

Icariin Synergizes with Arsenic Trioxide to Suppress Human Hepatocellular Carcinoma

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Abstract Hepatocellular carcinoma (HCC) is an aggressive malignancy with high chemoresistance to chemotherapeutics. Arsenic trioxide (ATO) is of therapeutic potential for the treatment of HCC; however, the therapeutic benefit of ATO is very limited due to narrow therapeutic window. Icariin is a natural compound which inhibits tumour cell growth and induces apoptotic cell death in a variety of cancer cells. This study was designed to determine whether Icariin can potentiate the antitumour activity of ATO in HCC treatment. Cell proliferation and apoptosis were measured using an MTT assay and flow cytometry respectively. Changes in reactive oxygen species (ROS) level and mitochondrial membrane potential were analysed by fluorescence signals. Protein expression was measured by western blotting and NF- κ B activity was determined by ELISA assay. In addition, the antitumour effect of combination treatment with Icariin and ATO on HCC was evaluated using a murine HCC cancer xenograft model. Icariin inhibited proliferation and induced apoptosis in both of the tested HCC cell lines in a dose-dependent fashion. Icariin enhanced the antitumour activity of ATO both

in vitro and in vivo. The antitumour activity of Icariin and its enhancement of the antitumour activity of ATO correlated with the generation of intracellular ROS and inhibition of NF- κ B activity. Our results showed that Icariin potentiated the antitumour activity of ATO in HCC. Therefore, we propose that the combination treatment with Icariin and ATO might facilitate the optimization of ATO therapy for patients with HCC.

Keywords Hepatocellular carcinoma · Icariin · Arsenic trioxide · ROS · NF- κ B · Synergistic effect

Introduction

Hepatocellular carcinoma (HCC) remains one of the leading causes of cancer deaths with more than 500,000 new cases diagnosed worldwide every year [1]. Although, surgical resection or liver transplantation offers a cure to patients when the malignancy is diagnosed at early stage, approximately 70 % of patients are inoperable due to coexisting advanced cirrhosis, multifocal disease, invasion and extrahepatic metastases [2]. Currently, systemic chemotherapy remains the main therapeutic option for majority of patients with unresectable HCC [3]. However, low response rate (0–20 %) and extremely poor prognosis (5-year survival rate less than 5 %) has been reported for single-agent chemotherapy because of the aggressive biological behaviour and resistance [4]. Therefore, combination therapies with two or more agents have attracted a lot of interest. Combination therapy with agents including doxorubicin, cisplatin, fluorouracil and interferon have been investigated, and clinical results have showed that these combination therapies were more effective in treating HCC [5].

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Arsenic trioxide (ATO), a traditional Chinese medicine, has been widely used in the treatment of acute promyelocyte leukaemia (APL) in China since 1970s [6]. Clinical data showed that 80–90 % patients with APL as well as 60–90 % of all-trans-retinoic acid (ATRA)-refractory patients reached complete remission after receiving ATO treatment [7–9]. In addition to APL, the antitumour activity of ATO has been reported in a variety of solid tumour cell lines including hepatocellular, gastric, oesophageal, prostate and colorectal cancers [10–14]. In vitro studies showed that ATO could induce apoptosis and genotoxicity in HCC cells by generating oxidative stress [15]. A recent study by Li and Cao [16] also reported that ATO could inhibit the migration and invasion in HCC SMMC-7721 cells probably by down-regulation of CD147 and MMP-2. Although, in vitro study demonstrated high potency of ATO in treating HCC, data from phase II trials indicated that 0.16–0.24 mg/kg per day was not active against advanced HCC as a single agent, and 7–8 mg/m² injection was required for management of primary hepatocarcinoma; both the dosages were much higher than the effective dose for treatment of haematological malignancies [17, 18]. Given the narrow therapeutic window of ATO [6], the required high dosage for HCC treatment was associated with the risk of severe adverse effects such as leukopenia, anaemia, fever and vomiting, which limited the application of ATO in the treatment of solid tumour [17]. Therefore, novel strategies of treatment which can potentiate the antitumour activity and alleviate toxicity are needed for employment of ATO on patients with HCC.

Icariin, the major active ingredient of traditional Chinese medicine E Herba, is a flavonoid with significant antiproliferative effect on human tumour cell growth in vitro including lung cancer, gastric cancer, leukaemia cells, breast cancer, HCC and gallbladder cancer [19–23]. It has also been reported that Icariin can induce apoptosis in human HCC cells via a ROS/JNK-dependent mitochondrial pathway [20]. A recent study by Chen's group showed that increasing intracellular ROS level could sensitize HCC cells to ATO treatment [24]. Therefore, it was considered important to verify our hypothesis that Icariin could potentiate the antitumour activity of ATO in treating HCC. Results presented in this study indeed showed that Icariin sensitized HCC cells to ATO, enhancing ATO-induced growth inhibition and apoptosis. Moreover, we also examined the anticancer activity of Icariin in combination with ATO in a murine HCC model, and found that Icariin treatment potentiated the cytotoxicity of ATO in vivo. This synergistic anticancer activity is associated with ROS generation and modulation of NF- κ B activity and its downstream genes.

Materials and Methods

Cell Culture and Drugs

The human HCC cell line SMMC-7721 and HepG2 were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Primary murine hepatocytes were prepared as previously described [25]. All cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) containing 10 % foetal bovine serum (Invitrogen). Cells were maintained in a humidified incubator at 37 °C and 5 % CO₂. ATO was purchased from Sigma (St. Louis, MO, USA) and Icariin was obtained from the National Institute for the control of pharmaceutical and biological products (Beijing, China).

Cell Growth Assay

Cell proliferation was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma, St. Louis, MO), as previously described [26]. Briefly, cells were plated at a density of 5×10^3 cells/well in 96-well culture plates. After treatment, 10 μ L of MTT solution [5 mg/ml in phosphate-buffered saline (PBS)] was added to each well and incubated for 4 h. MTT formazan was dissolved in 150 μ L of isopropanol, and the absorbance was measured at 570 nm with an ELISA reader (Sunrise, Tecan Group Ltd, Austria).

Cell Apoptosis Analysis

Cell apoptosis was determined using a FITC Annexin V apoptosis kit (BD Pharmingen, Franklin Lakes, NJ) according to the manufacturer's instructions. In brief, cells were washed with ice-cold PBS and resuspended in binding buffer (10 mmol/L HEPES, pH 7.4, 140 mmol/L NaCl, and 2.5 mmol/L CaCl₂) at a concentration of 1×10^6 cells/ml. Cells were stained with annexin V-FITC and propidium (PI) for 15 min in the dark before analysis by a flow cytometer (Beckman Coulter Inc, Miami, Florida, USA). Annexin V⁺/PI⁻ cells were considered as apoptotic cells.

Western Blot Analysis

After treatment, cells were washed with ice-cold PBS, harvested and lysed in 100 μ L of lysis buffer (Cell Signalling, Danvers, MA) containing protease inhibitors (Sigma). Extracted proteins were separated by SDS-PAGE, and then transferred electrophoretically onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA).

Proteins were probed with primary and secondary antibodies (Santa Cruz, CA, USA) as previously described [27]. The extraction of mitochondrial and cytoplasm fractions was performed with mitochondrial extraction kit (Beyotime Biotech., Jiangsu, China).

Detection of Intracellular ROS Detection

Intracellular ROS level was measured by oxidation-sensitive fluorescent dye 2,7-Dichlorodihydrofluorescein diacetate (DCF-DA) (Invitrogen, USA) method [28]. Briefly, after washing once with PBS, treated cells were incubated with 20 μ M DCF-DA in serum-free DMEM at 37 °C for 30 min before analysis by flow cytometry. Intracellular glutathione (GSH) measurement Au: provide details of the methodology.

Measurement of Mitochondrial Membrane Potential

Mitochondrial membrane potential was measured using 3,3'-dihexyloxycarbocyanine iodide [DiOC6(3)] (Invitrogen) by flow cytometry. After treatment, cells were washed once with ice-cold PBS and then incubated with 50 nm DiOC6(3) in serum-free DMEM for 30 min before flow cytometry analysis.

NF- κ B p65 Activity Analysis

The nuclear extract was prepared from cells using a nuclear extract kit (Active Motif, Carlsbad, CA), and the activity of NF- κ B p65 was examined using an ELISA kit (Active Motif, Carlsbad, CA). Results were expressed as relative change to control.

Tumour Implantation and Evaluation of In Vivo Antitumour Effects in Murine Model

The animal experiments were performed in accordance with CAPN (China Animal Protection Law), and the protocols were approved by the Animal Care and Use Committee of the Jining No. 1 People's Hospital. Female BALB/c (nu/nu) mice, 6-weeks-old, purchased from the Animal Centre of China (Beijing, China), were housed with a light/dark cycle of 12/12 h and allowed free access to rodent chow and water. HepG2 cells were harvested from subconfluent cultures and washed in serum-free medium before resuspending in PBS. The mice were anesthetized using ethyl ether and tumour was established by subcutaneously injection of 10^6 HepG2 cells into the flank of mice. Tumour volume was measured along the longest orthogonal axes and calculated as volume = (length \times width²)/2, where width was the shortest measurement. Tumour appearance was inspected twice per

week. Once tumour masses became established (tumour volume about 100 mm³) and palpable, animals were randomized into four groups ($n = 15$ per group) to receive daily intraperitoneal injections as follows: (A) vehicle (0.9 % sodium chloride + 1 % DMSO), (B) Icaritin (40 mg/kg, dissolved in vehicle) alone [23], (C) ATO (2.5 mg/kg, dissolved in vehicle) [29] alone, or (D) Icaritin and ATO in combination for 20 days. Tumour volumes and body weight were measured every 4 days. All mice were sacrificed 3 days after the last injection, blood samples were collected via cardiac puncture and tumours were excised.

Statistical Analysis

All data are expressed as the mean \pm SEM and represent the results of three separate experiments performed in quadruplicate unless otherwise stated. Student's *t* test was used to evaluate the difference between two groups, and a Oneway ANOVA followed by LSD (least significant difference) was used for comparison among three or more groups. $P < 0.05$ was considered to be statistically significant.

Results

Icaritin Inhibits Cell Growth and Induces Apoptosis in HCC Cells

To examine the ability of Icaritin to inhibit the growth of HCC cells, the viability of the cells was measured using the MTT assay after the cells had been treated with increasing concentrations of Icaritin for 24 h. As shown in Fig. 1a, Icaritin inhibited HCC cell growth in a dose-dependent manner. Compared to the control, SMMC7721 cell viability was reduced up to 21.4 ± 4.2 after 40 μ M Icaritin treatment for 24 h. HepG2 cells were less sensitive to Icaritin than SMMC7721 cells, showing a reduced viability of 33.6 ± 4.0 with 40 μ M Icaritin treatment for 24 h. The ability of Icaritin to induce apoptosis in SMMC7721 and HepG2 cells was also analysed. As shown in Fig. 1b, Icaritin significantly triggered apoptosis in both the cell lines, indicating that the loss of cell viability was in part due to Icaritin-induced apoptosis. In contrast, even at the maximum dose tested, Icaritin did not significantly affect the viability of normal mouse hepatocytes.

Icaritin Synergizes with ATO to Reduce Cell Viability of HCC Cells

To explore whether Icaritin could enhance the chemosensitivity of HCC cells to ATO, we examined the effect of

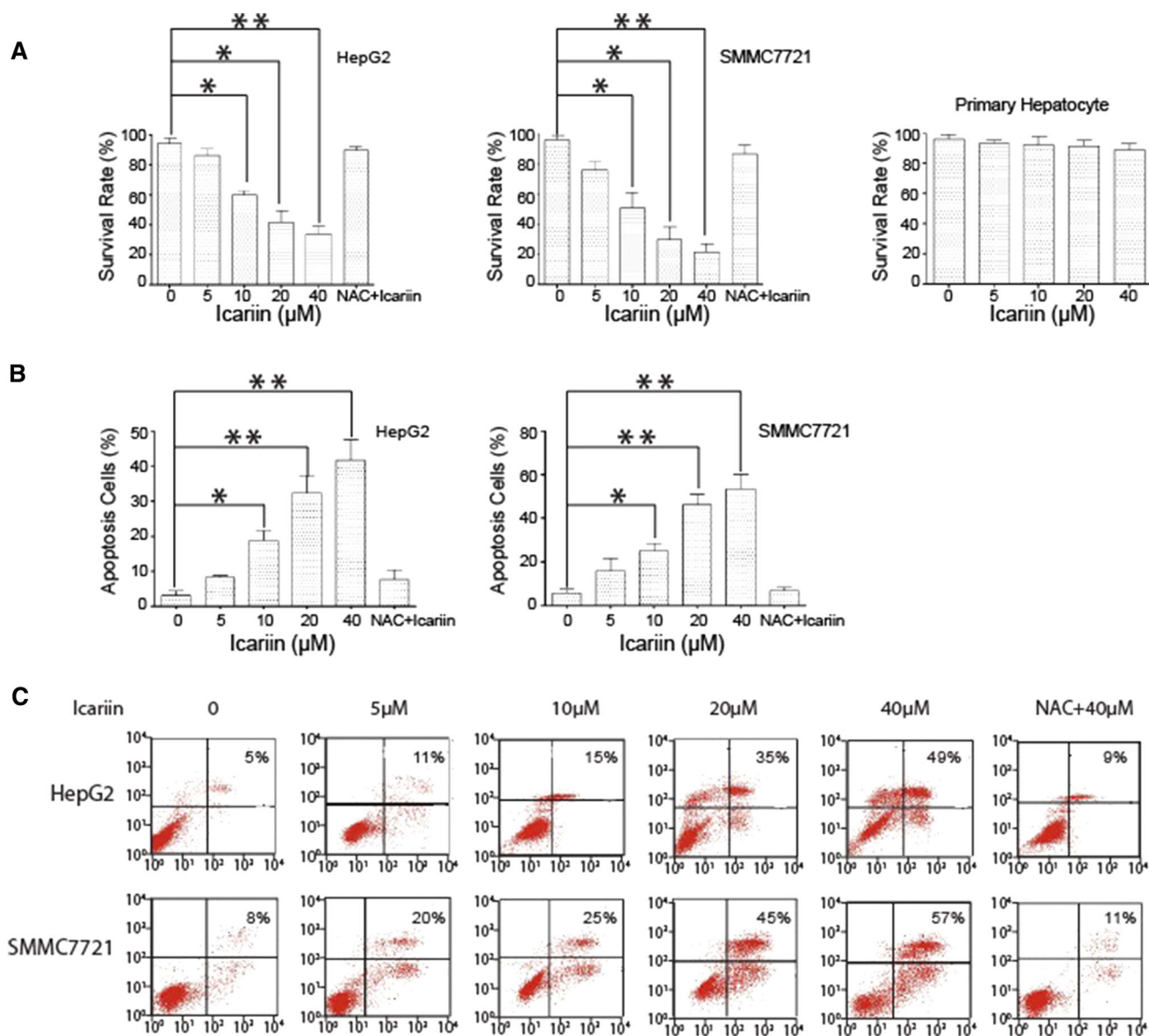


Fig. 1 Effect of Icaritin on cell growth inhibition and apoptosis. **a** HepG2 cells, SMMC7721 cells and normal mouse hepatocytes were treated with indicated concentration of Icaritin for 24 h and cell viability was evaluated by MTT assay. **b** HepG2 and SMMC7721

cells were treated with indicated concentration of Icaritin for 24 h and cell viability was evaluated via flow cytometry. **c** Representative histograms were shown for cytometrically analysed cells

treatment with ATO alone or in combination with Icaritin on the growth of SMMC7721 and HepG2 cells. Since Icaritin at 10 μM did not affect the viability of normal mouse hepatocytes while significantly inhibiting cell growth in both the tested cell lines, we used 10 μM Icaritin in the combinational treatment with ATO. As shown in Fig. 2a, treatment with 1 and 2 μM ATO caused 20 and 29 % loss of viable SMMC cells respectively. Combination treatment with ATO and Icaritin dramatically reduced the viable SMMC7721 cells to 22 % (Icaritin + 1 μM ATO) and 11 % (Icaritin + 2 μM ATO). A similar pattern was found in HepG2 cells when treated with ATO, Icaritin or

both. To determine whether the effects of ATO and Icaritin were additive or synergistic, we calculated the combination index value according to Chou's method [30]. A CI value less than, equal to or >1 indicates that the agents are synergistic, additive or antagonistic, and <0.7 indicates a significantly synergistic effect [31]. The CI for HepG2 cells treated with 10 μM Icaritin with 2 and 4 μM ATO was 0.658 and 0.587 respectively. Similarly, the CI for SMMC7721 cells treated with 10 μM Icaritin with 1 and 2 μM ATO was 0.713 and 0.624 respectively. The results indicated that Icaritin and ATO had significant synergistic effects in inhibiting the viability of both HCC cells.

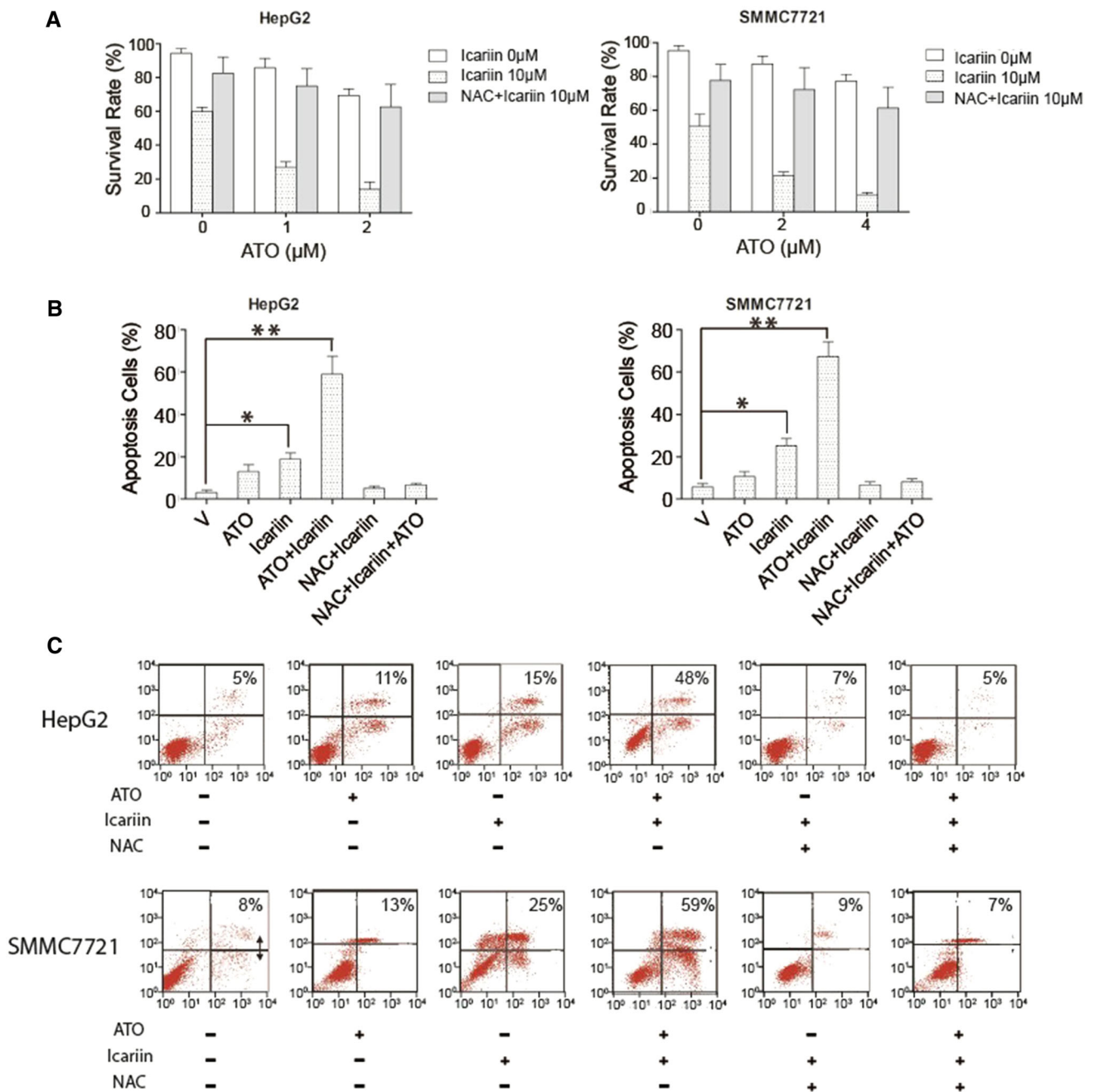


Fig. 2 Enhanced antiproliferative and pro-apoptotic effect of ATO by Icaritin on cell growth inhibition and apoptosis. **a** HepG2 and SMMC7721 cells were treated with ATO (2 μM), Icaritin (10 μM), ATO + Icaritin, NAC (5 mM) + Icaritin and NAC + ATO + Icaritin for 24 h, and cell viability was evaluated by MTT assay. **b** HepG2

and SMMC7721 cells were treated with same agents as in MTT assay for 24 h and cell apoptosis was evaluated by flow cytometry. **c** Representative histograms were shown for cytometrically analysed cells

Icaritin Sensitized SMMC7721 and HepG2 Cells to Apoptosis Induced by ATO

SMMC7721 and HepG2 cells were incubated with Icaritin, ATO or ATO + Icaritin for 12 h and scored for apoptotic cell death. As shown in Fig. 2a, 1 μM ATO slightly increased the apoptosis rate of HepG2 cells compared to control, but the

difference was not statistically significant; whereas the combination of 2 μM ATO and 10 μM significantly increased the apoptosis rate to 63.7 %. While incubation of SMMC7721 cells with 4 μM ATO resulted in approximately twofold increase in the apoptosis rate, incubation with ATO + Icaritin led to a 12-fold increase in the apoptosis rate compared to control, and a 6.2-fold increase compared to

ATO alone. Representative histograms for the above cytometrically analysed cells are shown in Fig. 2b.

Icariin Elevated Intracellular ROS and Diminished Mitochondrial Membrane Potential

The deregulation of cellular redox status has been suggested as one of the potent mechanism for cell death. As shown in Fig. 3a, Icariin treatment significantly promoted ROS generation in SMMC7721 cells ($P < 0.05$) while ATO treatment induced little increase in intracellular ROS level. The combination of ATO and Icariin elicited a highly significant increase in ROS generation, compared to cells treated with vehicle or ATO alone. ROS generation induced by combination therapy was also greater than Icariin alone, but was not significantly higher than Icariin treatment alone. An ROS inhibitor, NAC, was used to confirm the role of ROS in the pro-apoptotic effect of Icariin. As shown in Fig. 3a, pretreatment with NAC

dramatically reduced ROS level in cells treated with Icariin or Icariin plus ATO. Furthermore, NAC also reduced the antiproliferative and pro-apoptotic effect of Icariin and combination therapy (Fig. 1), suggesting that ROS generation was at least partly responsible for the synergistic effect of these two agents. ROS generation is accompanied by rapid depletion and disruption of mitochondrial membrane potential. Therefore, the change in mitochondrial membrane potential was also measured following treatment with ATO, Icariin or co-treatment with these two agents. As shown in Fig. 3b, though ATO also cause little change in mitochondrial membrane potential, Icariin and combination treatment significantly diminished the mitochondrial membrane potential. Our results also show that pretreatment with NAC inhibited the disruption of mitochondrial membrane potential following Icariin or combination treatment. Together, these results suggested ROS generation activated oxidative stress signalling pathways and triggered mitochondrial apoptotic pathways.

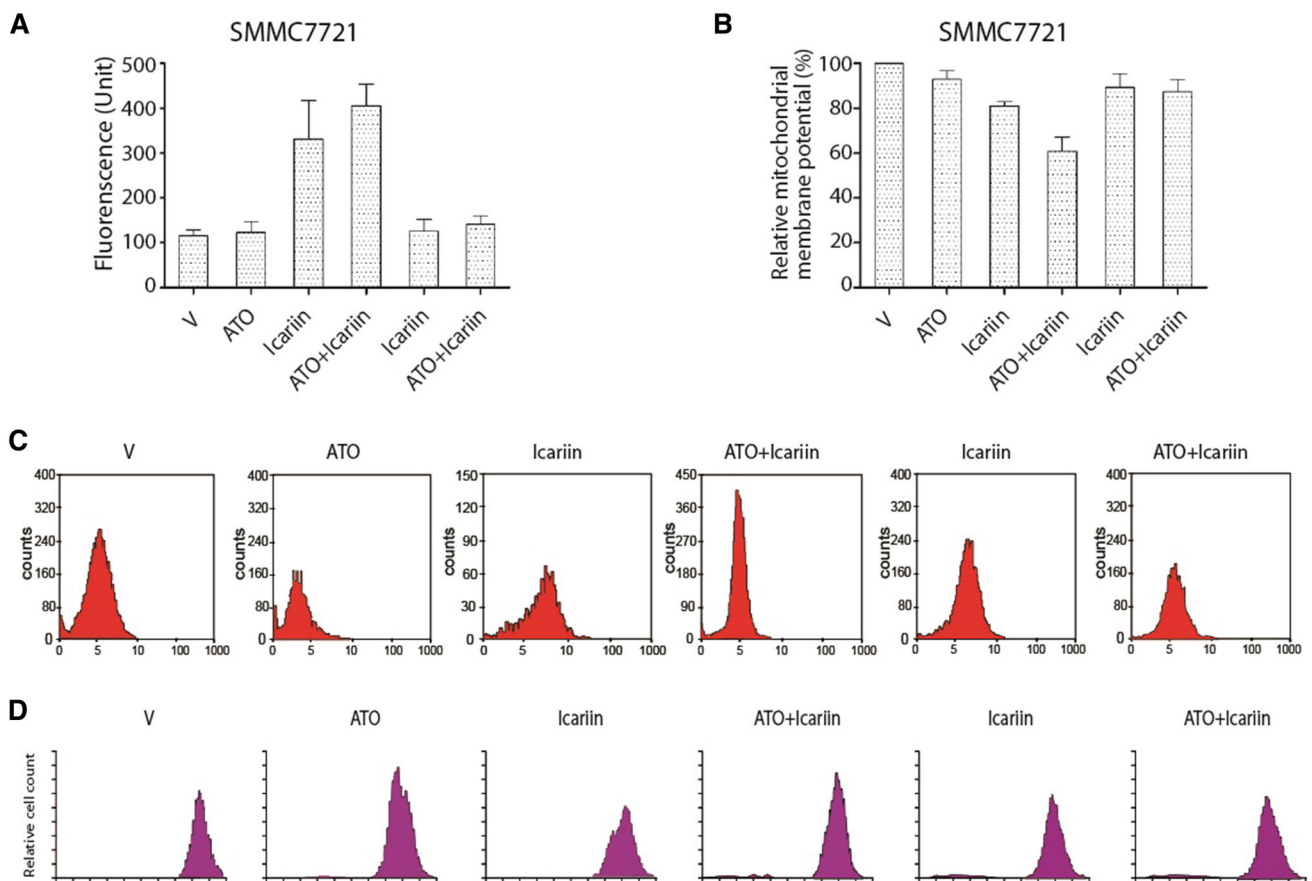


Fig. 3 Increase in intracellular ROS and changes in mitochondrial membrane potential in vitro. **a** HepG2 and SMMC7721 cells were treated with ATO (2 μ M), Icariin (10 μ M), ATO + Icariin, NAC (5 mM) + Icariin and NAC + ATO + Icariin for 24 h, and intracellular ROS level was evaluated by measuring fluorescent signals. **b** HepG2 and SMMC7721 cells were treated with same agents as in

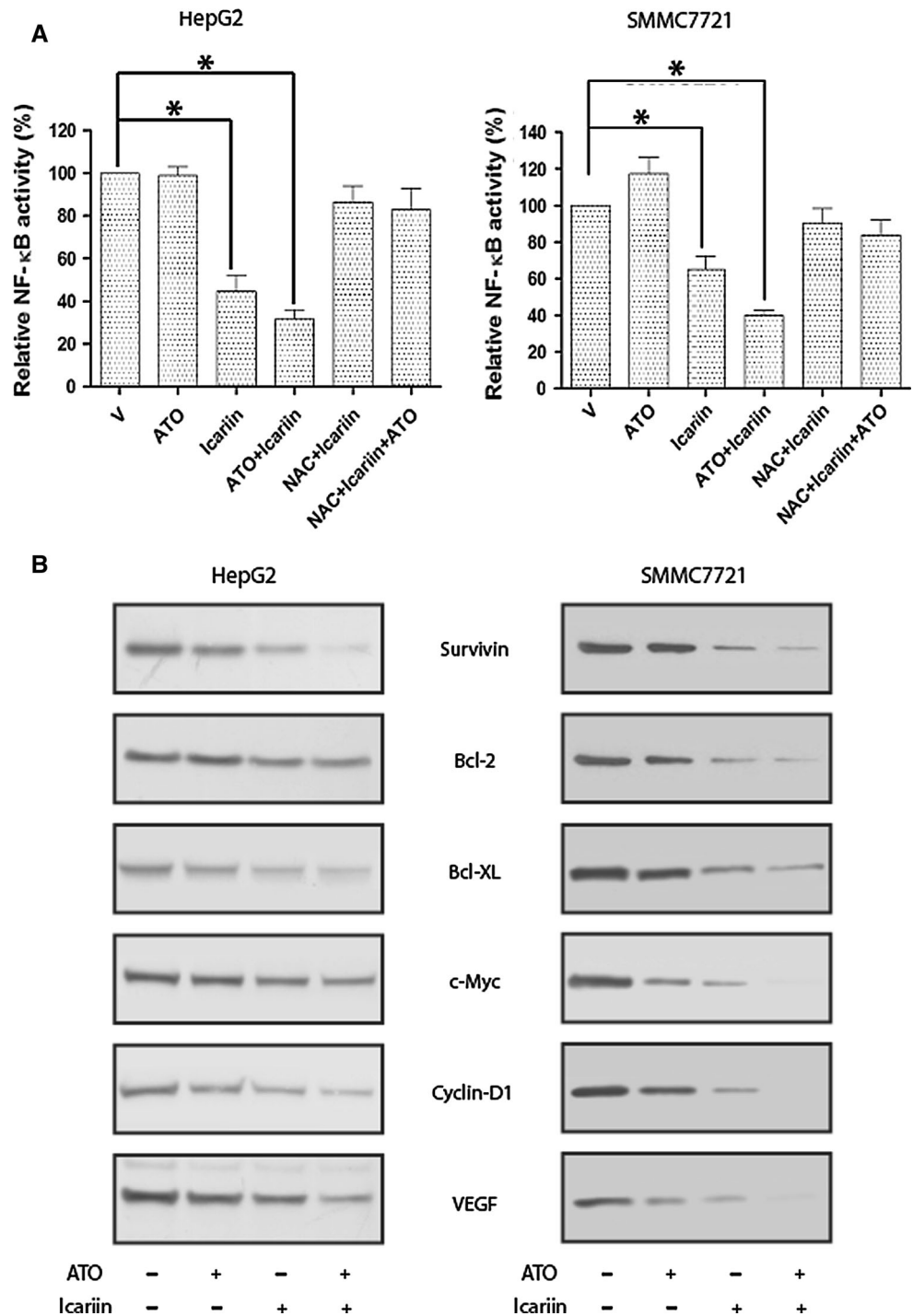
ROS assay for 24 h and changes in mitochondrial membrane potential was evaluated by flow cytometry. **c** Representative histograms were shown for cytometrically analysed cells for intracellular ROS level. **d** Representative histograms were shown for cytometrically analysed cells for changes in mitochondrial membrane potential

Icariin Synergizes with ATO to Inhibit NF-κB Activity and Regulate Its Downstream Genes

NF-κB activity in SMMC7721 cells were analysed to investigate the effect of combination strategy on this signalling pathways. Our results demonstrate that the combined therapy with ATO and Icariin significantly inhibited the activity of

NF-κB compared to control group (Fig. 4a). In addition, NAC could partly abrogate the inhibitory effect of Icariin or combination treatment on NF-κB activity, suggesting that there might be a crosstalk between ROS and NF-κB signalling pathway [32]. Furthermore, we tested whether Icariin co-treatment with ATO significantly decreased Bcl-2, Bcl-xL, c-myc, Cyclin-D1, survivin and VEGF expression.

Fig. 4 Changes in NF-κB activity and expression of NF-κB-target genes. **a** HepG2 and SMMC7721 cells were treated with ATO (2 μM), Icariin (10 μM), ATO + Icariin, NAC (5 mM) + Icariin and NAC + ATO + Icariin for 24 h, and NF-κB activity was assessed using ELISA kit. **b** HepG2 and SMMC7721 cells were treated with same agents as in NF-κB activity assay for 24 h, and gene expression was detected by western blotting. Representative histograms were shown for protein expression level



Synergistic Antitumour Activity of ATO and Icariin in Xenograft Murine Model

To evaluate in vivo antitumour activity of the combination therapy, the tumour size in the murine model was measured. As shown in Fig. 5, the tumour size of mice in ATO group (treated with 2.5 mg/kg ATO) reached $1860.5 \pm 302.3 \text{ mm}^3$ after treatment, which indicated that ATO at this dose was not sufficient to significantly inhibit the tumour growth ($P < 0.05$ vs Control). In contrast, Icariin alone significantly reduced tumour volume compared to control group ($P < 0.05$ vs Control). Mice treated with both the agents showed a smaller tumour size than those treated with ATO or Icariin alone, reaching $710.6 \pm 149.5 \text{ mm}^3$ at the end of the study. The CI value was 0.498, suggesting that Icariin and ATO synergistically suppressed the growth of tumour established by SMMC7721 cells. Changes in body weight, WBC count, liver function (serum AST and ALT level) and renal function (serum BUN and Cr level) were examined to assess the cytotoxicity of treatment. Results showed that ATO at dose 2.5 mg/kg, Icariin at 40 mg/kg or combined treatment did not cause significant changes in these parameters.

Discussion

Owing to the late diagnosis and aggressive behaviour, HCC remains one of the fatal diseases, with a life expectancy of about 6 months from the time of diagnosis. Because HCC is relatively chemoresistant, synthetic antineoplastic compounds showed very limited efficacy in clinical studies. In this respect, a number of novel therapeutic strategies with natural compounds have been investigated to improve the prognosis for patients with unresectable HCC.

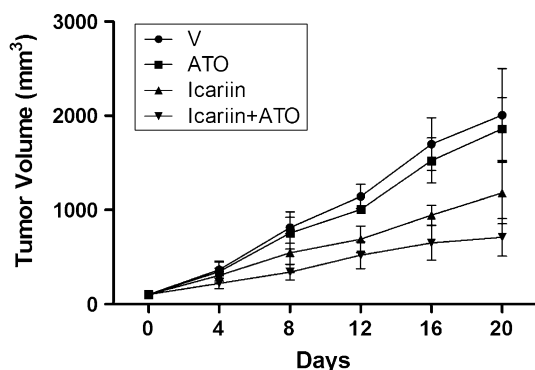


Fig. 5 Evaluation of antitumour activity of ATO, Icariin and the two-agent combination in murine HCC model. HepG2 tumour bearing mice received daily injection of 200 μL 0.9 % NaCl, equal volume of ATO at dose of 2.5 mg/kg, Icariin at dose of 40 mg/kg or the combination of the two drugs for 20 days. The tumour volumes were measured every 4 days

Furthermore, more and more researchers paid much attention to combination treatment of natural compounds because they might overcome drug resistance and achieve high efficacy without causing serious side effects. The present study has demonstrated that Icariin can potentiate the effect of ATO against human HCC cells. Icariin synergizes with ATO to inhibit tumour cell growth and induce apoptosis of HCC cells. Icariin in combination with ATO also showed synergistic antitumour efficacy in vivo in xenograft mice model. Moreover, this study demonstrated that the synergistic antitumour effect resulted from ROS-mediated mitochondrial dysfunction and modulation of apoptotic and survival signalling pathways.

Cancer cells seem to have unregulated antioxidant capacity in adaption to the relatively higher endogenous oxidative stress than normal cells, which help to keep a balanced intracellular redox status. When excess ROS production and/or antioxidant depletion occurs, this critical balance is disrupted [33]. Mounting evidence also suggested that many chemotherapeutic agents may be selectively toxic to tumour cells because of their ability to increase oxidant stress and enhance these already stressed cells beyond their limit [34–36]. Previous studies have showed that both Icariin and ATO could induce apoptotic cell death via ROS-mediated pathway. In this study, our results confirmed that treatment with Icariin could lead to ROS generation in HCC cells. Although, ATO treatment at low dose induced no detectable level of ROS generation, the elevated ROS levels by Icariin potentiate the cytotoxicity of ATO. The role of ROS in this synergistic effect was confirmed by the observations that antioxidant (NAC) could diminish the enhanced antiproliferative and pro-apoptotic effect of combinational treatment. That elevated ROS levels by Icariin interrupted the balanced intracellular redox status in HCC cells, and sensitize cells to ATO explains the synergistic effect.

NF- κB is a major stress-inducible antiapoptotic transcription factor which resides in the cytoplasm and respond to various inflammatory stimuli, environmental pollutants, prooxidants, carcinogens, stress and growth factors [37, 38]. Previous studies also showed that NF- κB was one of the major culprits involved in the development of drug resistance in HCC [39]. In addition, a number of researches have reported that inhibiting NF- κB could augment the efficacy of ATO treatment in colon cancer cells, leukaemic cells and HCC cells [29, 40–44]. In agreement with these studies, we found that the sensitization of HCC cells to ATO by Icariin correlated with suppressed NF- κB activity. Moreover, a couple of recent researches demonstrated the ability of agents to exert antimetastatic effect on hepatocellular carcinoma through inhibiting NF- κB activity [45, 46]. Therefore, by suppressing NF- κB activity, Icariin might also inhibit migration and invasion capacities of

HCC cells, in addition to inducing apoptosis and suppressing cell growth.

A number of proteins involved in cell survival, apoptosis and metastasis, including cyclin D1, Bcl-2, Bcl-xL, COX-2, survivin and VEGF, are associated with chemoresistance; all of which are regulated by NF- κ B [37, 47–49]. To determine whether the potentiating effect of Icariin on ATO is associated with down-regulating the expression of downstream genes of NF- κ B, we examined the effect of Icariin alone or in combination with ATO on the expression of these genes. We found that Icariin down regulated the constitutive expression of cyclin D1, Bcl-2, Bcl-xL, COX-2, survivin and VEGF in both HepG2 and SMCC7721 cells, and combination treatment showed a greater suppressive effect. Taken together, our results indicated that Icariin in vitro potentiates the inhibitory effect of ATO on NF- κ B DNA-binding activity and its downstream gene products, which is at least partly responsible for the enhanced therapeutic efficacy by the combined treatment.

To investigate whether the enhanced antiproliferative effect and induction of apoptosis could be recapitulated in vivo, we treated a hepatocellular carcinoma murine model with Icariin, ATO or a combination of Icariin and ATO. Combination treatment resulted in a significant inhibition of tumour growth compared to treatment with either agent alone. More importantly, no significant change in body weight, renal function, liver function and WBC count was found at dose of ATO we used in the in vivo study, so the combination of ATO with Icariin could achieve a greater therapeutic effect without causing systemic toxicity.

In conclusion, our results demonstrated for the first time that Icariin could potentiate the antitumour activity of ATO on human HCC cells both in vitro and in vivo. The underlying mechanisms may be, at least in part, due to Icariin-induced ROS generation and suppression of NF- κ B with NF- κ B-regulated gene products. Given the low toxicity of Icariin to normal tissue and narrow therapeutic window of ATO, Icariin may help broaden the application of ATO in treatment of HCC. However, further research is warranted to verify the therapeutic efficacy in HCC treatment.

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