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ORIGINAL RESEARCH

Discovery of a novel anticancer agent with both anti-topoisomerase I and II activities in hepatocellular carcinoma SK-Hep-1 cells in vitro and in vivo: *Cinnamomum verum* component 2-methoxycinnamaldehyde

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Abstract: *Cinnamomum verum* is used to make the spice cinnamon and has been used as a traditional Chinese herbal medicine for various applications. We evaluated the anticancer effect of 2-methoxycinnamaldehyde (2-MCA), a constituent of the bark of the plant, and its underlying molecular biomarkers associated with carcinogenesis in human hepatocellular carcinoma SK-Hep-1 cell line. The results show that 2-MCA suppressed proliferation and induced apoptosis as indicated by mitochondrial membrane potential loss, activation of caspase-3 and caspase-9, increase in the DNA content in sub-G1, and morphological characteristics of apoptosis, including blebbing of plasma membrane, nuclear condensation, fragmentation, apoptotic body formation, and long comet tail. In addition, 2-MCA also induced lysosomal vacuolation with increased volume of acidic compartments, suppressions of nuclear transcription factors NF-κB, cyclooxygenase-2, prostaglandin E_2 (PGE_2), and both topoisomerase I and II activities in a dose-dependent manner. Further study reveals the growth-inhibitory effect of 2-MCA was also evident in a nude mice model. Taken together, the data suggest that the growth-inhibitory effect of 2-MCA against SK-Hep-1 cells is accompanied by downregulations of NF-κB-binding activity, inflammatory responses involving cyclooxygenase-2 and PGE_2, and proliferative control involving apoptosis, both topoisomerase I and II activities, together with an upregulation of lysosomal vacuolation and volume of acidic compartments. Similar effects (including all of the above-mentioned effects) were found in other tested cell lines, including human hepatocellular carcinoma Hep 3B, lung adenocarcinoma A549, squamous cell carcinoma NCI-H520, colorectal adenocarcinoma COLO 205, and T-lymphoblastic MOLT-3 (results not shown). Our data suggest that 2-MCA could be a potential agent for anticancer therapy.

Keywords: 2-methoxycinnamaldehyde, anticancer, SK-Hep-1 cells, topoisomerase I, topoisomerase II

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies.[1] However, HCC is not sensitive to traditional chemotherapeutic agents, and there is a need for better treatment of the disease.

The genus *Cinnamomum* belongs to the Lauraceae family and comprises over 250 aromatic evergreen trees distributed mostly in Asia. *Cinnamomum verum* is a small evergreen tree in the genus and native to Sri Lanka. The bark of this plant is used to make the spice cinnamon and has long been used as a traditional Chinese herbal

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medicine for various conditions, such as improvement of the complexion, making it more youthful, alleviation of fever, inflammation, cough, induction of perspiration, and circulatory disorders.[2,3] In our ongoing study to identify anticancer agents from natural resources, 2-methoxycinnamaldehyde (2-MCA), a constituent of the bark of the plant, was discovered to have growth-inhibitory effect in human HCC SK-Hep-1 cells, both in vitro and in vivo.

Cancer is a hyperproliferative disorder. Numerous genetic and epigenetic changes are needed to drive normal cells toward neoplastic transformation. These alterations control various signaling pathways that cooperate to endow cancer cells with a wide range of biological capabilities necessary for growing, disseminating, and finally killing their host.[4] Although anticancer drugs may act differently, apoptosis is the most common and preferred mechanism through which many anticancer agents kill and eradicate cancer cells.[5]

Topoisomerases are enzymes that regulate the topological states of DNA and play an important role in maintaining genomic integrity.[6] These enzymes relax supercoiled DNA by transient protein-linked cleavages of either one (topoisomerase I) or both (topoisomerase II) of the sugar–phosphate backbones of double-stranded DNA strands.[7] In addition to apoptosis, topoisomerase is another major target of anticancer agents.[8–11] The transcription factor nuclear factor κB (NF-κB) plays an important role in the regulation of cell survival and is activated in many malignant tumors. In addition, the inhibition of NF-κB shifts the balance of death/survival toward apoptosis.[12]

NF-κB pathway is involved in the expression of cyclooxygenase-2 (COX-2).[13] Dysregulated expression of COX-2 and prostaglandin cascade plays an important role in carcinogenesis. Expression of constitutive COX-2-catalyzed prostaglandin is induced by most cancer-causing agents, and COX-2 expression is a characteristic feature of all premalignant neoplasms. In addition, COX-2 expression intensifies with stage at detection, cancer progression, and metastasis. Furthermore, various essential features of carcinogenesis are linked to COX-2-driven prostaglandin E_2 (PGE_2) biosynthesis, and COX-2 inhibitors reduce the risk of human cancer and precancerous lesions.[14]

This diversity of mechanisms of carcinogenesis suggests that there are probably multiple processes that could be effective targets for the prevention of cancer. In an attempt to understand the effects and underlying mechanisms of 2-MCA in SK-Hep-1 cells, we performed a series of experiments to answer the following questions: 1) what is the effect of 2-MCA on the growth in SK-Hep-1 cells? 2) What are the effects of 2-MCA on topoisomerase I and II activities? 3) How these activities are affected? Our results indicate that 2-MCA inhibited the growth in SK-Hep-1 cells, with decreased NF-κB DNA-binding activity and decreased COX-2 and PGE_2 expressions. In addition, 2-MCA inhibited both topoisomerase I and II activities and induced lysosomal vacuolation and increased the volume of acidic compartments (VACs). Finally, 2-MCA induced apoptosis, resulting in the suppression of cell growth, both in in vitro and in vivo.

Materials and methods

Materials

Minimum essential medium and fetal calf serum were purchased from Thermo Fisher Scientific (Waltham, MA, USA). 2-MCA, dimethyl sulfoxide (DMSO), propidium iodide (PI), and RNase were purchased from Sigma-Aldrich (St Louis, MO, USA).

Cell culture

Human HCC cell line, SK-Hep-1 cells (ATCC HTB-52; American Type Culture Collection, Manassas, VA, USA) were cultured in minimum essential medium, supplemented with 1.0 mM sodium pyruvate, 10% (v/v) fetal bovine serum, 10 U/mL penicillin, 10 µg/mL streptomycin, and 0.25 µg/mL amphotericin B at 37°C with 5% CO_2. The I-Shou University Institutional Review Board has reviewed and approved the exemption of the protocol regarding human cell lines as this research does not meet the definition of human subject research.

XTT assay for cell viability

Cells were seeded in 96-well culture plates (1×10^4 cells/well). After incubated for 24 hours, the cells were treated with different concentrations of 2-MCA for 24 hours, 48 hours, and 72 hours. The cell viability was determined by Cell Proliferation Kit II (XTT) (Hoffman-La Roche Ltd., Basel, Switzerland) following the manufacturer's protocol. The absorbance was measured using Tecan infinite M200 spectrophotometer (Tecan, Männedorf, Switzerland) at 492 nm with a reference wavelength of 650 nm.

Nuclear fragmentation assay

Acridine orange (AO) is a nucleic acid-selective metachromatic dye useful for cell cycle determination. When AO intercalates into dsDNA, the dye fluoresces green. On the contrary, it fluoresces red when interacts with ssDNA or RNA. Apoptotic cells (with a larger fraction of DNA in the denaturated form) display a red fluorescence and a reduced green emission when compared to nonapoptotic interphase cells. In addition, when AO enter acidic compartments, such

as lysosomes, the dye become protonated and sequestered. In these low pH conditions, the dye will emit orange light when excited by blue light.[15] Nuclear fragmentation assay is based on the characteristics of AO and observed under a fluorescent microscope. Briefly, the cells were treated with different concentrations of 2-MCA for 24 hours and stained with 5 μg/mL AO at room temperature. Then the cells were observed under fluorescent microscope.[16]

Comet assay

DNA strand breaks were evaluated using single cell gel electrophoresis (comet) assay following the procedure of Olive and Banath.[17]

Assay for volume of acidic compartment

VAC assay for cell lysosomal vacuolation was carried out as described previously.[16]

Flow cytometric analysis

To determine the effect of 2-MCA on cell cycle distribution, 5×10^5 cells were plated in 60 mm dishes and treated with different concentrations of 2-MCA for 24 hours. Then, the cells were harvested by trypsinization, washed with phosphate-buffered saline, and then fixed in chilled 70% ethanol for 2 hours on ice. The cells were then centrifuged to remove the fixative, washed and suspended in phosphate-buffered saline containing 1 mg/mL RNase and 50 μg/mL PI, incubated in the dark at room temperature for 30 minutes, and analyzed by CyFlow SL Flow Cytometer (Cytecs GmbH, Gorlitz, Germany). A total of 10,000 cells were counted for each determination. The data were analyzed using MultiCycle AV DNA Analysis Software (Phoenix Flow System, San Diego, CA, USA).

Assay for caspase activity

The assay is based on the detection of the chromophore AFC after cleavage from the labeled substrate DEVD- and LEHD-AFC by caspase-3 and caspase-9, respectively. Free AFC emits a yellow-green fluorescence (λ_{max} =505 nm). SK-Hep-1 cells were treated with different concentrations of 2-MCA for 24 hours, and caspase-3 and caspase-9 activities were detected using Fluorometric Assay Kit from BioVision (Milpitas, CA, USA) following the manufacturer's protocol. The AFC light emission was quantified using Tecan infinite M200 spectrophotometer. Results are represented as the percentage of change in activity compared with the untreated control.

Mitochondrial membrane potential assay

Mitochondrial membrane potential was determined using the mitochondrial-specific fluorescent probe JC-1 (Thermo Fisher Scientific) based on the method of Reers et al.[18] JC-1 exists as monomer when membrane potentials ($\Delta\Psi_m$) is lower than 120 mV and fluoresces green (540 nm) following excitation by blue light (490 nm) and as dimer (J-aggregate) at membrane potentials >180 mV and fluoresces red (590 nm) following excitation by green light (540 nm). SK-Hep-1 cells were plated in a 96-well plate and treated with different concentrations of 2-MCA for 12 hours, the cells were stained with 25 μM JC-1 at 37°C for 30 minutes. Fluorescence was monitored with the Tecan infinite M200 spectrophotometer. Changes in the ratio of red (590-nm emission) to green (540-nm emission) fluorescence are indicative of the mitochondrial membrane potential changes.[19]

Assay for topoisomerase I and II activities

These assays were performed by the method of Har-Vardi et al.[20]

Assay for NF-κB DNA-binding activity

For analyzing transcription factor NF-κB-binding activity to DNA, nuclear proteins were prepared as described previously,[21] and binding activity was quantified using TF ELISA kit (Panomics, Fremont, CA, USA) following the manufacturer's protocol. This method is faster, easier, and significantly more sensitive than the electrophoretic mobility shift assays and does not require the use of radioactivity.[22]

Assay for COX-2 activity

After incubation, SK-Hep-1 cells were harvested and spun down at 1,500× *g* at 4°C for 10 minutes and washed once with saline. Then the cells were suspended in cell lysis buffer (Sigma-Aldrich), supplemented with protease and phosphatase inhibitors (Hoffman-La Roche Ltd.), and sonicated before centrifugation at 12,500× *g* at 4°C for 20 minutes. The supernatants were collected and used for quantitative analysis of COX-2 activity using ELISA kit (USCN LIFE, Wuhan, People's Republic of China) following the manufacturer's protocol.

Assay for PGE_2 expression

After incubation, the culture medium was collected for measurement of PGE_2 expression by using ELISA kit (R&D System, Minneapolis, MN, USA) following the manufacturer's protocol.

In vivo tumor xenograft study

Male nude mice (BALB/c Nude; 6 weeks old) were purchased from the National Science Council Animal Center

(Taipei, Taiwan) and maintained in pathogen-free conditions in accordance with relevant guidelines and regulations for the care and use of laboratory animals of I-Shou University. SK-Hep-1 cells (5×10^6 cells in 200 μL) were injected subcutaneously into the flanks of nude mice. Tumors were allowed to develop for approximately 20 days until they reached approximately 75 mm^3, and then treatment was started. Thirty-two mice were randomly separated into four groups. The mice in the 2-MCA-treated group were injected intratumorally with different concentrations of 2-MCA in a 200 μL volume daily. The control group was treated with an equal volume of vehicle. After transplantation, tumor size was monitored at weekly intervals using calipers, and tumor volume was estimated by the hemiellipsoid model formula: tumor volume = $1/2(4\pi/3) \times (l/2) \times (w/2) \times h$, where l is the length, w is the width, and h is the height.

Specimens were analyzed by fluorescent TUNEL assay using Quick Apoptotic DNA Ladder Detection Kit (Chemicon, Temecuba, CA, USA) following the manufacturer's protocol.

Statistical analysis

Data were presented as means ± standard error. The evaluation of statistical significance was determined by one-way analysis of variance followed by Bonferroni *t*-test for multiple comparisons. A *P*-value <0.05 was considered statistically significant.

Results

Effects of 2-MCA on cell morphological changes

When SK-Hep-1 cells were exposed to 10 μM of 2-MCA, vacuolation of cells was observed, >40 μM of 2-MCA. In addition, plasma membrane blebbing, cell shrinkage, and cell detachment occurred (Figure 1).

2-MCA inhibits SK-Hep-1 cell proliferation

We investigated the potential cell proliferation-inhibitory activity of 2-MCA in SK-Hep-1 cells by the XTT. As shown in Figure 1E, 2-MCA inhibited cell proliferation in SK-Hep-1 cells in a dose- and time-dependent manner. The inhibitory concentration (IC_{50}) value following 48 hours of incubation was 25.72 μM.

Nuclear fragmentation assay

AO is a nucleic acid-selective metachromatic stain useful for cell cycle determination, measuring apoptosis, detecting intracellular pH gradients, and the measurement of proton pump activity.[23] The dye differentially stains single-stranded nucleic acids orange and double-stranded nucleic acids green. In addition, in living cells, it serves as a pH indicator, trapped in acidic compartments, such as lysosomes, which then fluoresces to brilliant orange–red.[24] When SK-Hep-1 cells were treated with 10 μM of 2-MCA for 24 hours, the result of AO staining demonstrated that a part of SK-Hep-1 cells died by apoptosis with nuclear condensation, fragmentation, and apoptotic bodies. In addition, orange-staining lysosomal vacuoles appeared. No significant nuclear fragmentation in control group was observed.

In addition, DNA strand breakage was investigated by the single cell gel electrophoresis assay (also known as comet assay) at 48 hours following treatment with different concentrations of 2-MCA. Fluorescent comets with tails were evident when SK-Hep-1 cells were treated with 10 μM of 2-MCA for 48 hours. Figure 2 shows representative examples of DNA strand breaks in SK-Hep-1 cells treated with 10 μM of 2-MCA for 48 hours. Treatment with 5 μM of MCA did not show an obvious difference from controls, which mostly appeared spherical (result not shown).

Blebbing of plasma membrane, nuclear condensation, fragmentation, and apoptotic body formation are characteristic morphologic features of apoptosis.[25] The morphological changes observed in our study suggest that 2-MCA did induce apoptosis in SK-Hep-1 cells (Figures 1D and 2B and D).

2-MCA increases volume of acidic compartments in SK-Hep-1 cells

Neutral Red has been used to stain lysosomes and quantify the VAC in cells.[16,26,27] Figure 3 demonstrates that 2-MCA treatment resulted in acidic vacuoles in SK-Hep-1 cells with positive neutral red staining. As shown in Figure 3D, the VAC of 2-MCA-treated SK-Hep-1 cells increased in a dose-dependent manner.

2-MCA induces apoptosis in SK-Hep-1 cells

Flow cytometer was used to examine the mechanism responsible for the inhibition of cell proliferation by 2-MCA. DNA content histogram analysis obtained from PI-stained SK-Hep-1 cells demonstrated that treatment with 2-MCA led to elevated sub-G1. The results shown in Figure 4 reveal that the percentage of cell population with reduced (hypodiploid) DNA content increased from untreated cells to cells exposed to 20 μM 2-MCA for 24 hours in a dose-dependent manner. The percentage DNA content in sub-G1 region increased from 1.602% in untreated control to 21.923% in cells treated with 20 μM 2-MCA for 24 hours as mentioned earlier (Figure 4B).

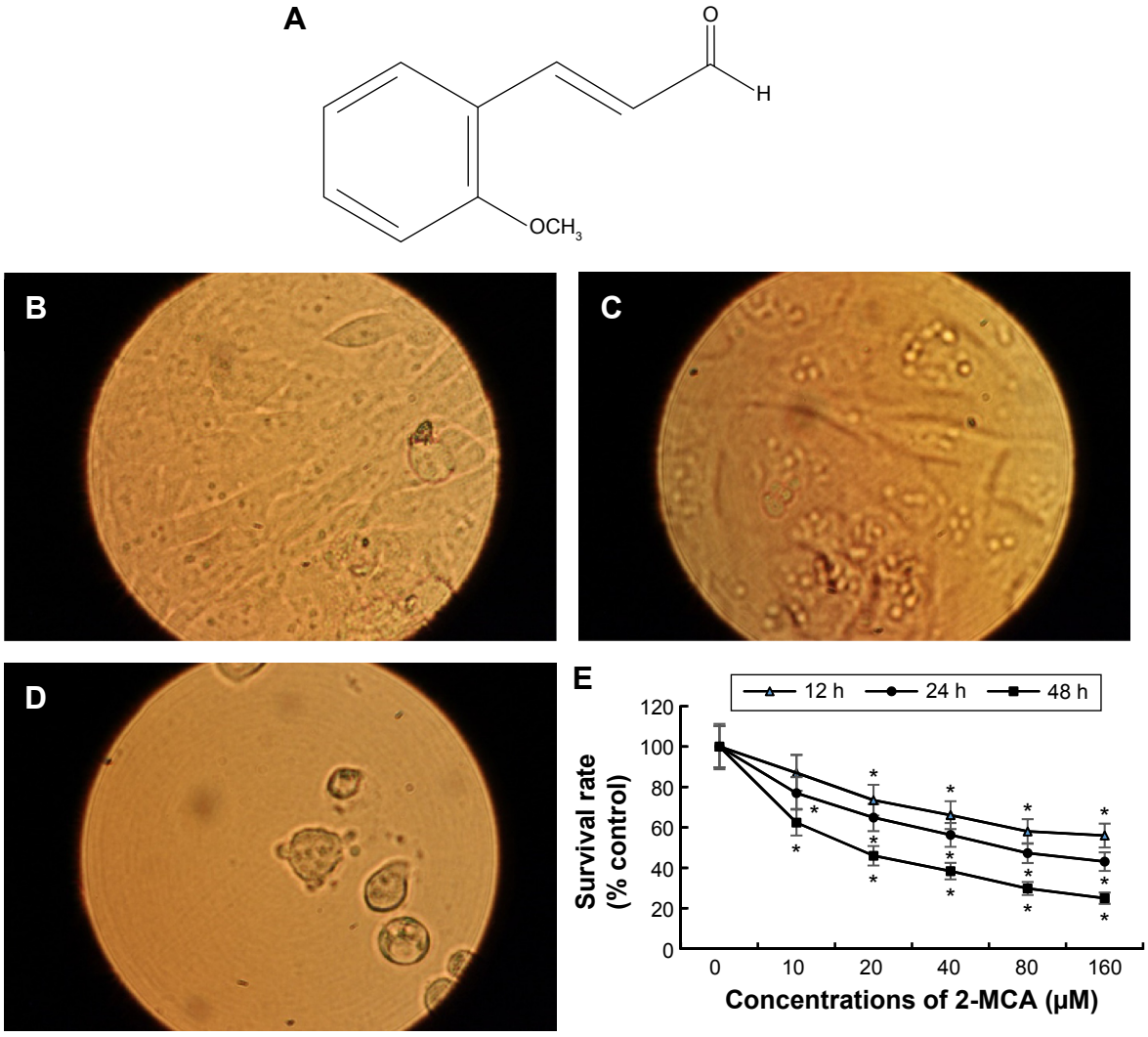


Figure 1 Structure and effects of 2-MCA on cell morphology and growth in SK-HEP-1 cells.
Notes: Structure (**A**) and effects of 2-MCA on cell morphology (**B**–**D**) and growth (**E**) in SK-HEP-1 cells. (**B**–**D**) Effect of 2-MCA on cell morphology. Cells were treated with 0 μM (**B**), 10 μM (**C**), and 40 μM (**D**) of 2-MCA, respectively, for 24 h. When SK-Hep-1 cells were exposed to 10 μM of 2-MCA, vacuolation of cells occurred. At 40 μM of 2-MCA, plasma membrane blebbing, cell shrinkage, and cell detachment were observed. (**E**) 2-MCA-inhibited proliferation. SK-Hep-1 cells were treated with 2-MCA at the indicated conditions. Cell proliferation-inhibitory activity was evaluated by the XTT assay. Data are expressed as means ± standard error of mean, n=5. *Indicates a significant difference ($P<0.05$) from control.
Abbreviations: 2-MCA, 2-methoxycinnamaldehyde; h, hours.

Then, we further investigated the role of mitochondria in the 2-MCA-induced apoptosis in SK-Hep-1 cells. Since early apoptotic cell death often involves mitochondrial depolarization and release of cytochrome *c* from mitochondria into cytosol, we initially investigated mitochondrial dysfunction, by measuring mitochondrial membrane potential $\Delta\Psi_m$ in 2-MCA-treated SK-Hep-1 cells using the mitochondria-specific dye JC-1, both microscopically and spectrophotometrically. As shown in Figure 5, 2-MCA induced loss of mitochondrial membrane potential as indicated by decreased $\Delta\Psi_m$ in a dose-dependent manner.

Caspases, or cysteine-aspartic proteases, are a family of cysteine proteases that play important roles in apoptosis. As shown in Figure 5D, the activities of both caspase-3 and caspase-9 increased in a dose-dependent manner in 2-MCA-treated SK-Hep-1 cells. This is consistent with the mitochondrial depolarization and release of cytochrome *c* from mitochondria into the cytosol.

2-MCA inhibits topoisomerase I activity in SK-Hep-1 cells

Inhibition of topoisomerase I activity in SK-Hep-1 cells by 2-MCA was performed in the presence of increasing concentration of 2-MCA (Figure 6) or camptothecin (CPT) (lane 6), a known specific inhibitor of topoisomerase I.[28–30] Figure 6A shows that the conversion of the supercoiled plasmid pUC 19 to the relaxed form decreased in a dose-dependent manner in the presence of 2-MCA or CPT (compare lanes 3–6

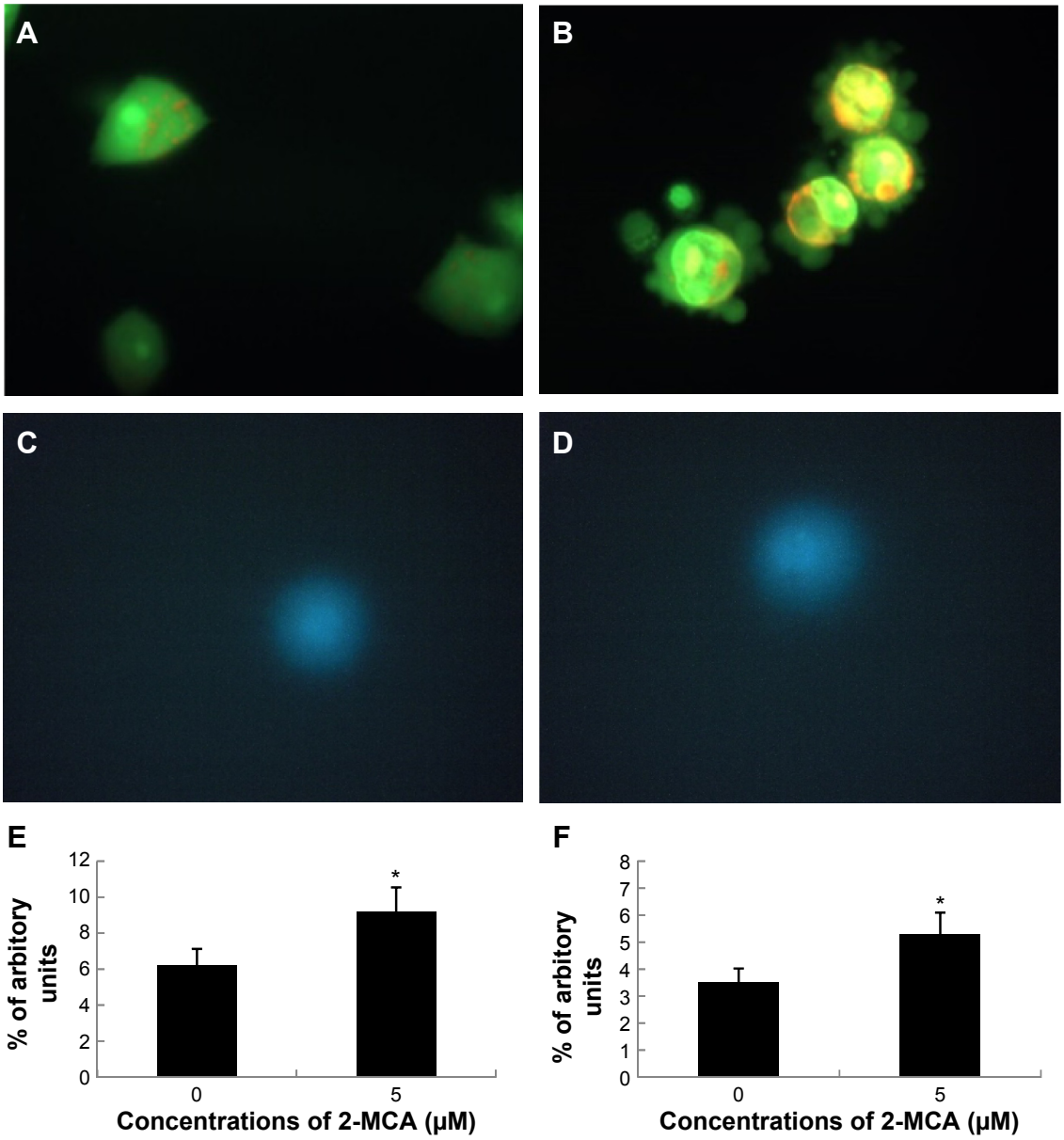


Figure 2 2-MCA-induced nuclear fragmentation in SK-Hep-1 cells.
Notes: (**A** and **B**) Acridine orange staining: cells were treated with 2-MCA 0 μM and 10 μM, respectively, for 24 h, then stained with acridine orange. Orange vacuoles in cells showed that they were acidic. (**A**) Control group; (**B**) test group treated with 10 μM 2-MCA in which nuclear fragmentation and lysosomal vacuolation were observed. (**C** and **D**) Comet assay: cells were embedded in agarose and DNA was then unwound in an alkaline solution and subjected to electrophoresis. Cells were then stained with DAPI and examined under a fluorescent microscope. (**C**) and (**D**) Representative SK-Hep-1 cells treated with 0 μM and 10 μM of 2-MCA, respectively for 48 h. (**E**) and (**F**) Tail intensity and moment of SK-Hep-1 cells treated with 0 μM and 10 μM of 2-MCA, respectively for 48 h. Data are expressed as means ± standard error of mean, n=5. *Indicates a significant difference ($P<0.05$) from control.
Abbreviations: 2-MCA, 2-methoxycinnamaldehyde; h, hours.

with lane 2). These results show that the DNA relaxation activity of SK-Hep-1 cell nuclear proteins is inhibited by 2-MCA.

2-MCA inhibits topoisomerase II activity in SK-Hep-1 cells

Inhibition of topoisomerase II activity in SK-Hep-1 cells by 2-MCA was examined in the presence of increasing concentration of 2-MCA (Figure 6B, lanes 3–5) or 60 μM VP-16 (lane 6), a known inhibitor of topoisomerase II.[29] Figure 6B, upper part, shows that the conversion of the supercoiled plasmid pUC 19 to the relaxed form decreased in a dose-dependent manner in the presence of 2-MCA or VP-16 (compare lanes 3–6 with lane 2). These results show that the DNA relaxation activity of SK-Hep-1 cell nuclear proteins is inhibited by 2-MCA. In addition, the effect of 2-MCA on topoisomerase II in SK-Hep-1 cells was further evaluated by decatenation assay. Decatenation activity is the releasing of monomers (minicircle DNA) from the kDNA (a large network of plasmid). Nuclear proteins

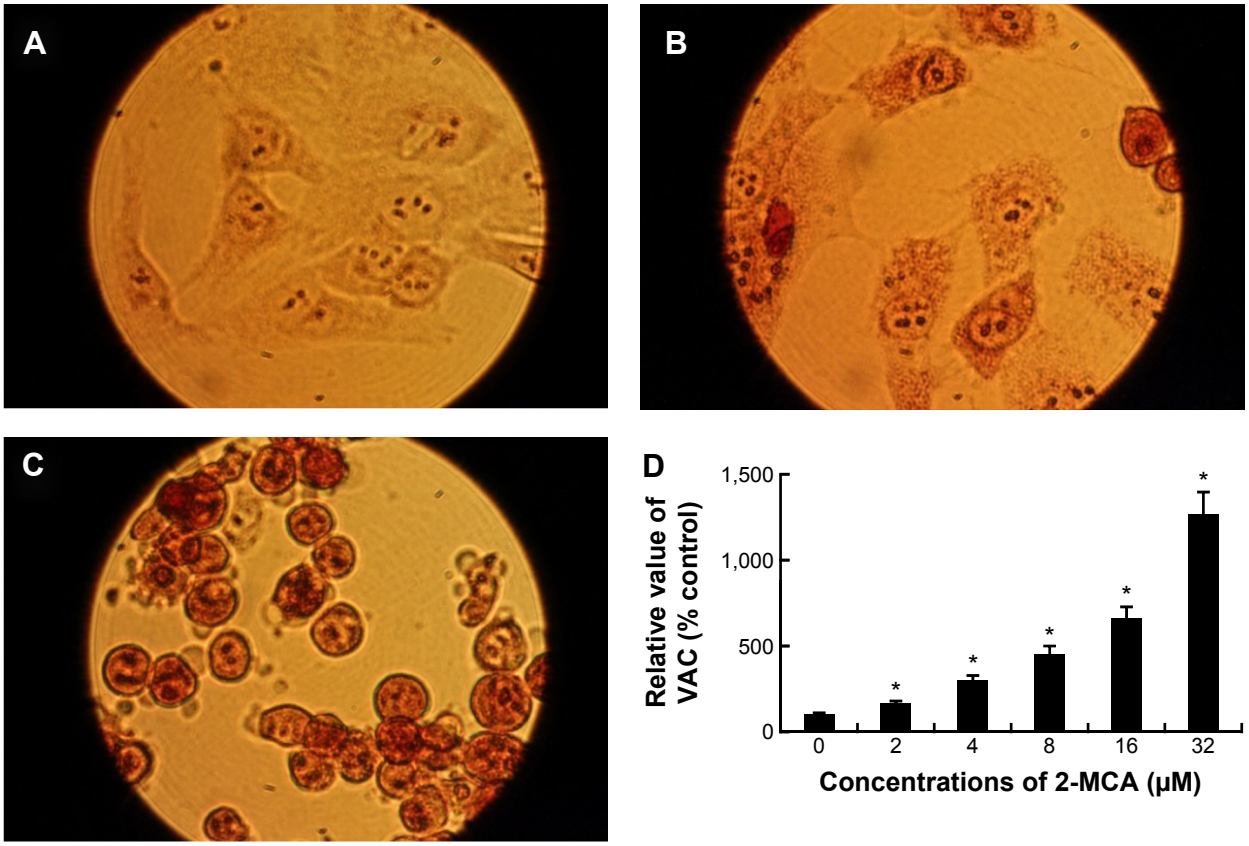


Figure 3 2-MCA increased VAC in SK-Hep-1 cells.
Notes: SK-Hep-1 cells were treated with 0 μM, 15 μM, or 30 μM of 2-MCA for 24 h, and then stained with neutral red. (**A**) Control group: there were no obvious vacuoles in cells. (**B**) and (**C**) SK-Hep-1 cells treated with 15 μM and 30 μM of 2-MCA, respectively. The acidic red vacuoles in cells were observed. (**D**) 2-MCA increased VAC in a dose-dependent manner. SK-Hep-1 cells were incubated with the indicated concentration of 2-MCA for 24 h, and data were analyzed with spectrophotometer. The images were obtained by using a light microscope with a yellow filter inserted in the light path. Data are expressed as means ± standard error of mean, n=5. *Indicates a significant difference ($P<0.05$) from control.
Abbreviations: 2-MCA, 2-methoxycinnamaldehyde; VAC, volume of acidic compartment; h, hours.

extract from SK-Hep-1 cells contained topoisomerase II, which converted a kinetoplast DNA to monomer DNA molecules (Figure 6B, lower part, compare lane 2 with lane 1). The conversion of kinetoplast DNA to monomers decreased in a dose-dependent manner in the presence of 2-MCA (compare lanes 3–5 with lane 2) or VP-16 (compare lane 6 with lane 2). These results show that the decatenation activity of SK-Hep-1 cell nuclear proteins is inhibited by 2-MCA.

2-MCA inhibits NF-κB, COX-2, and PGE_2 levels

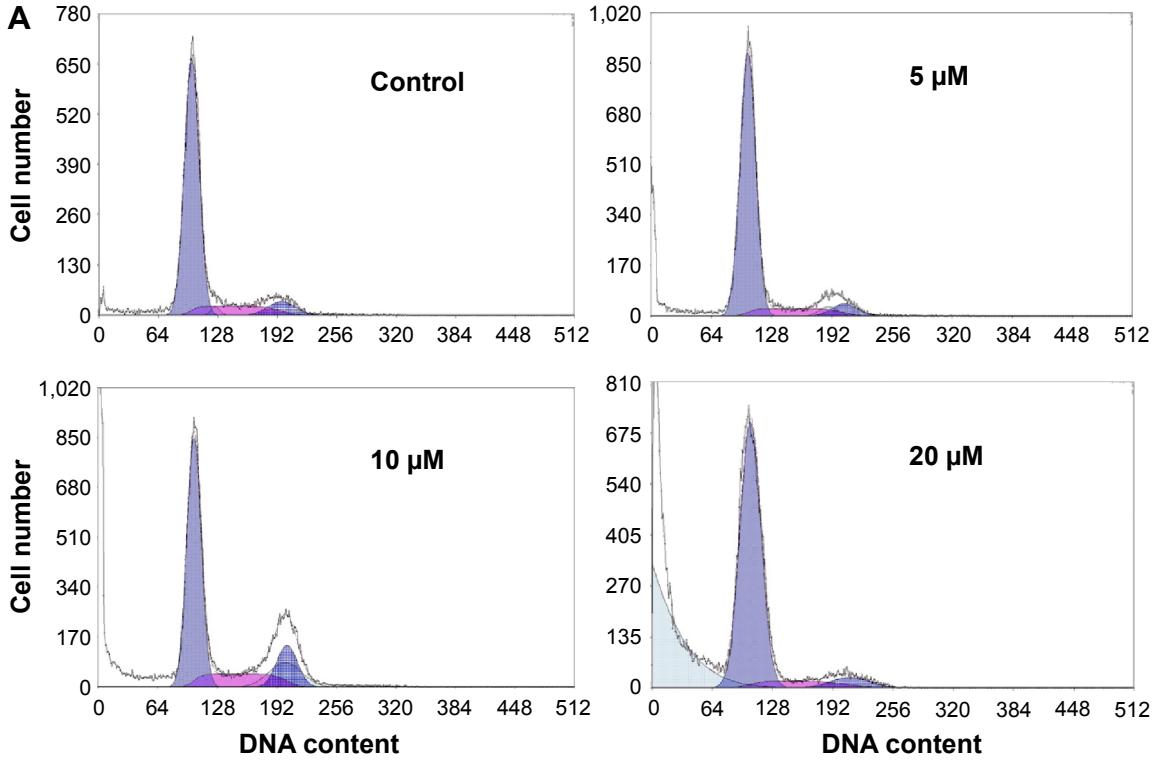
We then examined the effect of 2-MCA treatment on cellular factors associated with tumorigenesis in SK-Hep-1 cells. SK-Hep-1 cells were incubated without or with various concentrations of 2-MCA for 24 hours. After incubation, the NF-κB DNA-binding activity was quantified by ELISA. As shown in Figure 7, 2-MCA decreased NF-κB DNA-binding activity in a dose-dependent manner. The IC_{50} value of 2-MCA in inhibiting the binding activity was 42.16 μM.

To investigate the effects of 2-MCA on COX-2 activity in SK-Hep-1 cells, SK-Hep-1 cells were incubated with different concentrations of 2-MCA for 24 hours. Then, the COX-2 activity was determined by ELISA. As shown in Figure 7B, 2-MCA decreased COX-2 activity in a dose-dependent manner. The IC_{50} value of 2-MCA in inhibiting COX-2 activity was 407.92 μM.

In addition, as shown in Figure 7C, 2-MCA decreased PGE_2 level in a dose-dependent manner. The IC_{50} value of 2-MCA in inhibiting PGE_2 expression was 415.31 μM.

2-MCA inhibits growth of SK-Hep-1 xenograft in nude mice

To determine whether 2-MCA suppresses growth of SK-Hep-1 xenograft, equal numbers of SK-Hep-1 cells were injected subcutaneously into both flanks of the nude mice. Tumor growth suppression was noticed in all groups of 2-MCA-treated (5 mg/kg/d, 10 mg/kg/d, or 20 mg/kg/d of 2-MCA, respectively) mice. However, significant growth suppression was observed only in mice treated with 10 mg/kg/d



B

Flow cytometer analysis of SK-Hep-1 cells treated with 2-MCA

2-MCA concentration (μM)	DNA content (%) in sub-G1
0 (control)	1.60
5	3.29*
10	13.04*
20	21.92*

10,000 cells were counted for each preparation

Figure 4 Flow cytometric analysis of 2-MCA-treated SK-Hep-1 cells.
Notes: (**A**) SK-Hep-1 cells were treated with the indicated concentrations of 2-MCA for 24 h. The induction of apoptosis was determined by PI staining and analyzed using CyFlow SL Flow Cytometer. (**B**) Data are expressed as means ± standard error of mean, $n = 5$. *Indicates a significant difference ($P<0.05$) from control.
Abbreviations: 2-MCA, 2-methoxycinnamaldehyde; h, hours; PI, propidium iodide.

or 20 mg/kg/d of 2-MCA, where ~70% reductions in tumor size were found. No significant difference between these two groups was found (Figure 8). None of the 2-MCA treatments caused any significant decrease in diet consumption or body weight change (data not shown) compared with control mice. To gain insight into the mechanism of antitumor effect of 2-MCA in vivo, we harvested the SK-Hep-1 xenograft from vehicle- and 2-MCA-treated mice and assessed cell death by TUNEL analysis. As shown in Figure 8A, right parts, compared with tumors of vehicle-treated mice (upper part), elevated TUNEL-positive cells, suggesting apoptosis, were found in tumors of the 2-MCA-treated mice (lower part).

Discussion

Epidemiological and experimental studies have consistently shown that there is a correlation between regular consumption of fruits and vegetables and prevention of developing lifestyle disorders, such as cardiovascular disorders and cancer.[31,32] Phytochemicals, such as polyphenols and flavonoids that are abundant in fruits and vegetables, seem to possess many of the desirable qualities for anticancer and could have great potential as chemopreventive and antiproliferative agents.[33–38] *C. verum* has been traditionally used for treating dyspepsia, blood circulation, and inflammatory disorders, including gastritis.[39,40] 2-MCA, a constituent of the bark of the plant, could to be such a natural agent. Very few studies about 2-MCA have been reported. Moreover, to the best of our knowledge, there has been no report to date with regard to its effects on topoisomerase I and II activities. The current study was aimed at investigating the antiproliferative activity of 2-MCA and elucidating the underlying mechanisms of action.

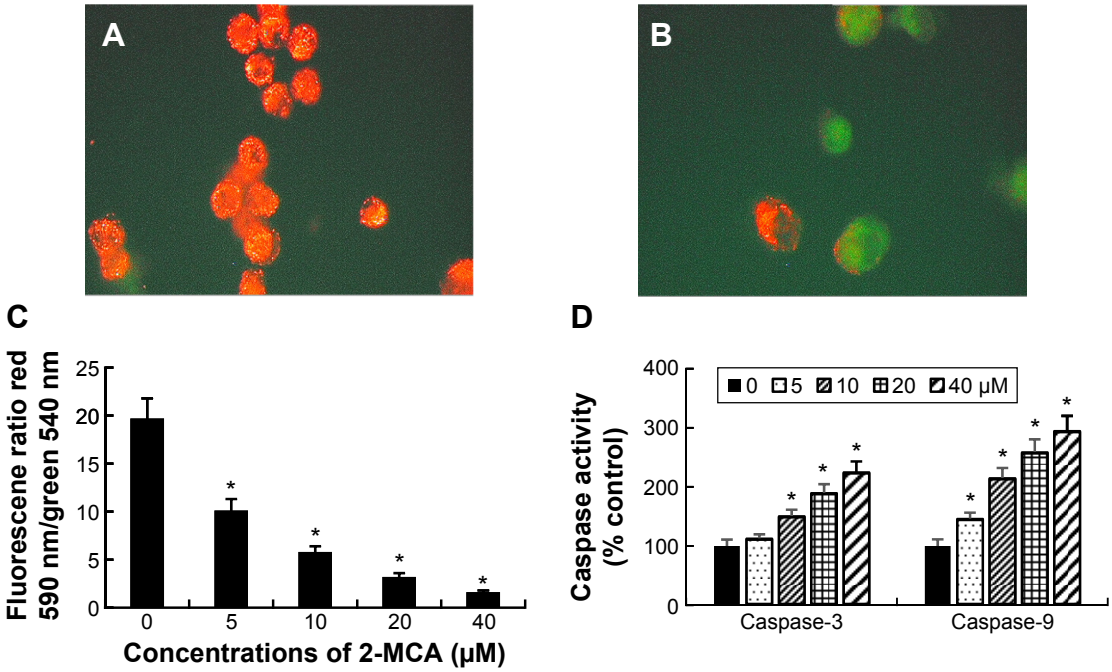


Figure 5 2-MCA induced apoptosis through the mitochondrial pathway in SK-Hep-1 cells.
Notes: (**A** and **B**) Cells were treated with 0 μM and 20 μM of 2-MCA, respectively, for 48 h, and $\Delta\Psi_m$ was analyzed by JC-1 using fluorescent microscopy (**A**) depicts control cells with intact mitochondria fluorescencing red; (**B**) shows most 2-MCA-treated cells fluorescencing green, suggesting the loss of $\Delta\Psi_m$. (**C**) Cells were treated with the indicated concentrations of 2-MCA for 12 h and $\Delta\Psi_m$ was analyzed by JC-1 using spectrophotometer. (**D**) Activations of caspase-3 and caspase-9. Cells were treated with the indicated concentrations of 2-MCA for 24 h, and activities of caspase-3 and caspase-9 were determined fluorometrically using fluorescent-labeled synthetic substrates. Data are expressed as means ± standard error of mean, n=5. *Indicates a significant difference ($P<0.05$) from control.
Abbreviations: 2-MCA, 2-methoxycinnamaldehyde; h, hours.

In this study, we first elaborately examined the effects of 2-MCA on the growth of human HCC SK-Hep-1 cells. We found that 2-MCA suppressed the proliferation of SK-Hep-1 cells in a dose- and time-dependent manner. Although cells can die by nonapoptotic mechanisms, apoptosis is the most common and preferred mechanism through which many chemotherapeutic agents kill and eradicate tumor cells.[5] In addition, apoptosis has been reported as the major mechanism of cancer cell death induced by selected polyphenols.[41–44] Our results suggested that 2-MCA did induce apoptosis as indicated by loss of $\Delta\Psi_m$, activations of caspase-3 and caspase-9 (Figure 5), increased DNA content in sub-G1 region as shown in flow cytometric analysis (Figure 4), and morphological characteristics of apoptosis, including blebbing of plasma membrane, nuclear condensation, fragmentation, and apoptotic body formation as shown in various stainings and comet assay (Figures 1–3).

Mitochondria are crucial to multicellular life and apoptosis-inducing agents that target mitochondria may affect them in various ways. They may induce the formation of membrane

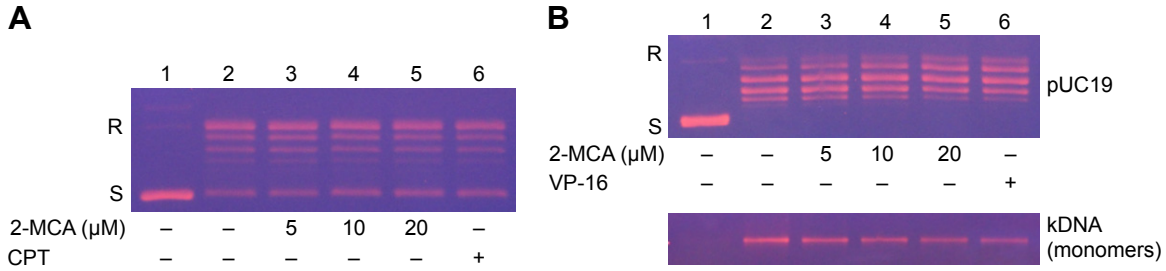


Figure 6 Inhibition of SK-Hep-1 topo I and II activities by 2-MCA.
Notes: (**A**) 2-MCA inhibited topo I activity in SK-Hep-1 cells. Nuclear proteins from SK-Hep-1 cells were added to a specific topo I reaction mixture in the presence of the indicated concentrations of 2-MCA (lanes 3–5) or 60 μM CPT (lane 6) or the vehicle (1% DMSO, lane 2). Lane 1: pUC19 DNA only. (**B**) 2-MCA inhibits topo II activity in SK-Hep-1 cells. DNA relaxation assay (upper part) and decatenation assay (lower part): nuclear proteins from SK-Hep1 cells were added to a specific topo II reaction mixture in the presence of the indicated concentrations of 2-MCA (lanes 3–5) or 60 μM VP-16, a specific topo II inhibitor (lane 6), or the vehicle (1% DMSO, lane 2). (Lane 1) Supercoiled pUC19 DNA (upper part) or kDNA (lower part) only. kDNA is a large network of plasmids, when it is analyzed by gel electrophoresis, it penetrates only slightly into agarose gel (result not shown). Upon decatenation by topo II, mini circles monomers of DNA are formed (lower part, lanes 2–6).
Abbreviations: 2-MCA, 2-methoxycinnamaldehyde; CPT, camptothecin; DMSO, dimethyl sulfoxide.

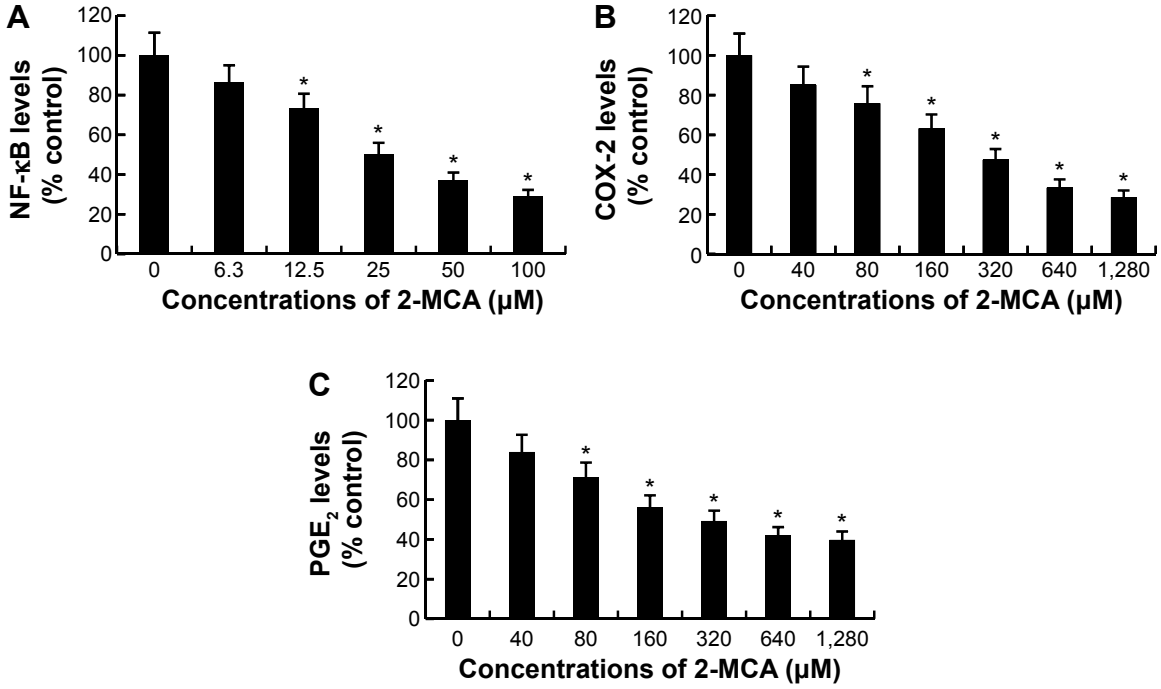


Figure 7 2-MCA-inhibited DNA-binding activity of NF-κB and expressions of COX-2 and PGE_2 in SK-Hep-1 cells.
Notes: SK-Hep-1 cells were incubated with or without the indicated concentrations of 2-MCA for 24 h. After incubation, nuclear extracts and culture media were analyzed for NF-κB DNA-binding activity (**A**), COX-2 (**B**), and PGE_2 (**C**) expressions, respectively. Data are expressed as means ± standard error of mean, n=5. *Indicates a significant difference ($P<0.05$) from control.
Abbreviations: 2-MCA, 2-methoxycinnamaldehyde; NF-κB, nuclear factor κB; COX-2, cyclooxygenase-2; PGE_2, prostaglandin E_2; h, hours.

pores, leading to mitochondrial swelling, or increase the permeability of mitochondrial membrane, resulting in releasing of apoptotic effectors from mitochondrial into cytosol. The released cytochrome *c* binds to apoptotic protease activating factor-1 and dATP, which then bind to pro-caspase-9 to create a protein complex called apoptosome. The apoptosome cleaves the pro-caspase-9 to its active form of caspase-9. The activated caspase-9 in turn activates effector caspase-3, thereby initiating a cascade of proteolytic events.[45–47] The present study with regard to the key events in induction of apoptosis demonstrate that 2-MCA induced the collapse of $\Delta\Psi_m$, upregulated activities of most upstream protease of intrinsic apoptotic pathway, caspase-9, and the effector caspase-3 suggested the involvement of these proteins in 2-MCA-induced apoptotic cell death.

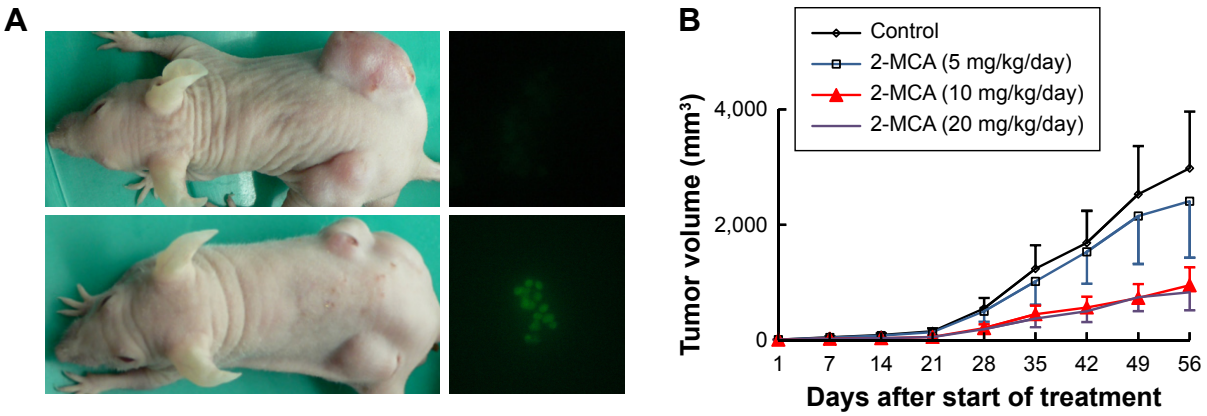


Figure 8 2-MCA suppressed growth and induced apoptosis in SK-Hep-1 xenograft.
Notes: Animals bearing preestablished tumors (n=8 per group) were injected intratumorally with the indicated concentration of 2-MCA. During the 56-day of 2-MCA treatment, tumor volumes were monitored using calipers and apoptosis was assessed by TUNEL assay. (**A**) Left: representative tumor-possessing nude mice from the control (upper) and 10 mg/kg/d of 2-MCA-treated (lower) groups. (**A**) Right: 2-MCA-induced apoptosis in SK-Hep-1 xenograft by TUNEL assay. Representative TUNEL assay of tumors from the control (upper) and 10 mg/kg/d of 2-MCA-treated (lower) groups. (**B**) Mean of tumor volume measured at the indicated number of days after start of treatment. Data are expressed as means ± standard error of mean, n=8.
Abbreviation: 2-MCA, 2-methoxycinnamaldehyde.

In addition, our results suggested that 2-MCA induced vacuolation with elevated VAC. Increase of VAC has been reported to be a common phenomenon of cells that undergo either apoptotic or necrotic cell death and could be a hallmark of dying cells.[48] Since apoptosis is an ordered process, the increase of VAC could be responsible for the self-digestion during the course of cell death.[48]

Type I topoisomerases act by creating a transient single-stranded break in the DNA double helix molecule, followed by either a single-stranded DNA passage or controlled rotation about the break. Type I topoisomerases are involved in all DNA processes that involve tracking systems and play important roles in maintaining genomic integrity.[6] Furthermore, elevated levels of topoisomerase I mRNA, protein, and catalytic activity are seen across human tumors.[49]

Type II topoisomerases act by generating a transient double-stranded DNA break, followed by a double-stranded DNA passage event. Type II topoisomerases function in numerous DNA processes and are required for recombination, the separation of daughter chromosomes, and proper chromosome structure, condensation, and decondensation.[6] The enzyme is increased drastically during cell proliferation and peak in G2/M. The resulting transient double-stranded break could lead to fragmentation of the genome with chromosomal translocations and other DNA aberrations.[7,50]

In addition to cell cycle regulation, topoisomerase is another major target of anticancer agents.[8–11] Chemotherapeutic agent etoposide kills cells by stabilizing the transient intermediate cleavage complex. The accumulation of cleavage complexes leads to the generation of permanent DNA strand breaks that fragments the genome, resulting in the activation of death pathways.[51] Apoptosis has been demonstrated to be the most efficient death pathway in tumor cells after topoisomerase II had been inhibited.[52] Our results documented that 2-MCA inhibited topoisomerase I and II activities in a concentration-dependent manner (Figure 6), which in parts, could be a mechanism driving the cells to apoptosis. While the majority of topoisomerase inhibitors are selectivity against either topoisomerase I or II,[53] our study obviously shows that 2-MCA inhibited both topoisomerase I and II activities in SK-Hep-1 cells. However, further works are needed to elucidate the specific underlying mechanism of the inhibition, possible mutagenic effect, and others for clinical usage as a chemopreventive or anticancer agent against HCC and/or other malignances.

It is generally accepted that carcinogenesis is a multistep process. Studying the effects of 2-MCA on both topoisomerase I and II in SK-Hep-1 cells in this process may provide new information on the pathological process of cancer.

Many diseases are due to the aberrant activation and expression of genes involved in the initiation and progression of pathogenesis. In general, these genes are quiescent or have low activity in normal physiological status, but under certain conditions are turned on by preexisting genetic switches.[40] These genetic switches are partially controlled by transcription factor NF-κB. NF-κB, a heterometric complex consisting of p50, p65, and IκBα, is present in its inactive state in the cytoplasm. When NF-κB is activated, IκBα is degraded and p50-p65 heterodimer is translocated to the nucleus, binds the κB-regulatory elements at the promoter region, and activates genes. NF-κB is involved in the regulation of cell proliferation, differentiation, immunity, inflammation, and apoptosis. The aberrant activation of NF-κB signaling results in the transcription of genes, generating biologically active proteins such as mitogen-activated protein kinase and COX-2. Cohesive scientific evidence from molecular, animal, and human studies suggests the hypothesis that aberrant induction of COX-2 and prostaglandin cascade play an important role in inflammation, aging, and carcinogenesis. Therefore, inhibition of the process has strong potential for cancer prevention and treatment.[14] Our results in this study clearly demonstrate that 2-MCA suppressed NF-κB DNA-binding activity and COX-2 and PGE_2 levels in SK-Hep-1 cells in a dose-dependent manner. It has been shown that COX-2 was highly expressed in the HCC tissue.[54] Treatment that inhibits COX-2 may be a promising targeted approach in HCC.

Therapy-induced cytotoxicity and other associated side effects of anticancer drugs are major concerns of chemotherapy. Therefore, ideal drugs should selectively kill cancer cells and not damage the healthy. None of the 2-MCA treatments caused any significant decrease in diet consumption or body weight change compared with control mice. These results convincingly indicate the protective effect of 2-MCA treatment against SK-Hep-1 xenograft growth in nude mice without any observable toxicity. Indeed, similar effects (including all of the above-mentioned effects) were found in other tested cell lines, including human HCC Hep 3B, lung adenocarcinoma A549, squamous cell carcinoma NCI-H520, colorectal adenocarcinoma COLO 205, and T-lymphoblastic MOLT-3 (results not shown), suggesting an antiproliferative action of 2-MCA in SK-Hep-1 cells and the agent as a potential source of antiproliferative agent for cancer.

Conclusion

Collectively, our data clearly indicate that 2-MCA induced apoptosis, suppressed tumor cells growth and the associated biomarkers. The molecular events associated with the tumor suppression effect of 2-MCA including downregulation of cell proliferative controls, involving apoptosis, transcription factor NF-κB, both topoisomerase I and II, and inflammatory responses involving COX-2 and PGE_2. The 2-MCA efficacy observed in the present study in terms of a shrinkage of tumor size would have potential clinical significance.

In conclusion, the present study provides fundamental information on the tumor suppression effect of 2-MCA in SK-Hep-1 cells, both in vitro and in vivo, suggesting a short-term model for evaluation of potential chemopreventive pharmacological modulators against hepatoma. Our results provide a focus for the rational development of 2-MCA as an anticancer agent against HCC.

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Disclosure

The authors report no conflicts of interest in this work.

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