocellular carcinoma.
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SURGERY is a central aspect of treatment for the majority of solid cancers, including hepatocellular carcinoma.[1,2] However, perioperative care and anesthetic management are increasingly being recognized as treatment approaches that may influence cancer recurrence, metastasis, and patient survival.[3–6] Recent retrospective studies have shown that regional anesthesia (epidural, intrathecal, and paravertebral), epidural analgesia, and perioperative ketorolac are associated with improved outcomes after surgery for different cancers.[7–9] Several hypotheses have emerged to explain these findings, including reduced immunosuppression, decreased perioperative surgical stress responses, reduced doses of opioids, antiinflammatory effects, and local anesthetic effects.[10,11] There is growing evidence that local anesthetics may effectively inhibit cell proliferation and invasion in the treatment of cancer.[12–15]

Questions have been raised regarding the possible mechanisms of local anesthetic-induced tumor suppression. Previous studies have suggested that the apoptosis-inducing activity of local anesthetics is mediated, at least in part, by the activation of caspases and the regulation of mitogen-activated protein kinase (MAPK) signaling pathways.[16,17] Activated extracellular signal-regulated kinase (ERK) and p38 are implicated in the induction of proliferation, apoptosis, and autophagy by chemotherapeutic drugs in hepatocellular carcinoma.[18,19] The intrinsic pathways of

What We Already Know about This Topic

- Lidocaine may exert antitumor activity by suppressing cell proliferation, inducing apoptosis, and inhibiting migration
- Several retrospective studies have reported that the use of regional anesthesia is associated with improved outcomes after surgery

What This Article Tells Us That Is New

- Either lidocaine (30 mg/kg intraperitoneally, twice a week) or cisplatin (3 mg/kg intraperitoneally, once a week) treatment alone markedly suppressed human hepatocellular carcinoma HepG2 xenograft tumor growth in male athymic nude mice compared with the control
- The combination of lidocaine and cisplatin exerted a therapeutic effect that was significantly better than the effects in all the other experimental groups

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apoptosis that increase the Bax/Bcl-2 ratio lead to the activation of caspase-3.[20] Other recent evidence has indicated that local anesthetics may also influence tumor progression by increasing demethylation.[21,22] However, the majority of research studies have focused on *in vitro* studies and the lack of animal experiments.

Lidocaine is commonly used for regional anesthesia and pain relief, and it is increasingly being used for intravenous infusion in the context of multimodal treatment regimens. Intraarterial administration of lidocaine provides effective pain control during transarterial chemoembolization (TACE) for hepatocellular carcinoma.[23,24] The rationale for TACE includes infusion of both lipiodol and anticancer drugs, such as cisplatin, into the hepatic artery, followed by administration of embolic agents, which results in a strong and sustained high drug concentration in the tumor.[25] Therefore, a combination of lidocaine and cisplatin in the treatment of hepatocellular carcinoma may be useful. An effective course of chemotherapy for advanced hepatocellular carcinoma normally persists for only a few months because of the rapid development of drug resistance. Therefore, resistance to chemotherapeutic drugs remains a crucial challenge to hepatocellular carcinoma treatment.[26] Previous studies have reported that lidocaine suppresses proliferation and induces apoptosis in breast cancer cells.[21,22] Moreover, lidocaine enhances the sensitization of breast cancer cells to chemotherapeutic agents.[27] However, the function and underlying mechanisms of lidocaine in hepatocellular carcinoma cells *in vitro* and in xenograft experiments *in vivo* have been poorly studied and remain unclear.

The aim of this study was to determine the potential effects of lidocaine on the biologic behavior of cancer cells with respect to cell proliferation and apoptosis in the human HepG2 cells. We assessed the molecular mechanisms involved in lidocaine-induced cell death. Furthermore, we used a xenograft model to investigate the *in vivo* effects of lidocaine on tumor growth and whether lidocaine may increase the antitumor activity of HepG2 cells in response to cisplatin.

Materials and Methods

Cell Cultures and Reagents

Hepatocellular carcinoma HepG2 cells were obtained from the Chinese Type Culture Collection (China). Cells were routinely cultured in high glucose in Dulbecco Modified Eagle medium supplemented with 10% (v/v) fetal bovine serum (Invitrogen, USA) and maintained at 37°C in a humidified atmosphere of 5% CO_2 plus 95% air. Lidocaine and *cis*-diammineplatinum dichloride (cisplatin) were purchased from Sigma-Aldrich (USA). An ERK inhibitor (PD98059) and a p38 inhibitor (SB203580) were purchased from Selleck Chemicals (USA). Antibodies for Western blotting, including those specific to phospho (p)-ERK1/2, p-p38, p–c-Jun N-terminal kinase (p-JNK), ERK1/2, p38, JNK, caspase-3, cleaved caspase-3, Bcl-2, and Bax, were purchased from Cell Signaling Technology (USA). Other antibodies, including anti-glyceraldehyde-3-phosphate dehydogrenase and anti–β-actin, were purchased from Santa Cruz Biotechnology, Inc. (USA).

Drug Treatment

HepG2 cell monolayers were incubated with lidocaine (0.1, 0.5, 1, 2, 5, and 10 mM) in the viability assay for 24, 48, and 72 h. HepG2 cells were treated with lidocaine (1 or 5 mM) in the colony-forming assay, cell cycle analysis, apoptosis analysis, Western blot, and caspase-3 colorimetric assay. To assay whether lidocaine sensitizes HepG2 cells to cisplatin, the cells were treated with 10 μM cisplatin for 24, 48, or 72 h in the absence or presence of 5 mM lidocaine. For some experiments, cells were pretreated with the ERK1/2 inhibitor PD98059 (10 μM) or p38 inhibitor SB203580 (10 μM) for 2 h followed by exposure to lidocaine (5 mM) for 24 h.[28]

Viability Assay

Cell viability was quantified using the Cell Counting Kit-8 assay kit (Dojindo Molecular Technologies, Japan) according to the manufacturer's instructions. HepG2 cells were cultured at 3,000 cells per well in 96-well plates and exposed to lidocaine or cisplatin (alone or in combination) at the indicated concentrations. Cell Counting Kit-8 was added to each well, and the plate was incubated for 3 h at 37°C. The absorbance value (OD) was read at 450 nm using a spectrophotometer, and the data were analyzed using Softmax Pro software (Molecular Devices LLC, USA). Each experiment was performed in triplicate.

Colony-forming Assay

HepG2 cells were seeded on a six-well tissue culture plate at 100 cells per well. After 24 h of incubation, the cells were treated with the indicated concentrations of lidocaine. After 14 days, the cells were stained with 0.5% crystal violet (in methanol), and the colonies were counted.

Cell Cycle and Apoptosis Analysis

The cell cycle analysis was conducted by flow cytometry using the Muse Cell Analyzer (Merck Millipore, USA). The cells were harvested, washed twice with 0.01 M phosphate-buffered saline (PBS), and fixed with 70% ethanol. After the cells were fixed overnight at 4°C, they were washed twice with 0.01 M PBS, and 200 μl Muse Cell Cycle (Merck Millipore) reagent was added to each tube at room temperature for 30 min in the dark. The percentages of the nuclei in HepG2 cells at each phase of the cell cycle (G_0/G_1, S, and G_2/M) were calculated using the Lysis software program (Becton Dickson, USA).

The analysis of apoptosis was performed by flow cytometry using the Annexin V-FITC/PI kit (Thermo Scientific, USA). The cells were harvested and washed twice with 0.01 M PBS, and fluorescein isothiocyanate, conjugated Annexin V and propidium iodide were then added at room

temperature for 30 min in the dark. Apoptosis was determined according to the manufacturer's protocol.

Western Blot

Total protein was extracted using a radioimmunoprecipitation assay buffer containing a protease inhibitor cocktail (Roche Boehringer Mannheim Diagnostics, Switzerland) and was quantified using a bicinchoninic acid Protein Assay Kit (Thermo Scientific) according to the manufacturer's instructions. Equal amounts of proteins (40 μg/lane) were separated on 8 to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Millipore, USA). The membranes were incubated with primary antibodies at 4°C overnight and then incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. The blots were detected using the electrochemiluminescence system (Pierce Biotechnology, USA).

Caspase-3 Colorimetric Assay

Caspase-3 activity was measured using a caspase-3 activity assay kit (Thermo Scientific) according to the manufacturer's instructions. Briefly, HepG2 cells were treated with lidocaine in the absence or presence of cisplatin at the indicated concentrations for the specified time periods. The cells were extracted using a radioimmunoprecipitation assay buffer containing a protease inhibitor cocktail and cell lysates, and they were quantified as described above. Cell lysates were mixed with a reaction buffer and the caspase-3 substrate Ac-DEVD-*p*NA in 96-well plates. Lysates were incubated at 37°C for 2 h in the dark. OD was read at 405 nm using a spectrophotometer.

Xenograft Experiments in Nude Mice

All animal experiments were performed in accordance with protocols approved by the Animal Research Committee of Sun Yat-Sen University Cancer Center (Guangzhou, China) and according to standard institutional guidelines. Male BALB/c nude mice (weighing approximately 20 g) between 4 and 6 weeks of age were purchased from Shanghai Institutes for Biological Sciences (China). As shown in figure 1, HepG2 cells (2×10^6) were subcutaneously injected into the right side of the armpits of BALB/c nude mice. To assess the antitumor activity and improve the chemotherapy effects of lidocaine in a xenograft model *in vivo*, the mice were randomly divided into control, lidocaine, cisplatin, and lidocaine plus cisplatin groups (n = 8 per group). After palpable tumors developed a volume of approximately 50 mm^3, the mice were injected with a PBS solution as a control, lidocaine (30 mg/kg, twice a week), cisplatin (3 mg/kg, once a week), or a combination of lidocaine and cisplatin through intraperitoneal injections, and the tumor growth was examined during the course of 30 days. The tumor width and length were measured by a micrometer every 3 days. The tumor volumes were calculated as (length × width^2)/2. The body weights were also recorded. Tumor growth was observed for 30 days from the first treatment until tumors reached approximately 1,000 mm^3 in total volumes, at which time mice were euthanized. The major diameters of the formed tumors were measured macroscopically, and the tumors were weighed with an electronic balance at the end of the experiment.[29,30]

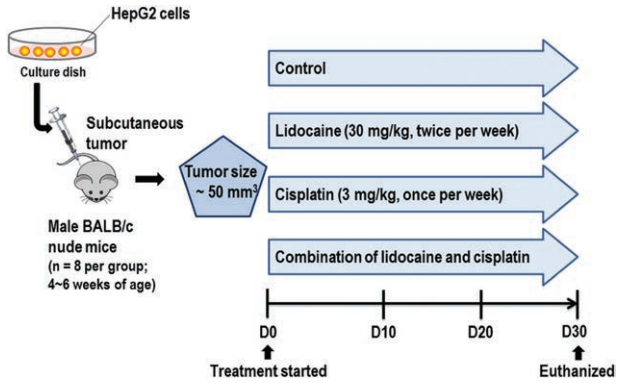

Fig. 1. Experimental design. HepG2 cells (2×10^6) were subcutaneously injected into the right side of the armpits of BALB/c nude mice. After palpable tumors developed a volume of approximately 50 mm^3, the mice were injected with a phosphate-buffered saline solution as a control, lidocaine (30 mg/kg, twice a week), cisplatin (3 mg/kg, once a week), or a combination of lidocaine and cisplatin through intraperitoneal injections. Tumor growth was observed for 30 days from the first treatment until the tumors reached approximately 1,000 mm^3 in total volume, at which time the mice were euthanized. D0 = day 0; D10 = day 10; D20 = day 20; D30 = day 30.

Histology and Immunohistochemistry

Formaldehyde-fixed, paraffin-embedded sections of hepatocellular carcinoma xenograft samples were subjected to hematoxylin and eosin staining and immunohistochemistry according to a standard protocol. The tumor samples were immunostained with antiproliferating cell nuclear antigen (anti-PCNA) and anticleaved caspase-3 antibodies. A primary antibody was added to the sections, and the sections were incubated at 4°C overnight. The sections were then incubated for 1 h at room temperature with the secondary antibody. Diaminobenzidine was used for staining. The samples were counterstained with hematoxylin and photographed.

Statistical Analysis

Nude male BALB/c mice (4 to 6 weeks of age) were randomly assigned into four experimental groups based on computer-generated random number: control, lidocaine, cisplatin, and lidocaine plus cisplatin. The researchers were not blinded to the experimental conditions.

We did not conduct *a priori* power analysis before starting the study. The sample size used in the study was based on previous research.[31–33] There were no lost or missing data in this study.

The statistical analysis was performed with SPSS software, version 16.0 (SPSS, Inc., USA). Values are expressed as the mean ± SD. The data were compared using a one-way ANOVA followed by a Dunnett *post hoc* test, as appropriate; the results were demonstrated by charts using SigmaPlot

version 8.0 (Systat Software, Inc., USA) or GraphPad Prism version 5.04 (GraphPad Software, Inc., USA). Other data were evaluated with a two-way ANOVA to detect differences between different treatment groups. Bonferroni *post hoc* testing was applied to detect differences between different treatment groups. All the experiments *in vitro* were performed a minimum of three times. Differences were considered statistically significant when $P < 0.05$.

Results

Effect of Lidocaine on Cell Growth and Apoptosis of Human HepG2 Cells

Cell proliferation and apoptosis play a central role in hepatocellular carcinoma progression. First, we assessed the effect of lidocaine on the treatment of HepG2 cells (one-way ANOVA, $P < 0.0001$). Treatment with 0.5, 1, 5, or 10 mM lidocaine resulted in significant reductions in cell viability (both $P < 0.05$; n = 3), whereas lower concentrations of lidocaine (0.1 mM) had no effect ($P > 0.05$; n = 3; fig. 2A). Lidocaine inhibited the growth of HepG2 cells in a dose- and time-dependent manner. Furthermore, the effects of lidocaine on hepG2 cells also caused a significant dose-dependent reduction in cell colony formation (359 ± 18 *vs.* 247 ± 1 at 1 mM [$P = 0.0031$] and 359 ± 18 *vs.* 108 ± 13 at 5 mM [$P = 0.0003$]; n = 3). The results are shown in figure 2B.

As shown in figure 2C, the cell cycle status of the HepG2 cells was measured after a lidocaine treatment of 1 or 5 mM. After lidocaine treatment for 24 h, the sub-G0/G1 population significantly increased from 63.7 ± 1.7% (control) to 72.4 ± 3.2% (5 mM); $P = 0.0143$; n = 3. Correspondingly, the percentage of cells in S phase decreased from 24.0 ± 2.2% (control) to 16.8 ± 2.1% (5 mM); $P = 0.0147$; n = 3. In addition, we used fluorescein isothiocyanate, conjugated Annexin V/propidium iodide dual staining to further assess lidocaine-induced apoptosis in HepG2 cells. After lidocaine treatment for 72 h, the apoptosis rate significantly increased from 1.7 ± 0.3% (control) to 5.0 ± 0.7% (5 mM); $P = 0.0009$; n = 3; fig. 2D. These results suggest that 5 mM lidocaine (higher concentrations) have significant antitumor activity.

Expression of Apoptosis-related Proteins and Caspase-3 Activities in Human HepG2 Cells Treated with Lidocaine

We investigated the molecular mechanisms underlying the induced apoptosis of HepG2 cells treated with lidocaine. Immunoblotting was used to evaluate caspase-3, cleaved caspase-3, Bcl-2, and Bax expression, and the results demonstrated that the level of apoptosis-related proteins was altered in HepG2 cells treated with lidocaine. Apoptosis is associated with the activation of intracellular cysteine proteases known as caspases. The activation of caspase-3 plays a pivotal role in the execution phase of apoptosis. As shown in figure 3A, quantitative analysis revealed that caspase-3 was activated in HepG2 cells as early as 24 h after exposure to 5 mM lidocaine ($P = 0.0493$; n = 3). Spectrometry demonstrated that the treatment of HepG2 cells with lidocaine (5 mM) for 0 to 72 h resulted in increased caspase-3 activity (at 24 h, $P = 0.0091$; at 48 h, $P = 0.0447$; at 72 h, $P = 0.0314$; n = 3; fig. 3B). The maximum increase in caspase-3 activity was observed at 24 h. The Bcl-2 family is a critical regulator of the apoptotic process and consists of inhibitors (*e.g.*, Bcl-2) and promoters (*e.g.*, Bax). We observed that treatment with lidocaine resulted in a decrease in the Bcl-2 levels with a concomitant increase in the Bax levels (fig. 3A). Quantitative analysis revealed that lidocaine (5 mM) significantly decreased Bcl-2 expression and increased Bax expression ($P = 0.0488$ and $P = 0.0105$; n = 3; fig. 3A). Collectively, these results clearly indicate that caspase-3 activation and the Bcl-2 family play an important role in HepG2 apoptosis induced by 5 mM lidocaine (higher concentrations).

MAPK Signaling in HepG2 Cells Treated with Lidocaine

The activation of MAPK pathways plays a central role in the proliferation and apoptosis of cancer cells. We determined whether modulation of the ERK1/2, p38 MAPK, and JNK signaling pathways account for the antitumor activity of lidocaine. As shown in figure 4A, after a 3-h treatment with lidocaine, a dose-dependent increase in phosphorylation of ERK1/2 and p38 was detected in HepG2 cells, whereas the total ERK1/2 and p38 protein amounts remained unchanged. Lidocaine had no marked effect on total JNK or the p-JNK. Furthermore, the activation of ERK1/2 and p38 was significantly increased by lidocaine in a time-dependent manner (fig. 4B). In the experiments (fig. 4C) involving the ERK inhibitor (PD98059) and p38 inhibitor (SB203580), analysis with a two-way ANOVA showed a significant effect of lidocaine ($P < 0.0001$). A Bonferroni *post hoc* analysis revealed a significant reduction in lidocaine-induced cleaved caspase-3 activation in the presence of 10 μM PD98059 or SB203580 ($P = 0.0033$ and $P = 0.0024$ *vs.* lidocaine group; n = 3; fig. 4C). Spectrometry demonstrated that a significant decrease in lidocaine-induced cleaved caspase-3 activity in the presence of 10 μM PD98059 or SB203580 (both $P = 0.0013$ *vs.* lidocaine group; n = 3, fig. 4D). The results suggested that the inhibition of cleaved caspase-3 by lidocaine is mediated through the inactivation of the ERK1/2 and p38 signaling pathways in HepG2 cells.

Lidocaine Enhances the Cytotoxicity of Cisplatin against HepG2 Cells In Vitro

Cisplatin is a cytotoxic drug that is widely used for hepatocellular carcinoma treatment. To investigate whether lidocaine sensitizes hepatocellular carcinoma cells to cisplatin, we determined the effect of lidocaine in combination with cisplatin on cell proliferation and apoptosis in HepG2 cells. The effect reached statistical significance in a two-way ANOVA ($P < 0.0001$) with lidocaine in the absence or presence of cisplatin. Bonferroni *post hoc* testing was conducted to detect differences between the groups. The cell viability analysis in HepG2 cells showed that treatment with cisplatin in combination with lidocaine caused significantly decreased

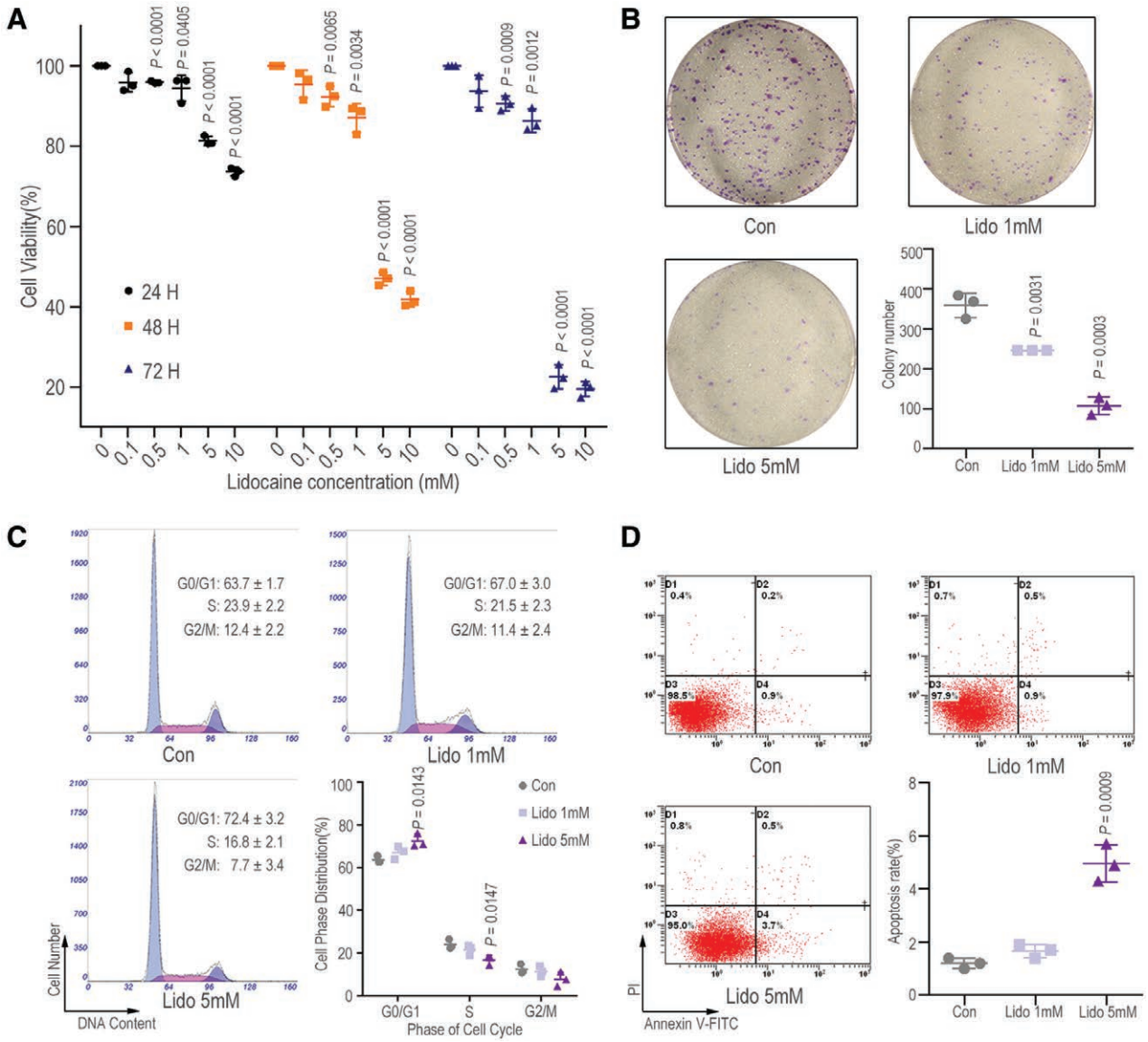

Fig. 2. Effect of lidocaine (Lido) on cell growth and apoptosis of human HepG2 cells. (*A*) Growth-inhibitory effects of Lido on HepG2 cells were determined by the Cell Counting Kit-8 assay. Cells were exposed to culture medium alone (control [Con]) or to Lido (0.1–10 mM) for 24, 48, and 72 h. Treatment with 0.5, 1, 5, or 10 mM of Lido resulted in significant reductions in cell viability, whereas lower concentrations of Lido (0.1 mM) had no effect. (*B*) The colony formation assay was scored after 14 days. Representative images and statistical analyses showing Lido-independent cell growth of HepG2 cells. (*C*) Representative image showing the effect of lidocaine on cell cycle progression. G_0/G_1 phase arrest was observed in HepG2 cells treated with lidocaine. (*D*) Apoptosis was assessed by flow cytometry using an Annexin V-FITC/PI kit. Lido induced apoptosis in a dose-dependent manner. Statistical differences were determined using a one-way ANOVA. These results are expressed as the mean ± SD of three independent experiments and are significantly different ($P < 0.05$) between the treatment group and the corresponding control group. FITC = fluorescein isothiocyanate.

cell viability compared with treatment with cisplatin or lidocaine alone (at 24 h, $P = 0.0002$ and $P < 0.0001$; at 48 h, $P = 0.0022$ and $P < 0.0001$; at 72 h, $P = 0.0116$ and $P < 0.0001$; n = 3; fig. 5A). The flow cytometry analysis in HepG2 cells showed that treatment with cisplatin in combination with lidocaine significantly increased cell apoptosis compared with treatment with cisplatin or lidocaine alone ($P = 0.0001$ and $P < 0.0001$; n = 3; fig. 5B). These data suggest that lidocaine enhanced cisplatin-induced apoptosis of HepG2 cells and exhibited a synergistic effect when combined with cisplatin.

To further analyze the effect of lidocaine on cisplatin-induced apoptosis, several apoptosis-related proteins were examined by Western blotting. As shown in figure 5C, lidocaine enhanced the cisplatin-induced levels of cleaved caspase-3 and Bax, whereas the caspase-3 levels remained unchanged. Lidocaine, compared with cisplatin or lidocaine alone, decreased the cisplatin-induced levels of Bcl-2

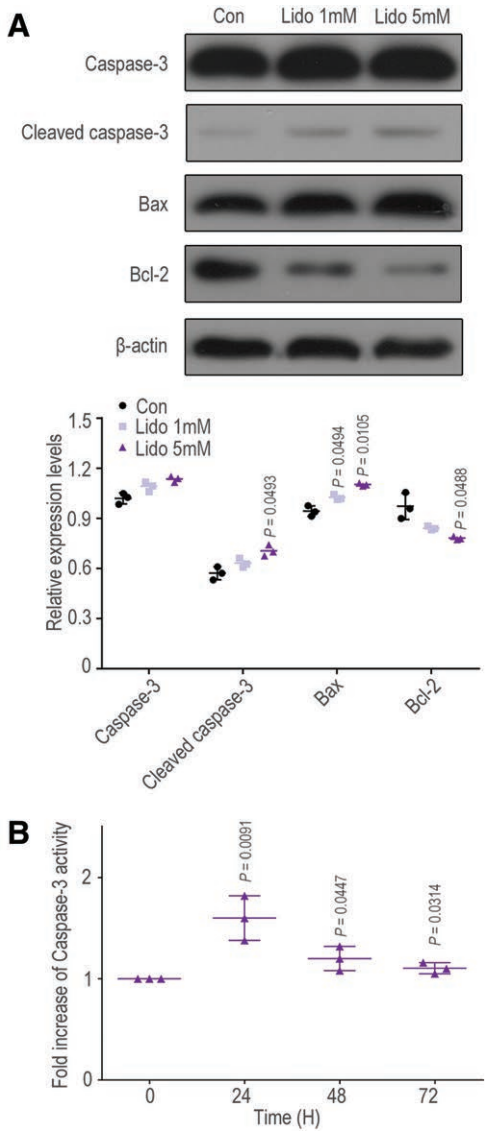

Fig. 3. Expression of apoptosis-related proteins and caspase-3 activities in human HepG2 cells treated with lidocaine (Lido). (*A*) Cells were treated with different concentrations of Lido (1 and 5 mM) for 24 h. Expression levels of apoptosis-related proteins (caspase-3, cleaved caspase-3, Bax, and Bcl-2) were assayed by Western blot analysis and quantified using the ImageJ program. β-actin was used as a loading control. The image is representative of three independent experiments yielding similar results. (*B*) Cells were treated with 5 mM lidocaine for 24, 48, or 72 h. When caspase-3 was activated, chromophoric groups were freed from caspase substrates and detected by spectrophotometry. OD values obtained by spectrophotometry represent caspase-3 activity. Statistical differences were determined using a one-way ANOVA. These results are expressed as the mean ± SD of three independent experiments and are significantly different ($P < 0.05$) between the treatment group and the corresponding control group.

in HepG2 cells. Quantitative analysis showed that 5 mM lidocaine increased 10 μM cisplatin-induced cleaved caspase-3 and Bax activation ($P = 0.0243$ and $P < 0.0448$ *vs.* cisplatin group; n = 3), and lidocaine attenuated 10 μM cisplatin-induced Bcl-2 activation ($P = 0.0243$ *vs.* cisplatin group; n = 3). Furthermore, a caspase colorimetric substrate assay showed that an increase in caspase-3 activity occurred in HepG2 cells treated with the combination of lidocaine and cisplatin compared with all the other experimental groups (at 24 h, $P < 0.0001$ *vs.* control group, $P = 0.0065$ *vs.* cisplatin group, and $P = 0.0001$ *vs.* lidocaine group; at 48 h, $P = 0.0009$ *vs.* control group, $P = 0.0163$ *vs.* cisplatin group, and $P = 0.0014$ *vs.* lidocaine group; at 72 h, $P = 0.0006$ *vs.* control group, $P = 0.0273$ *vs.* cisplatin group, and $P = 0.0009$ *vs.* lidocaine group; n = 3, fig. 5D). Collectively, these results indicate that lidocaine enhanced cisplatin cytotoxicity by increasing cisplatin-induced apoptosis in HepG2 cells.

Therapeutic Efficacy of Lidocaine in the Antitumor Activity of Cisplatin against Tumor Growth of HepG2 Cell Xenografts in Nude Mice

To determine the *in vivo* effects of lidocaine on tumor growth and whether lidocaine might increase the antitumor activity of HepG2 cells in response to cisplatin in a xenograft model, HepG2 cells were injected subcutaneously into 4- to 6-week-old male athymic nude mice (BALB/c-nu/nu). The mice were randomly divided into control, lidocaine, cisplatin, and lidocaine plus cisplatin groups (n = 8 per group). The tumor size and body weight were measured twice per week, and treatment began after the subcutaneous tumor reached a volume of approximately 50 mm^3. The mice were injected with a PBS solution as a control, lidocaine (30 mg/kg, twice a week), cisplatin (3 mg/kg, once a week), or a combination of lidocaine and cisplatin through intraperitoneal injection. Tumor growth was observed for 30 days after the first treatment until tumors reached approximately 1,000 mm^3 in total volume, at which time the mice were euthanized. The major diameters of the tumors were measured macroscopically, and the tumors were weighed with an electronic balance. As shown in figure 6A, lidocaine significantly inhibited subcutaneous tumor growth compared with the control groups ($P < 0.0001$). Lidocaine treatment did not affect body weight during the experiment (fig. 6B). Based on tumor weight, lidocaine attenuated the progression of hepatic tumors compared with PBS ($P = 0.0001$; fig. 6, C and D). Analysis of the results of xenograft experiments revealed that lidocaine suppresses tumor growth in a subcutaneous tumor model.

Moreover, consistent with the results of the *in vitro* experiments, either lidocaine or cisplatin treatment alone markedly suppressed xenograft tumor growth compared with the control (two-way ANOVA, $P < 0.0001$). No significant differences were found between the two treated groups (lidocaine alone and cisplatin alone, $P = 0.6110$). The combination of lidocaine and cisplatin exerted a dramatically therapeutic effect that was significantly better than the effects in all the other experimental groups ($P < 0.0001$ *vs.* control group, $P = 0.0002$ *vs.* cisplatin group, and $P = 0.0007$ *vs.* lidocaine

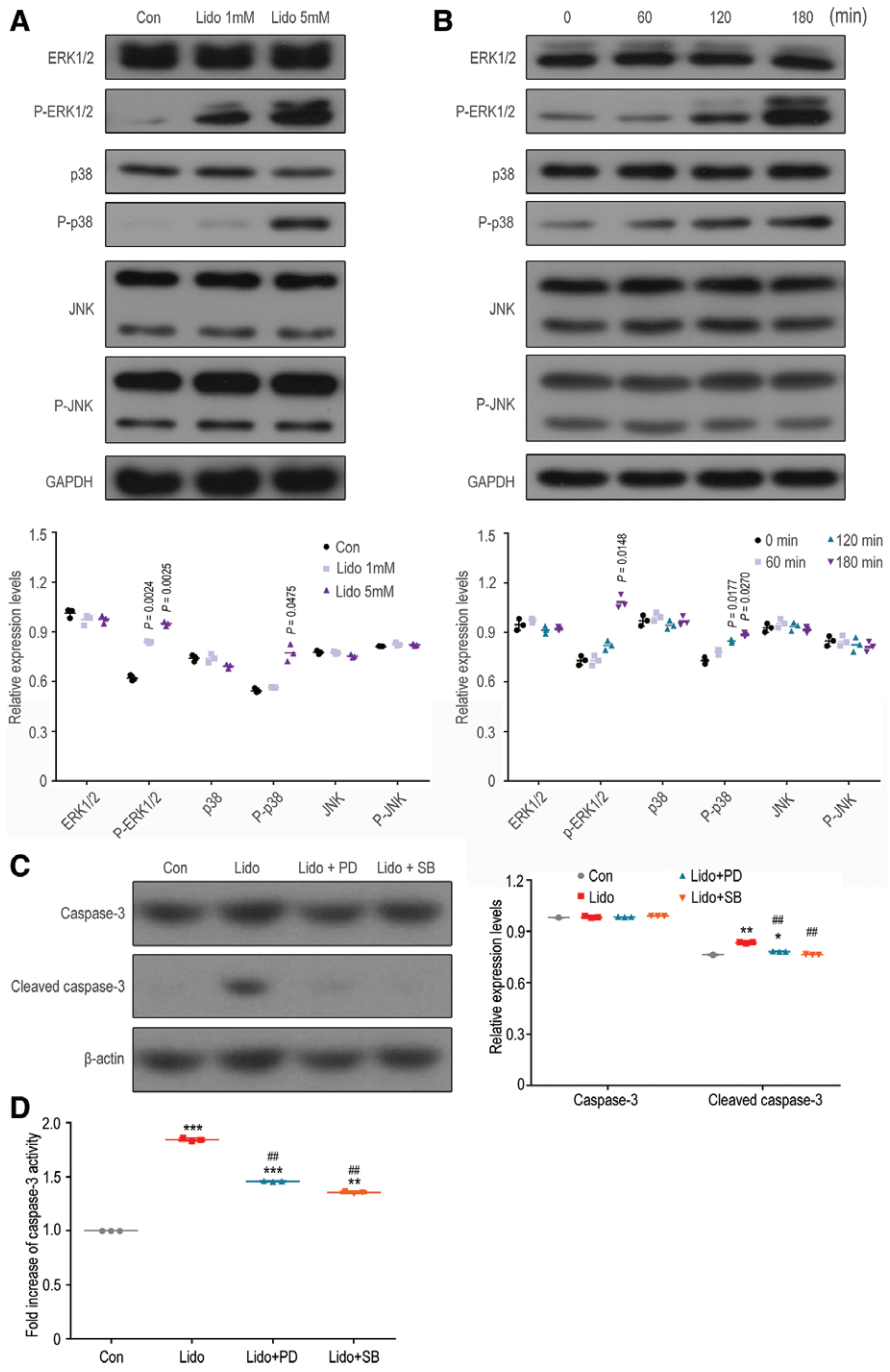

Fig. 4. Mitogen-activated protein kinase signaling in human HepG2 cells treated with lidocaine (Lido). (*A, B*) Cells were treated with different concentrations of Lido (1 and 5 mM) for 3 h and with 5 mM Lido for 60, 120, and 180 min. Expression levels of mitogen-activated protein kinase (extracellular signal-regulated kinase [ERK] 1/2, p38, and c-Jun N-terminal kinase [JNK]) and their activated forms (phospho (p)-ERK1/2, p-p38, and p-JNK) were assessed by Western blot analysis and quantified using the ImageJ program. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control (Con). Statistical differences were determined using a one-way ANOVA. (*C*) Cells were pretreated with or without the ERK1/2 inhibitor PD98059 (PD; 10 μM) or p38 inhibitor SB203580 (SB; 10 μM) for 2 h followed by exposure to Lido (5 mM) for 24 h. Expression levels of apoptosis-related proteins (caspase-3 and cleaved caspase-3) were assayed by Western blot analysis and quantified using the ImageJ program. β-actin was used as a loading Con. The image is representative of three independent experiments yielding similar results. (*D*) Cells were pretreated with or without the ERK1/2 inhibitor PD (10 μM) or p38 inhibitor SB (10 μM) for 2 h followed by exposure to Lido (5 mM) for 24 h. When caspase-3 was activated, chromophoric groups were freed from caspase substrates and detected with spectrophotometry. OD values obtained by spectrophotometry represent caspase-3 activity. Statistical differences were determined using a two-way ANOVA. These results are expressed as the mean ± SD of three independent experiments. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 different with the corresponding Con group; ##*P* < 0.01 Lido group *versus* Lido plus PD group or Lido plus SB group.

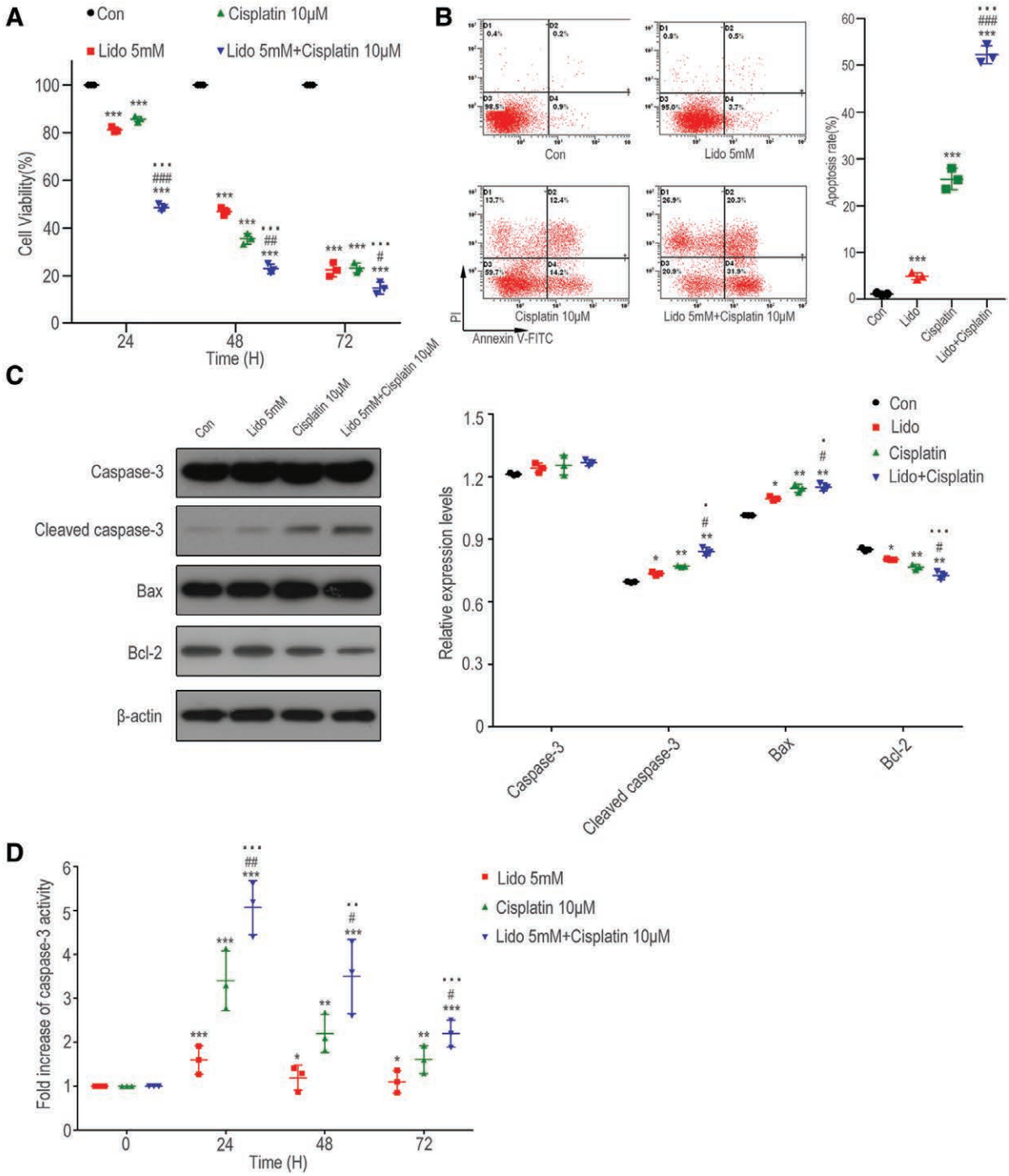

Fig. 5. Lidocaine (Lido) enhances cisplatin-induced apoptosis and activates apoptosis-related proteins. (*A*) Cell proliferation assays demonstrated that Lido increases the sensitivity of HepG2 cells to the cytotoxic effect of cisplatin. Cells were treated with 10 μM cisplatin for 24, 48, or 72 h in the absence or presence of 5 mM Lido. (*B*) Cells were treated with 10 μM cisplatin for 72 h in the absence or presence of 5 mM Lido. Lido in combination with cisplatin in HepG2 cells significantly facilitated apoptosis compared to cisplatin or Lido alone. (*C*) Expression levels of apoptosis-related proteins (caspase-3, cleaved caspase-3, Bax, and Bcl-2) were assessed by Western blot analysis and quantified using the ImageJ program. β-actin was used as a loading control (Con). The image is representative of three independent experiments yielding similar results. (*D*) Cells were treated with 10 μM cisplatin for 24, 48, or 72 h in the absence or presence of 5 mM Lido. When caspase-3 was activated, chromophoric groups were freed from caspase substrates and detected with spectrophotometry. OD values obtained with spectrophotometry represent caspase-3 activity. Statistical differences were determined using a two-way ANOVA. These results are expressed as the mean ± SD of three independent experiments. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 different with the corresponding Con group; #*P* < 0.05, ##*P* < 0.01, and ###*P* < 0.001 cisplatin group *versus* Lido plus cisplatin group; •*P* < 0.05, ••*P* < 0.01, and •••*P* < 0.001 Lido group *versus* Lido plus cisplatin group. FITC = fluorescein isothiocyanate; PI = propidium iodide.

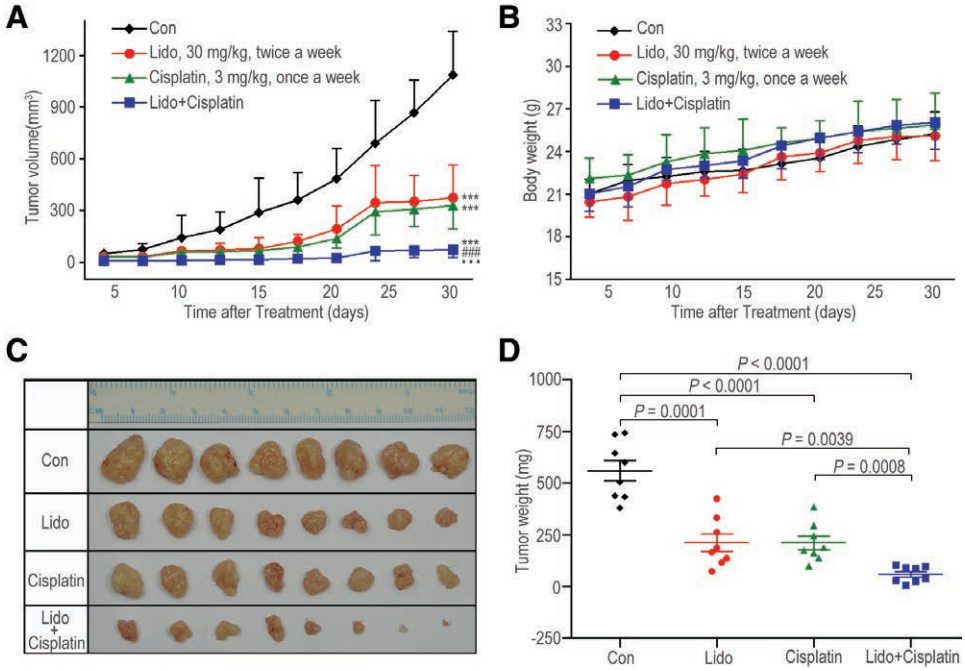

Fig. 6. Therapeutic efficacy of lidocaine (Lido) in the antitumor activity of cisplatin against tumor growth of HepG2 cell xenografts in nude mice. A hepatocellular carcinoma xenograft model was established by the implantation of HepG2 cells into BALB/c nude mice. When the tumors reached a volume of approximately 50 mm^3, the mice were injected with a phosphate-buffered saline solution as a control (Con), Lido (30 mg/kg, twice per week), cisplatin (3 mg/kg, once per week), or a combination of Lido and cisplatin by intraperitoneal injection (n = 8 each group). (*A*) The tumor width (W) and length (L) were measured by a micrometer every 3 days. Tumor volumes were calculated as (L x W^2)/2. (*B*) Administration of Lido, cisplatin, or their combination had no effect on the health of nude mice as assessed by body weight during and beyond the treatment period. (*C*) The macroscopic appearance of the dissected tumor tissue. (*D*) The measurement of individual tumor weight in each group by an electronic balance. Statistical differences were determined using a two-way ANOVA. Data represent the mean ± SD, ***$P < 0.001$ different with the corresponding Con group. ###$P < 0.001$ cisplatin group *versus* Lido plus cisplatin group. •••$P < 0.001$ Lido group *versus* lidocaine plus cisplatin group.

group; fig. 6A). In addition, the administration of lidocaine, cisplatin, or a combination of the two had no effects on the health of nude mice, which was assessed by body weight during and beyond the treatment period ($P > 0.05$; fig. 6B). Consistent with the growth curves, the tumor weights were markedly reduced in nude mice treated with lidocaine combined with cisplatin compared with nude mice treated with lidocaine or cisplatin alone ($P < 0.0001$ *vs.* control group, $P = 0.0008$ *vs.* cisplatin group, and $P = 0.0039$ *vs.* lidocaine group; fig. 6, C and D).

Furthermore, we performed immunohistochemistry to detect the expression of cleaved caspase-3 and anti-PCNA in randomly selected xenograft nude mice tumors, and the results demonstrated that the above treatment retarded HepG2 cell growth (fig. 7). Staining for cleaved caspase-3 confirmed that lidocaine promoted cisplatin-induced apoptosis of tumor cells. Moreover, anti-PCNA expression was significantly inhibited in the combined group, which exhibited a marked suppression of tumor growth compared with all the other experimental groups. These results indicate that lidocaine induces caspase-dependent apoptosis and suppresses tumor growth, and it enhances the cytotoxicity of cisplatin, thereby inhibiting HepG2 tumor growth in a xenograft model *in vivo*.

Discussion

Increasing evidence clearly demonstrates that lidocaine may exert antitumor activity in the treatment of cancer by suppressing cell proliferation, inducing apoptosis, and inhibiting migration.[12,13,16,17] Lidocaine antitumor effects on various breast tumors and other solid tumors have been extensively documented, but *in vitro* experiments are limited in their ability to model tumor responses *in vivo*. Recently, lidocaine has been shown to enhance the tumoricidal effects of conventional chemotherapeutics.[21,22,27] Our results demonstrated that lidocaine suppresses tumor growth and induces apoptosis in human HepG2 cells *in vitro* and were also supported, for the first time, by animal experiments that clearly showed lidocaine not only suppressed hepatocellular carcinoma development but also sensitized hepatocellular carcinoma to cisplatin *in vivo*. Therefore, we propose a plausible explanation for the antitumor activity of lidocaine to explore the effect of anesthesia on cancer behavior, and our explanation may provide a therapeutic approach for enhancing sensitivity to cisplatin in hepatocellular carcinoma.

Lidocaine may indirectly or directly influence the tumor biologic characteristics of cancer cells. The indirect effects of lidocaine include enhancing natural killer cell activity and

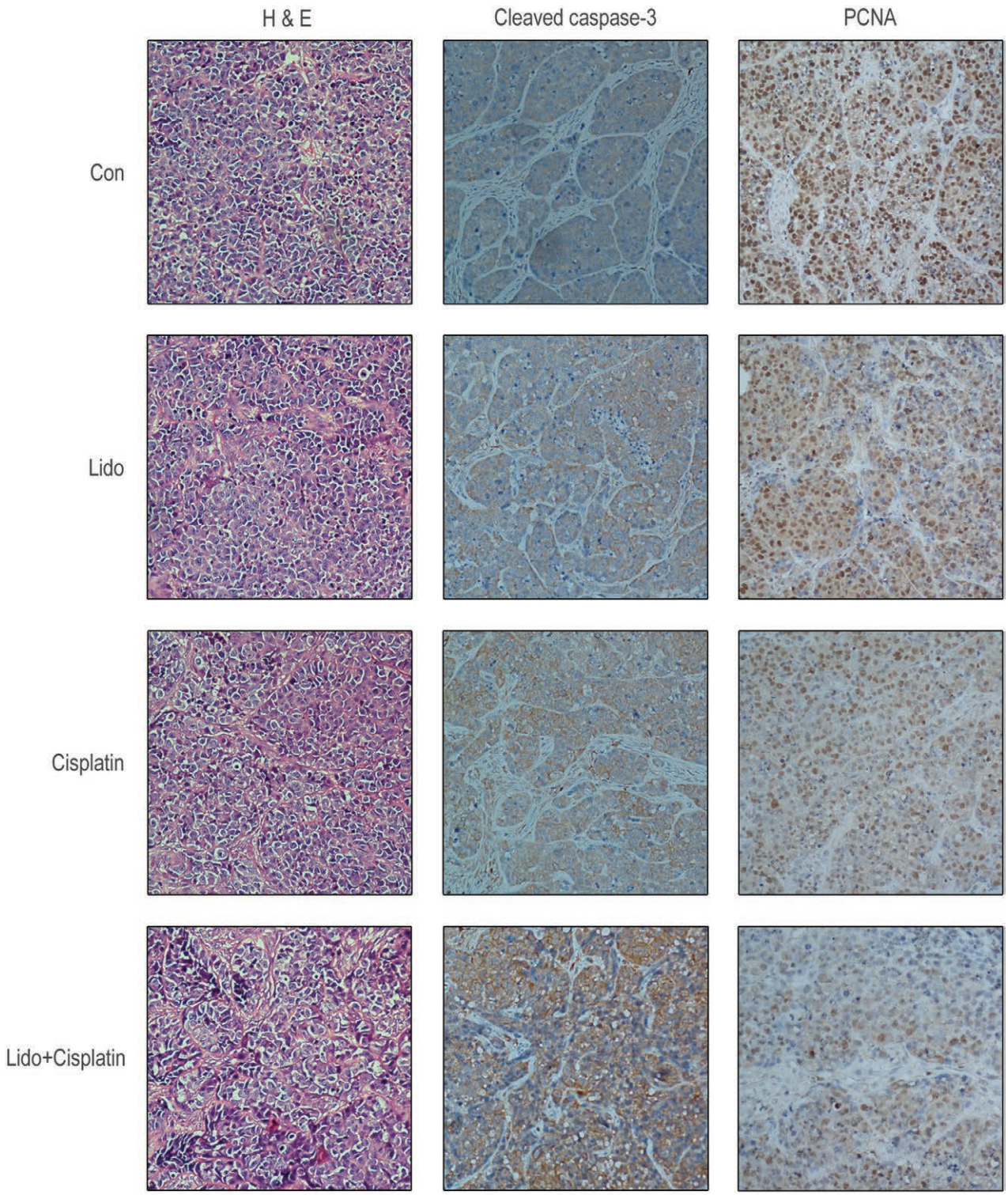

Fig. 7. Analysis of the impact of lidocaine (Lido) and cisplatin as single agents or in combination on cleaved caspase-3 and antiproliferating cell nuclear antigen (anti-PCNA) expression *in vivo* in human HepG2 cell xenografts. The same tumor sections were stained for hematoxylin and eosin (H&E) and immunostained for anti-PCNA and cleaved caspase-3. Magnification ×200. Con = control.

improving the preservation of immunocompetence.[34] The direct effects of lidocaine on cancer cells include inhibition of proliferation, induction of apoptosis, and suppression of metastatic efficiency.[13,16,17] Specially, proliferation and apoptosis are two important hallmarks of cancer cells, and the suppression of proliferation and induction of apoptosis are two promising strategies in the development of antitumor drugs.[35] In the current study, lidocaine, as a single

agent, suppressed the growth of HepG2 cells in a dose- and time-dependent manner, arrested cells in the G_0/G_1 phase of the cell cycle, and induced cell apoptosis. Although the lidocaine doses used in our *in vitro* studies were high, these doses are very similar to doses used in previous *in vitro* studies.[12,16,17] The lidocaine dose used in mice was higher than the allowable dose for humans. Presumably, the plasma level after this dose will be higher in the mice. However, none of our mice developed significant lidocaine toxicity. Also, it is not appropriate to extrapolate our findings in this study directly to humans. Nevertheless, our findings suggest that lidocaine has antihepatic tumor properties.

The underlying molecular mechanism of lidocaine that is involved in cytotoxicity remains unclear. Caspase-3 activation is considered a critical activator of apoptosis.[36] In the current study, we found that the expression levels of activated caspase-3 were increased in a dose-dependent manner in HepG2 cells, and these results revealed the participation of caspase-3 in lidocaine-induced apoptosis. More importantly, *in vivo* experiments not only prove that lidocaine could induce caspase-dependent apoptosis, but also confirm that lidocaine dramatically enhanced cisplatin-induced caspase-dependent apoptosis. In fact, it is likely that many apoptosis-associated proteins also promote lidocaine-induced apoptosis, such as cleaved poly adp-ribose polymerase and released cytochrome c.[27] Moreover, apoptosis is regulated by the balance between pro- and antiapoptotic proteins in the Bcl-2 family.[37] Our results suggest that lidocaine alters the protein levels of critical members of the Bcl-2 family, which may lead to the induction of HepG2 cell apoptosis by increasing the ratio of Bax/Bcl-2. Given that caspases and the Bcl-2 family play central roles as the main regulators of the process of apoptosis, these results strongly support the hypothesis that lidocaine treatment causes an increase in the Bax protein, activates caspase-3, and initiates a corresponding decrease in the Bcl-2 protein.

The MAPK signaling pathway is involved in the central regulation of cell proliferation, apoptosis, differentiation, and migration through several phosphorylation cascades in various cancers, including hepatocellular carcinoma.[38] The p44/42, also known as ERK 1/2, MAPK signaling pathway is part of the MAPK family and plays a key role in multiple biochemical signal pathways that could be activated in response to a series of extracellular stimuli, including transcription factors, that lead to the activation of growth factors and apoptotic factors that could ultimately influence cell survival.[39] Activated ERK1/2 and its downstream effectors are implicated in the induction of apoptosis by cisplatin in various cancers.[18,40,41] As shown in this study, lidocaine dramatically increased activation of the ERK1/2 cascade, which could be responsible for the suppressive effect of lidocaine on hepatocellular carcinoma cell proliferation. P38 and JNK kinases are preferentially activated by extracellular stress, which results in cell differentiation and apoptosis.[42,43] The p38 MAPK signaling pathway is a member of the MAPK family, and activated p38 is mainly implicated in inducing proliferation, differentiation, apoptosis, and autophagy by chemotherapeutic drugs in hepatocellular carcinoma.[19] Our study demonstrated that lidocaine treatment caused significant increases in the p-p38 level and had no effect on the p-JNK level. We demonstrated that the phosphorylation levels of ERK1/2 and p38 were remarkably upregulated by lidocaine. Previous studies reported that phosphorylated ERK1/2 and p38 are activated by external and internal stimuli, which could directly induce caspase-3 activation through the upregulation of Bax, which leads to apoptosis and programmed cell death.[41,44–47] Taking into account these findings and the current study, we propose that phosphorylated ERK1/2 and p38 participate in suppressed proliferation and facilitate apoptosis by lidocaine *via* the intrinsic pathway (fig. 8).

Lidocaine could sensitize cancer cells to the effects of chemotherapeutic drugs.[21,22] Our results demonstrating the effect of lidocaine in combination with cisplatin on the suppression of tumor growth in HepG2 cells and in mice with xenografts further supports this hypothesis. Previous studies demonstrated that lidocaine sensitizes breast cancer cells to the cytotoxic effect of chemotherapy agents. Cisplatin is a chemotherapeutic drug that is widely used in TACE for hepatocellular carcinoma. Cisplatin kills cancer cells by binding to and causing cross-linking of DNA and interfering with cellular DNA repair mechanisms, which ultimately induces cell apoptosis (programmed cell death).[48] As expected, we demonstrated that lidocaine dramatically increased cisplatin-induced cell apoptosis both *in vitro* and *in vivo*. Lidocaine may sensitize HepG2 cells to cisplatin in hepatocellular carcinoma treatment through multiple mechanisms. Our data showed that the combination of lidocaine and cisplatin led to increased expression of the proapoptotic protein Bax and cleaved caspase-3 and to decreased expression of the antiapoptotic protein Bcl-2. Previous studies revealed that cisplatin induced apoptosis in various cancer cells.[41,49,50] Apoptosis appears to be the final pathway shared by many chemotherapeutic drugs although it exerts its antitumor effects on cancer cells through a variety of mechanisms.[51–53] Lidocaine may improve chemotherapy effects by controlling apoptosis-related proteins and may result in efflux-independent chemotherapy resistance in HepG2 cells. Another underlying mechanism by which lidocaine may influence tumor growth is through interaction with the tumor epigenome.[54] Human malignancies undergo a crucial disruption of global genomic DNA methylation and histone modification patterns.[55] Unlike genetic alterations, aberrant epigenetic DNA methylation has excellent prospects for cancer prevention, detection, and therapy.[56,57] Increased DNA methylation frequently results in downregulation of tumor suppressor genes, which favors the development and progression of cancer.[58] Recently, lidocaine has been shown to sensitize the cytotoxicity of cisplatin in breast cancer cells *via* the upregulation of retinoic acid receptor β and Ras association

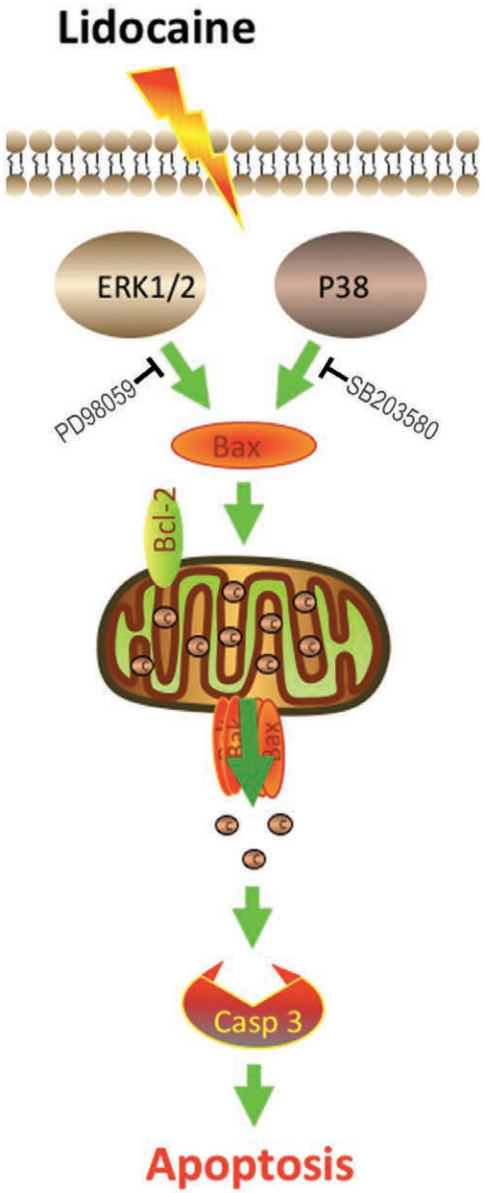

Figure 8. Mechanisms of extracellular signal-regulated kinase (ERK)/p38-mediated cell apoptosis and cell survival induced by lidocaine ERK1/2 and p38 may regulate apoptosis and cell survival by lidocaine at multiple points that include caspase-3 activation through the upregulation of Bax and the decrease of Bcl-2.

domain family 1A demethylation.[27] Therefore, a combined strategy that yields synergistic efficacy and restores the sensitivity of chemotherapeutic drugs is urgently required for treating hepatocellular carcinoma.

Based on multiple recent retrospective studies, the use of regional anesthesia is associated with improved outcomes after surgery.[7,8] Accumulating evidence demonstrates that reduced immunosuppression, decreased perioperative surgical stress responses, reduced doses of opioids, antiinflammatory effects, and local anesthetic effects may affect survival.[11] More specifically, the use of local anesthetics is associated with an improved prognosis, and these antiproliferative and antiinflammatory effects cannot be ignored. In this study, we observed a significant inhibitory role of lidocaine in HepG2 cell proliferation. Moreover, lidocaine significantly inhibited tumor growth in tumor-bearing mice. Lidocaine is commonly used for regional anesthesia and pain relief, and its administration *via* intravenous infusion during the perioperative stage is increasing. The intraarterial administration of lidocaine is effective for preventing or reducing pain before and during TACE for hepatocellular carcinoma.[23,24] Clinically, the combination of lidocaine and cisplatin could potentially be used during TACE. Acquired resistance to some existing chemotherapeutic agents for advanced or metastatic hepatocellular carcinoma limits the clinical application of these agents.[26] Therefore, combined chemotherapy remains an urgent medical need, especially in cases with drug-resistant solid tumors. More importantly, we demonstrated that lidocaine not only suppresses tumor growth but also sensitizes cells to cisplatin in hepatocellular carcinoma treatment *in vivo*. The data presented here provide experimental evidence, showing that novel therapeutic strategies for treatment of hepatocellular carcinoma could be developed to achieve better outcomes by combining lidocaine and cisplatin.

Taken together, these results suggest that lidocaine may exert potent anticancer activity in hepatocellular carcinoma by suppressing cell growth in parallel with inducing caspase-dependent apoptosis through the MAPK pathway. Our results may provide a plausible exploration of a relationship between the direct influence of lidocaine on cancer cell biologic characteristics and the impact of perioperative anesthetic management on postoperative cancer prognosis. Furthermore, our findings strongly demonstrated that lidocaine can sensitize hepatocellular carcinoma cells to cisplatin both *in vitro* and *in vivo*. These results provide a basis for further clinical trials to study the combination of lidocaine and cisplatin to treat hepatocellular carcinoma, especially in TACE therapy for hepatocellular carcinoma.

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Competing Interests

The authors declare no competing interests.

Correspondence

Address correspondence to Dr. Zeng: Cancer Center, Sun Yat-Sen University, No. 651, Dongfeng Road East, Guangzhou, Guangdong, China 510060. zengwa@mail.sysu.edu.cn. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made free-

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