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ORIGINAL PAPER

Caffeine enhances the anti-tumor effect of 5-fluorouracil via increasing the production of reactive oxygen species in hepatocellular carcinoma

Zhilei Wang[1] · Chengxin Gu[1] · Xinrui Wang[2] · Yating Lang[2] · Yanqin Wu[2] · Xiaoqin Wu[2] · Xifei Zhu[2] · Kunyuan Wang[1] · Hui Yang[1]

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Abstract

The development of drug resistance affecting the prognosis of patients with hepatocellular carcinoma (HCC) leads to low survival rate of HCC patients. Caffeine is reported to have a function of protecting the liver and anti-tumor activity. Therefore, caffeine may be an ideal enhancer for HCC chemotherapy regimens. Our study showed that the combination of caffeine and 5-FU significantly inhibited the proliferation of HCC cells in vivo and in vitro comparing with caffeine or 5-FU monotherapy. The CI values of caffeine (0.5 mM) combined with 5-FU (25, 50 μM) were all less than 1, confirming that the utilization of drug combination has a synergistic inhibitory effect on the proliferation of HCC cells. Meanwhile, results of Western blot and TUNEL assays demonstrated that the apoptotic level of HCC cells in the combined group was significantly increased. The protein expression level of cleaved PARP was up-regulated, while the protein level of Bcl-2 and Bcl-xL was down-regulated. In addition, we found that ROS levels were increased in the 1 mM caffeine and 25 μM 5-FU combination group comparing with the control or single drug group. Taken together, this is the first study to demonstrate that the combination of caffeine and 5-FU inhibits HCC cells proliferation and promotes cellular apoptosis by regulating intracellular ROS production. The present data provides a basis for the application of caffeine combined with 5-FU as a novel chemotherapy regimen for HCC.

Keywords Caffeine · 5-Fluouracil · Hepatocellular carcinoma · Proliferation · Apoptosis · Reactive oxygen species

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in the clinic. According to global cancer statistics in 2018, the incidence of HCC ranks the sixth among malignant tumors, and it is the fourth leading cause of cancer-related deaths worldwide [1]. Although the therapy approaches of HCC have been improved in the recent years, most patients with HCC are in advanced stage at clinical diagnosis, losing the opportunity for surgery. The use of systemic chemotherapy has been shown to play an important role in the treatment of advanced HCC [2]. However, the therapeutic effect is limited due to the instinct or extrinsic chemical resistance of HCC cells. Therefore, it is important to find a combination that enhances the cytotoxicity of traditional chemotherapy regimens.

Caffeine, one of the most common food ingredients, can be found in coffee, soda, tea, chocolate, and energy drinks. It is also a psychoactive drug that affects the brain function or mental state, which can improve performance during exercise and cognitive functions [3, 4]. Moreover, increasing evidence suggests that coffee consumption reduces the risk of multiple cancers, such as glioblastoma [5, 6], colorectal cancer [7], skin cancer [8], and liver cancer [9]. It has been reported that caffeine and its analog CGS 15943 inhibit the progression of HCC via Akt signaling pathway [10, 11]. And caffeine has the function of increasing the anti-tumor effect of cisplatin on cell proliferation and apoptosis in HCC cell lines [12], which indicates that caffeine-assisted chemotherapy is useful for HCC treatment.

✉ Kunyuan Wang
kalawang@foxmail.com

✉ Hui Yang
yanghui@gzhmu.edu.cn

[1] Department of Gastroenterology, The Second Affiliated Hospital of Guangzhou Medical University, 250 Changgang East Road, Guangzhou 510260, Guangdong, People's Republic of China

[2] The Second Clinical Medicine School of Guangzhou Medical University, Guangzhou 510260, Guangdong, People's Republic of China

Previous investigations have shown that 5-fluorouracil (5-FU) alone or in combination with other therapeutic drugs have been widely used for clinical treatment of various cancers [13–16]. However, 5-FU-based chemotherapy has limited anti-cancer efficacy in clinic due to multidrug resistance and dose-limiting cytotoxicity [17–19]. To overcome these problems, a novel combination of 5-FU and other anticancer drugs with different mechanistic action is required. In the present study, we explored whether caffeine could enhance the anti-tumor effect of 5-FU on HCC cells in vitro and in vivo.

Materials and methods

Cells and reagents

Human HCC cell lines SMMC-7721 and Hep3B were obtained from the Cell Bank of Type Culture Collection (CBTCC, Chinese Academy of Sciences, Shanghai, China) and the American Type Culture Collection (ATCC, Manassas, VA, USA), respectively. Cells were cultured in Dulbecco's modified Eagle medium (Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA; cat. no. 8117220) supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin (Gibco, Thermo Fisher Scientific, Inc.; cat. no. 15-140-122) and maintained at 37 °C in a 5% CO_2 incubator. 5-Fluorouracil (5-FU) was purchased from Selleck Chemicals (Houston, TX, USA; cat. no. S1209). Caffeine was purchased from Sigma-Aldrich (St. Louis, MO, USA; cat. no.93784-250G).

Cell viability assays

HCC cells were seeded into 96-well plates at 5000 cells per well. After overnight incubation, cells were treated with the indicated concentrations of 5-FU, caffeine or combination of 5-FU and caffeine for 48 h. Then, cell viability was determined by colorimetric MTS assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) Cell Titer 96® AQueous One solution (Promega, USA; cat. no. G3588). The cells were incubated with a solution containing 20 µl MTS and 100 µl DMEM for 2 h at 37 °C, and the absorbance at 490 nm was measured with Synergy H1/Epoch microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). Viability rate was calculated as follows: viability (%) = (average OD value of drug-treated sample/average OD value of control sample) × 100%. The concentrations of agents that caused cell growth inhibition by 50% (IC50) were calculated by Probit Regression.

Combined effect evaluation

The interaction between 5-FU and caffeine was quantified basing on multiple drug effect equation and determined by the combination index (CI) value [20]. The combination index was evaluated by Com-puSyn software (ComboSyn, Inc., Paramus, NJ), which was available at http://www.combosyn.com/feature.html. CI values < 1, $= 1$ and > 1 indicated synergy, addition, and antagonism, respectively.

Western blot analysis

The extraction of total protein lysate and SDS-PAGE were performed according to previously described [21]. The primary antibodies included anti-poly (ADP-ribose) polymerase (PARP; 1:1000; cat. no. 9532; Cell Signaling Technology, Inc.), anti-Bcl-2 (1:1000; cat. no. 2870; Cell Signaling Technology, Inc.), anti-Bcl-xL (1:1000; cat. no. 2764; Cell Signaling Technology, Inc.), and anti-GAPDH (1:1000; cat. no. 2118; Cell Signaling Technology, Inc.).

Cellular reactive oxygen species (ROS) detection

The level of intracellular ROS was determined by Reactive Oxygen Species Assay Kit(ROS Assay Kit, Beyotime). After drug treatment, cells were treated with DCFH-DA (10 µM) at 37 °C for 20 min and then examined by flow cytometer (BD Bioscience). The ROS production was analyzed by FlowJo 10.0 software.

TUNEL assay

The DeadEnd™ Fluorometirc TUNEL system (Promega, USA; cat. no. G3250)was used to detect the level of cell apoptosis. 1×10^4 cells per well in a 96-well plate were fixed with 100 µl 4% para-formaldehyde at 25 °C for 30 min. The cells were then washed twice with PBS and incubated with 0.2% Triton X-100. After adding the equilibration buffer for 5 min, a mixed reagent containing an equilibration buffer, a nuclear mixture, and rTdT was added. Cells were incubated at 37 °C for 60 min in the dark. Thereafter, the cells were washed with 2 × SCC for 15 min and incubated with 10 mg/ml DAPI for 20 min. Staining results were observed and imaged under a light microscope (Olympus BX 51; Olympus Corporation, Tokyo, Japan). TUNEL-positive cells, total cells, and the percentage of apoptotic cells were counted.

In vivo tumor assays

Male BALB/c nude mice (4 weeks) were obtained from the Guangdong Animal Center. A 200 µl phosphate-buffered

saline (PBS) cell suspension containing 1×10^7 SMMC-7721 was subcutaneously injected into the dorsal region to establish a subcutaneous tumor formation model in nude mice. When the tumor size reached 5×5 mm^2, mice were randomly divided into four groups ($n = 3$ in each group). Tumor-bearing mice were injected intraperitoneally with DMSO (as a control), 5-FU (20 mg/kg/day, 5 consecutive days), caffeine (20 mg/kg/day, every other day for 2 weeks), or a combination of 5-FU and caffeine, respectively. The tumor volume was measured by a caliper and calculated according to the formula (width$^2 \times$ length)/2. Mice were sacrificed, and tumors were collected within 3 h after the last injection. All the experiments in vivo were performed in accordance with the institutions of guidelines for the use of laboratory animals and approved by the second affiliated hospital of Guangzhou Medical University [21].

Statistical analysis

Statistics were analyzed by SPSS version 16.0 software (SPSS, Inc., Chicago, IL, USA). The quantitative data were shown as mean ± SD from three independent experiments. In all experiments, one-way analysis of variance (ANOVA) was used to calculate the significance of differences between groups. A value of $P < 0.05$ indicated a significant difference.

Results

Caffeine synergistically sensitizes HCC cells to 5-FU in vitro

To investigate the anti-proliferative effect of caffeine and 5-FU, the viability of HCC cells was assessed by MTS assay after treatment with caffeine or 5-FU. We found that caffeine inhibited the growth of SMMC-7721 and Hep3B cells in a dose-dependent manner (Fig. 1a). The IC50 value of caffeine was 2.211 mM and 2.026 mM for SMMC-7721 and Hep3B cells, respectively. As presented in Fig. 1b, 5-FU significantly inhibited the growth of HCC cells with the increase of dose, and its IC50 value was 135.2 μM for SMMC-7721 and 158.0 μM for Hep3B. Then, to explore the synergistic effect of caffeine and 5-FU, HCC cells were treated with different concentration of caffeine (0, 0.5, 1 mM) and 5-FU (0, 25, 50 μM) alone or in combination. The MTS results revealed that the anti-proliferative effect of the combined treatment of caffeine (1 mM) and 5-FU (25, 50 μM) was markedly higher in comparison with caffeine or 5-FU treatment alone (Fig. 1c, d). We found that the combination index (CI) values of caffeine and 5-FU combined groups were less than 1, which were shown in the Fa-Combination Index Plot (Fig. 1c, d). These results demonstrated there was a synergistic interaction between caffeine (0, 0.5, 1 mM) and 5-FU (0, 25, 50 μM) in SMMC-7721 and Hep3B cells.

The combination of caffeine and 5-FU induces apoptosis of hepatoma cells

To explore the apoptosis induced by caffeine and 5-FU, HCC cells were treated with caffeine, 5-FU or combination of caffeine and 5-FU for 48 h, and the expression levels of apoptosis-related proteins were detected by Western blotting. We observed that in the combination groups of caffeine (1 mM) and 5-FU (25, 50 μM), the expression level of pro-apoptotic protein cleaved PARP was higher than other groups. Conversely, the expression levels of anti-apoptotic proteins Bcl-2 and Bcl-xL were decreased in the combination groups (Fig. 2). Since the expression levels of apoptotic proteins were similar in 1 mM caffeine combined with 25 μM 5-FU group and 1 mM caffeine combined with 50 μM 5-FU group, the combination of 1 mM caffeine and 25 μM 5-FU was used for the subsequent research.

Meanwhile, a similar phenomenon was observed in the TUNEL assay. The number of apoptotic cells was highest in the caffeine and 5-FU combination group comparing with caffeine or 5-FU alone group (Fig. 3a, b). In addition, results of Western blotting assay showed that comparing with the monotherapy group, the protein expression level of cleaved PARP was significantly increased, while the expression levels of Bcl-2 and Bcl-xL were decreased in combination treatment group (Fig. 3c). Taken together, caffeine synergistically promoted cellular apoptosis induced by 5-FU in HCC cells.

Co-treatment with caffeine and 5-FU increases ROS production in hepatoma cells

Then, in order to clarify whether the combined treatment of caffeine and 5-FU caused oxidative stress, the intracellular reactive oxygen species (ROS) levels were detected in HCC cells after treated with single agent or the combination. Flow cytometry analysis showed that the number of cells stained with fluorochrome DCFH-DA was obviously increased in the caffeine and 5-FU combination group, comparing with caffeine or 5-FU treatment group ($P < 0.05$, Fig. 4a, b). These results suggested that caffeine increased the antitumor effect of 5-FU by increasing the production of ROS.

The combination of caffeine and 5-FU inhibits the growth of HCC cells in vivo

To verify the effect of caffeine and 5-FU combination on HCC cells proliferation in vivo, a subcutaneous xenograft tumor model was established by transplanting SMMC-7721 cells into nude mice. Tumor-bearing mice were subsequently treated with DMSO, 5-FU, caffeine, or 5-FU and caffeine

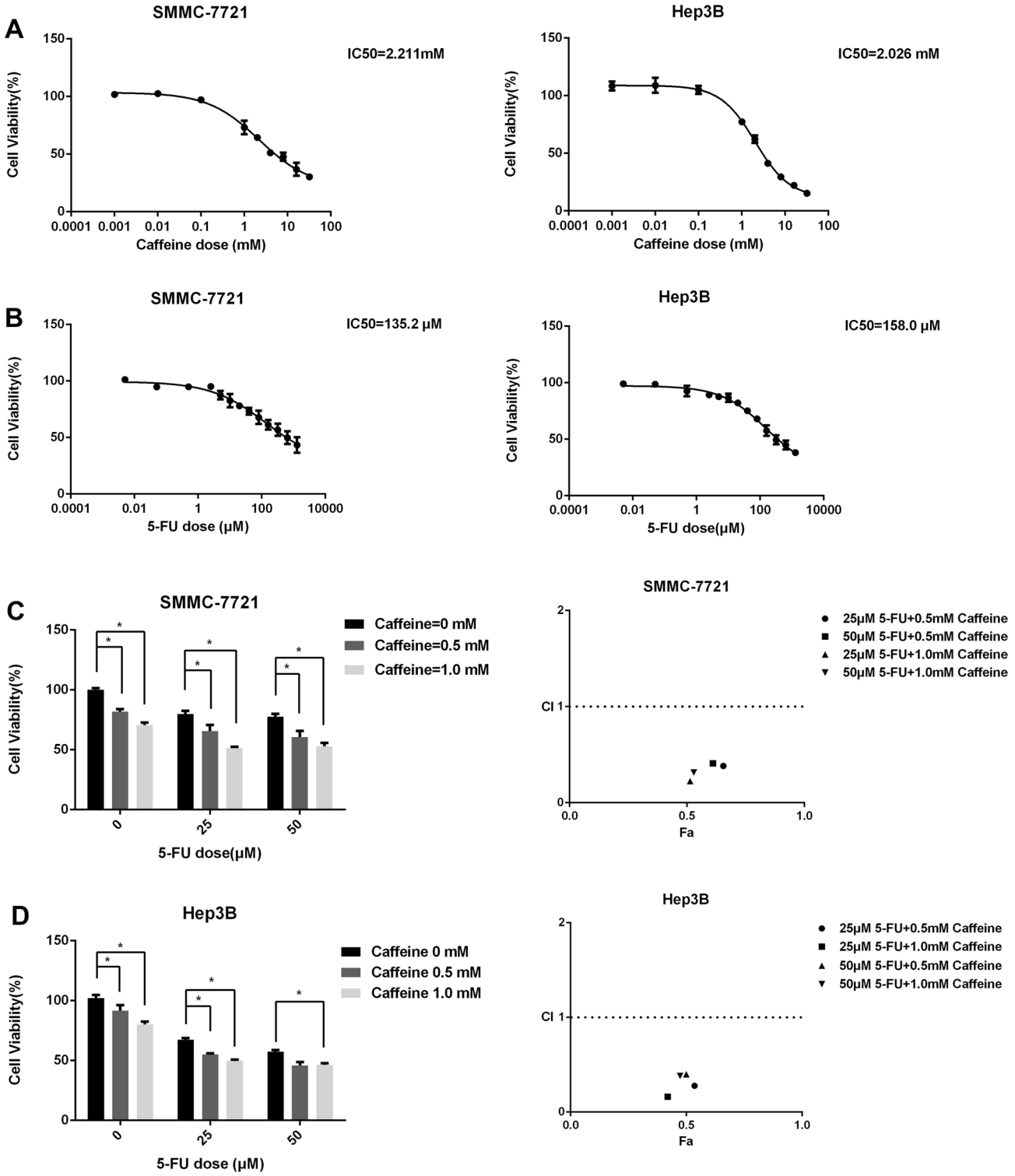

Fig. 1 Caffeine enhanced the anti-tumor effect of 5-FU on HCC cells in vitro. **a**, **b** SMMC-7721 and Hep3B cells were treated with different concentrations of caffeine (0, 0.001, 0.01, 0.1, 1, 2, 4, 8, 16, and 32 mM) and 5-FU (0, 0.005, 0.05, 0.5, 2.5, 5, 10, 20, 40, 80, 160, 320, 640, and 1280 μM) for 48 h, and cell viability was detected by MTS assay. (C and D) SMMC-7721 and Hep3B were co-treated with caffeine (0, 0.5, 1 mM) and 5-FU (0, 25, 50 μM), respectively. Cell viability was detected by MTS assay and the combination index was calculated by CompuSyn software. Points below the dotted line (CI values < 1) indicated synergy (*$P < 0.05$)

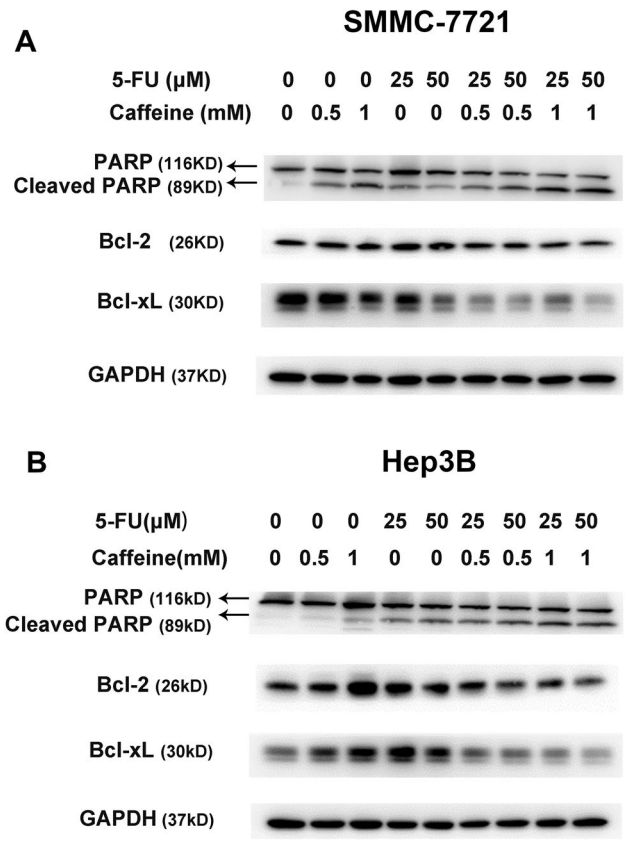

Fig. 2 Apoptosis was induced by caffeine and 5-FU in HCC cells. **a**, **b** SMMC-7721 and Hep3B cells were incubated with various concentrations of caffeine (0, 0.5, 1 mM) and 5-FU (0, 25, 50 μM) for 48 h. The expression of related apoptotic proteins was detected by Western blotting. GAPDH was used as a loading control

combination, respectively. The results revealed that the tumor sizes in the combined treatment group were markedly smaller than that of the other single treatment groups (Fig. 5a). The tumor weight and volume were significantly reduced in co-treated group as compared with other groups ($P < 0.01$, Fig. 5b). Moreover, we found that the expression level of cleaved PARP protein was up-regulated in the caffeine and 5-FU combination group, while the protein level of Bcl-2 and Bcl-xL was down-regulated in the combined group (Fig. 5c). The above results indicated that caffeine enhanced the chemosensitivity of 5-FU in vivo.

Discussion

In this study, the anti-tumor role of caffeine and 5-FU combination therapy on HCC was investigated in vitro and in vivo. Compared with caffeine or 5-FU monotherapy, caffeine together with 5-FU significantly inhibited the proliferation and induced the apoptosis of HCC cells. Our current data also demonstrated that ROS was involved in the anti-tumor

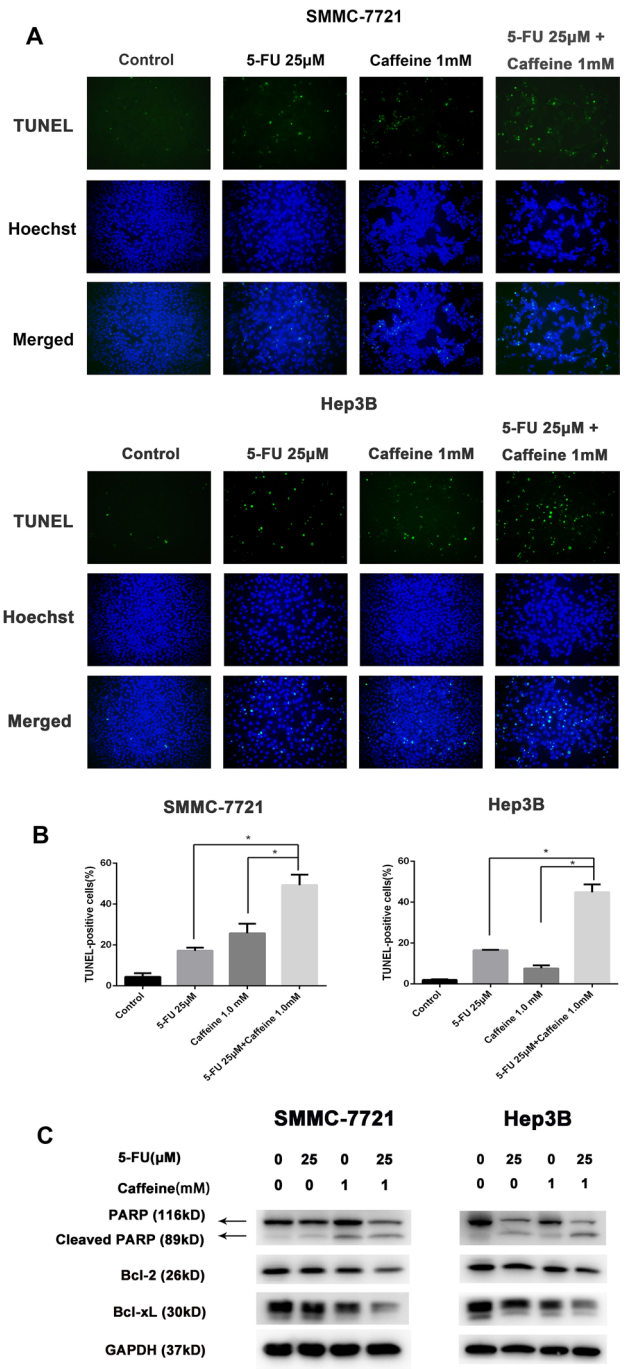

Fig. 3 Combination of caffeine and 5-FU induced apoptosis in HCC cells. **a** Apoptotic cells were examined by TUNEL assay in 1 mM caffeine and 25 μM 5-FU co-treated and single drug-treated groups (×200 magnification). **b** Quantitative analysis of TUNEL-positive HCC cells (green) was performed in each group. The value was presented as the mean ± SD of five random fields (*$P < 0.05$). **c** The expression of related apoptotic proteins in 1 mM caffeine and 25 μM 5-FU combined or single drug-treated groups was detected by Western blotting. GAPDH was used as a loading control

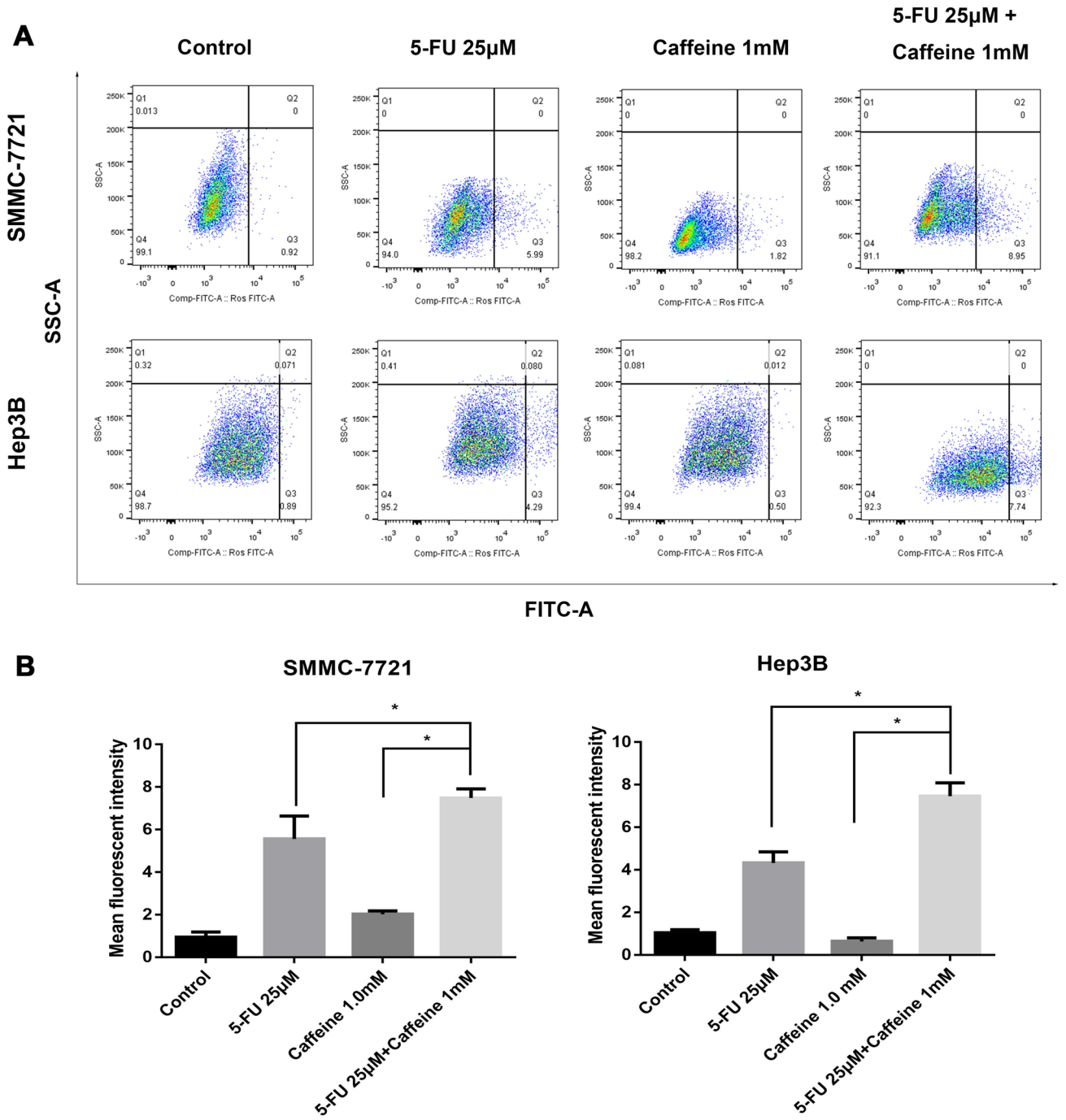

Fig. 4 The ROS levels were increased in caffeine combined with 5-FU treatment. **a** Fluorochrome DCFH-DA assay was used to measure the level of intracellular ROS. SMMC-7721 and Hep3B cells were treated with 1 mM caffeine, 25 μM 5-FU, or their combination for 48 h, respectively. **b** Each histogram showed the mean fluorescence intensity in the DCF (*$P < 0.05$)

effect of caffeine combined with 5-FU. These findings suggest the therapeutic value of caffeine and 5-FU combination therapy for patients with HCC.

5-FU is a fluoropyrimidine analog that exerts anti-tumor function by directly incorporating its metabolites into the DNA and RNA of cancer cells, which inhibits DNA synthesis and repair [22]. 5-FU alone or in combination with other chemotherapeutic agents is widely used for advanced HCC chemotherapy. It has been reported that transcatheter arterial chemoembolization with oxaliplatin, 5-fluorouracil, or Lipiodol chemoembolization is a safe and promising treatment for patients with HCC whose tumor diameter is larger

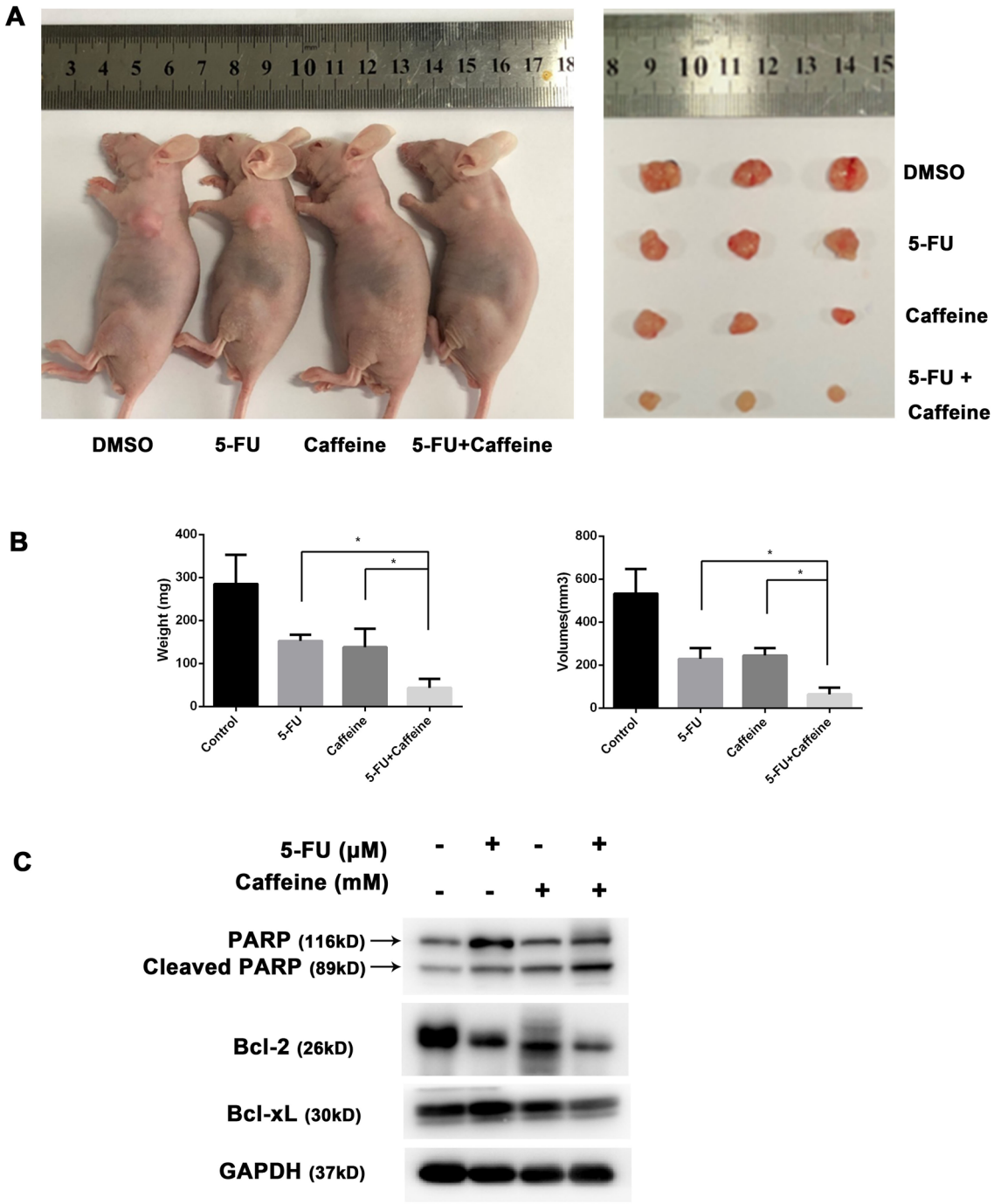

Fig. 5 The combination of caffeine and 5-FU significantly suppressed HCC tumor growth in vivo. **a** Approximately 1×10^7 SMMC-7721 cells were subcutaneously injected into the dorsal region of nude mice. Images of subcutaneous xenografts from mice receiving DMSO, caffeine, 5-FU, and combination treatments. **b** The weight and volume of xenograft tumors were shown as mean ± SD in DMSO, caffeine, 5-FU, and combination groups ($n = 3$, $^*P < 0.05$). (C) The expression of related apoptotic proteins was detected by Western blotting in each group. GAPDH was used as a loading control

than 10 cm [23]. Moreover, TAC-ECF treatment (transarterial infusion of epirubicin and cisplatin combined with systemic infusion of 5-fluorouracil) has higher overall survival rate and tumor response compared with the conventional TACE DOX in advanced HCC patients with portal vein tumor thrombosis [24]. However, the primary or acquired 5-FU resistance and the cytotoxicity of chemotherapeutic agents still prevent their clinical application in HCC therapy

[19, 25, 26]. Therefore, it is necessary to find agents with low cytotoxicity and use them in combination with 5-FU for patients with HCC. Our study demonstrated that caffeine combined with 5-FU enhanced the anti-tumor activity by inhibiting the proliferation and inducing the apoptosis of HCC cells. Meantime, we found no significant difference in anti-proliferative effects between 25 and 50 μM 5-FU combined with 1 mM caffeine, both of which had synergistic effects. This suggests that caffeine can sensitize HCC cells to low dose of 5-FU.

Recently, increasing evidence has suggested that coffee consumption can prevent HCC recurrence after orthotopic liver transplantation and reduce the risk of death associated with cirrhosis [9, 27]. Caffeine, a purine alkaloid and a major component of coffee, has anti-tumor effect and a protective role on HCC. For example, caffeine and its analog CGS 15943 block proliferation in HCC cell lines by inhibiting the PI3K/Akt pathway [11]. Caffeine attenuates lipid accumulation to prevent fatty liver from progressing to liver cancer [28]. In addition, caffeine has been shown to increase the apoptosis of HCC cells [29]. Similar to previous studies, our study showed that caffeine alone could suppress the growth of HCC cells and induce cellular apoptosis to some extent, whereas caffeine in combination with 5-FU has more significant anti-tumor activity than caffeine or 5-FU alone, which indicates that caffeine can increase the sensitivity of HCC cells to 5-fluorouracil.

Previous studies have verified that intracellular ROS plays a key role in 5-FU-mediated anti-cancer effects. And the combination of 5-FU and certain agents or techniques induces apoptosis through ROS-mediated mitochondrial pathways [30, 31]. Nevertheless, caffeine has been considered as an antioxidant agent, which has the ROS scavenging properties [32, 33]. To clarify whether the combination of caffeine and 5-FU induced apoptosis via increasing ROS generation, the staining assays with fluorochrome DCFH-DA was applied. The results showed that caffeine significantly enhanced the cytotoxic efficacy of 5-FU by the accumulation of ROS in HCC cells. This finding demonstrates that the combination of caffeine and 5-FU induces apoptosis via regulating the production of ROS.

In conclusion, our study demonstrates for the first time that combination therapy with caffeine and 5-FU inhibits proliferation and induces apoptosis of HCC cells in vitro and in vivo. ROS is involved in the regulation of anti-tumor activity of caffeine combined with 5-FU. These results suggest that caffeine combined with 5-FU may be a promising therapeutic strategy for patients with advanced HCC.

Author contributions ZW generated, analyzed, and interpretated the data and prepared the manuscript. CG, XW, YL, YW, XW, and XZ generated, analyzed, and interpretated the data. KW generated the idea, designed the study, analyzed and interpretated the data, and prepared the manuscript. HY generated the idea, designed the study, analyzed and interpretated the data, and edited the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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