Antiproliferative and apoptotic effects of paeonol on human hepatocellular carcinoma cells

Zhang Chunhu, Hu Suiyu, Cao Meiqun, Xiao Guilin and Li Yunhui

Paeonol, a major phenolic component of Moutan Cortex, is known to have antitumor effects through an unknown mechanism. In this study, we tried to elucidate the anticancer effects of paeonol on human hepatocellular carcinoma (HCC) cell lines BEL-7404, SMMC-7721, and MHCC97-H *in vitro*. Using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay, we compared the cytotoxicity of paeonol with the cytotoxicity of 5-fluorouracil (5-FU) in these three HCC cell lines. In addition, we examined the combined effect of paeonol and 5-FU over time, and found that the two compounds inhibited the proliferation of all three human HCC cell lines in a dose-dependent manner. The concentrations that inhibited the proliferation by 50% ranged from 11.39 to 56.23 mg/l for paeonol, and 6.47 to 37.87 mg/l for 5-FU. We determined that exposure to these compounds led to an upregulation of the anti-oncogene PTEN, and the downregulation of the oncogene AKT in the cell lines tested as determined by real-time quantitative reverse transcription-PCR and western blot. In addition, paeonol induced DNA fragmentation in the HCC cell line BEL-7404. These observations suggest that paeonol has the potential to be explored for use as an effective antitumor agent for HCC. *Anti-Cancer Drugs* 19:401–409 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer-related death worldwide [1]. Eighty percent of the burden is borne by countries in Asia and sub-Saharan Africa [2]. Although recent advances in management with a multidisciplinary approach result in improved local and regional disease control, the 5-year survival rate is still less than 10% [3]. Thus, it is imperative to develop more effective chemotherapy agents with low toxicity.

Recently, extracts of Chinese herbal medicines have become the focus of much research, and many kinds of extract drugs have come into the market. Paeonol (Fig. 1) is isolated from the herb *Pycnostelma paniculatum* K.S. and the root of the plant *Paeonia suffruticosa* Andrew. It is a major component of the Chinese herbal medicine, Moutan Cortex, with a white needle crystal appearance and a relatively low melting point of 51–52°C. The molecular weight of paeonol is 166.18 ku and the molecular formula is $C_9H_{10}O_3$. Recent studies support that paeonol (2-hydroxy-4-methoxyacetophenone) exhibits an antineoplastic activity [4]. In addition to its antineoplastic property, previous pharmacological experiments have shown that paeonol also has anti-inflammatory, antiaggregatory, and antioxidant effects [5,6]. Though the mechanism by which paeonol exerts some of these latter effects in part has been elucidated, the mechanism by which paeonol exerts its antitumor effect remains unknown.

The phosphatidylinositol 3-kinase (PI3K) pathway is a critical mediator of cell survival [7,8]. Some reports suggest that components of the PI3K pathway, such as the key cell survival kinase AKT, are negatively regulated by the tumor suppressor gene PTEN [9,10]. The PI3K pathway has been shown to be dysregulated in many cancers [11,12], and thus presents an appealing cancer therapeutic target.

The objective of this study was to investigate the anticancer effect of paeonol on HCC cell lines *in vitro*, and to determine if modulation of the protumorigenic PI3K pathway is involved in the antitumor effect of paeonol in these same cells.

Materials and methods

Drugs and reagents

Paeonol injection (10 mg/ml) was purchased from the First Pharmaceutical Factory of Shanghai (China). 5-Fluorouracil (5-FU) injection (0.25 g/ml) was provided by Shanghai Haipu Pharmaceutical Factory (China). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and hydroxyethyl piperazine ethanesulfonic acid

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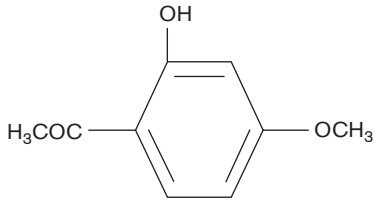
Fig. 1



Structure of paeonol (2-hydroxy-4-methoxyacetophenone).

were obtained from Sigma (St Louis, Missouri, USA). The Revert Aid First Strand cDNA Synthesis Kit was obtained from Promega (Madison, Wisconsin, USA). PTEN (N-19) goat polyclonal antibody and AKT1/2(H-136) rabbit polyclonal antibody were purchased from Santa Cruz Biotech (Santa Cruz, California, USA). Phospho-PTEN (Ser^{380}/$Thr^{382/383}$) rabbit polyclonal antibody and Phospho-AKT (Thr^{308}) monoclonal antibody were obtained from Cell Signaling Tech (Beverly, Massachusetts, USA). Rabbit IgG horseradish peroxidase (HRP)-conjugated rabbit anti-goat polyclonal antibody was purchased from KPL (Maryland, USA) and mouse IgG HRP-conjugated rabbit anti-mouse polyclonal antibody was purchased from Upstate (New York, USA). Radio immunoprecipitation assay protein lysis buffer was obtained from Santa Cruz Biotechnology. Hybond-P polyvinylidene difluoride was obtained from Amersham (Uppsala, Sweden) and phenylmethyl sulfonylfluoride was obtained from Sigma.

Cell lines and culture conditions

HCC cell lines BEL-7404 and SMMC-7721 were obtained from the Cell Biology Research Laboratory of the Xiangya Medical School of Central South University. MHCC97-H HCC cell line was kindly provided by the Institute of Hepatocellular Carcinoma of the Zhongshan Hospital of Fudan University. All cell lines were grown in PRMI 1640 medium (Gibco/BRL cell culture, Invitrogen, Karlsruhe, Germany) supplemented with 0.01 mol/l hydroxyethyl piperazine ethanesulfonic acid, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 5°C heat-inactivated fetal calf serum (Biochrome, Berlin, Germany). The cells were cultured on collagen-coated 7.5 cm^2 tissue culture flasks in a humidified atmosphere of 5% CO_2 at 37°C.

Cell proliferation assay

Inhibition of cell proliferation by paeonol was measured by the MTT assay [13,14]. Briefly, cells were plated on collagen-coated 96-well flat-bottom microtiter culture plates (Becton Dickinson, Heidelberg, Germany) at 1×10^4 cells/well. After incubation for 24 h, the cells were treated with 0, 2.6, 5.19, 10.39, 20.78, 41.55, 83.09, 166.18 mg/l paeonol and 0, 1.56, 3.13, 6.25, 12.51, 25.01, 50.01, 100.01 mg/l 5-FU. Medium (200 μl) containing various concentrations of drug was added to each well. The concentration range used in this study was equivalent to the approximate accumulation of plasma concentration of dietary phenols and polyphenols by an average adult human [15]. At certain time points, 20 μl of MTT reagent (dissolved in phosphate-buffered saline with a pH of 7.4 at a concentration of 5 mg/ml) was added to each well, and the cells were incubated for another 4 h. The medium was removed and 150 μl of dimethyl sulfoxide was added to each well followed by 10 min on a rocking platform. The absorbance of the dissolved formazan dye in each well was measured at 570 nm with a reference wavelength of 630 nm using an automated Dynatech MR7000 microplate reader (Dynatech, Alexandrina, Virginia, USA).

Each drug concentration was tested in five replicates from which the mean, standard deviation and coefficient of variation were calculated. Dose/time–response curves were also calculated. The drug concentration capable of 50% growth inhibition relative to untreated controls (IC_{50}) was calculated by the equation {[percentage viable cells ($>50\%$)] – 50}/{[percentage viable cells ($>50\%$)] – [percentage viable cells ($<50\%$)]} – (drug concentration above 50% viable cells–drug concentration below 50% viable cells) + (drug concentration below 50% viable cells).

Analysis of in-vitro drug interaction

The coefficient of drug interaction (CDI) was used to analyze the potential synergistic inhibitory effect of drug combinations [16]. CDI was calculated by the equation, $CDI = AB/(A \times B)$. According to the absorbance of each group, AB is the ratio of the combination groups to the control group; A or B is the ratio of the single-agent groups to the control group. Thus, a CDI value less than, equal to, or greater than 1 indicates that the drugs are synergistic, additive, or antagonistic, respectively. A CDI value less than 0.7 indicates that the drugs are significantly synergistic.

DNA fragmentation analysis

The isolation of fragmented DNA from cells cultivated in six-well plates was carried out according to the procedure of Hermann *et al.* [17] with some modifications. Briefly, after treatment with paeonol/5-FU/paeonol + 5-FU (48 h IC_{50}), 10^7 HCC cells were trypsinized and washed with phosphate-buffered saline once, and then collected by centrifugation (2000 *g*, 10 min). The pellet was resuspended in 0.5 ml DNA lysis buffer (2% SDS, 10 mmol/l EDTA, 10 mmol/l Tris–HCl, pH 8.5). The lysate was immediately incubated with 0.1 mg/ml proteinase K (Sigma) and then incubated for 3 h at 37°C. After addition of isopropanol the DNA was precipitated with 70% ethanol. The suspension was centrifuged and the DNA was treated with 100 μl of 10 mmol/l Tris–HCl with

a pH of 7.5 and 0.5 mg/ml RNase A (Boehringer Mannheim, Germany) at 37°C for 24 h. The sample was then loaded into a *2%* agarose gel and electrophoresed. The DNA band pattern was visualized under ultraviolet light using ethidium bromide staining.

Quantitative real-time reverse transcription-PCR analysis of PTEN, AKT1, and AKT2 mRNA

RNA was extracted with Trizol (Invitrogen, Carlsbad, California, USA). Reverse transcription-PCR (RT-PCR) was accomplished with the Access RT-PCR System (Promega, USA). Human PTENmRNA (1025–1748 bp; GenBank accession number XM_005867) was amplified using the forward primer 5′-CCAGACATGACAGC CATC-3′ and the reverse primer 5′-GAACTTGTC TTCCCGTCG-3′. Human AKT1 mRNA (953–1065 bp was amplified using the forward primer 5′-TCTAT GGCGCTGAGATTGTG-3′ and the reverse primer 5′-CTTAATGTGCCCGTCCTTGT-3′. Expression of AKT2 mRNA was determined by amplification of base pairs at positions 1122–1267 bp [18]. Amplification was carried out using the forward primer 5′-TGAAAACCTT CTGTGGG-ACC-3′ and the reverse primer 5′-TGG TCCTGGTTGTAGAAGGG-3′. Human β-actin mRNA was amplified as the control using the forward primer 5′-CATCCTGCCTCTGC-ACCT-3′ and the reverse primer 5′-TCAGGAGGAGCAATGATCTTG-3′. Each reaction mixture (final volume 25 µl) contained a reverse transcriptase template or a negative control (2 µl), $MgCl_2$ (3 mmol/l), primers (0.4 µmol/l), and LC DNA FastSTART DNA Master SYBR Green 1(2 µl) (Roche Diagnostics Corporation, Indianapolis, Indiana, USA). Each PCR reaction was inserted in a LightCycler capillary (Roche). The PCR cycling conditions chosen were; (i) 10 s at 95°C; (ii)5 s at 67°C (PTEN), and 58°C (AKT1 and AKT2); and (iii) 26 s (PTEN), and 6 s (AKT1 and AKT2) at 72°C. A melting curve was generated for each reaction and the conditions were: (i) 95°C, (ii) 30 s at the annealing temperature, and (iii) temperature increased to 95°C (0.2°C/s). Finally, the DNA concentration of each reaction was determined quantitatively using a standard curve. The PCR reaction products were applied to a 1.2% agarose gel and separated by electrophoresis for 45 min. Bands were visualized by ethidium bromide staining and compared with the 100-bp DNA Marker (TaKaRa Biotechnology, Dalian, China). PCR fragments were cloned and sequenced to confirm the corresponding sequence.

Protein extraction and western blot analysis

Radio immunoprecipitation assay protein lysis buffer was used to separate the cellular proteins, followed by quantification by the BCA Protein Assay Kit (Pierce, Rockford, Illinois, USA). We analyzed the protein expression of PTEN (Ser^{380}/$Thr^{382/383}$), AKT1/2, AKT phosphorylated at Thr^{308} (p-AKT), using Bio-Rad electrophoresis apparatus and protein transfer equipment (MiniProtean; Bio-Rad, Hercules, California, USA) and Kodak XOMAT AR film (PerkinElmer Life and Analytical Sciences, Inc., Waltham, Massachusetts, USA). The antibodies used to probe the western blot and their dilutions were as follows: PTEN 1 : 500, p-PTEN 1 : 1000, AKT1/2 1 : 200, p-AKT 1 : 1000.

Statistical methods

Statistical analyses were carried out using the SPSS 14.0 (SPSS Inc., Brookfield, Wiconsin, USA) software package. All experimental data were derived from experiments that were repeated at least three times. Results are expressed as the mean value ± standard deviation. The cytotoxicity of different drug combinations was compared by the one-way analysis of variance. Differences between experimental groups were determined by the Tukey's test. A level of $P < 0.05$ was accepted as statistically significant.

Results

Effect of paeonol on the growth of human hepatocellular carcinoma cell lines

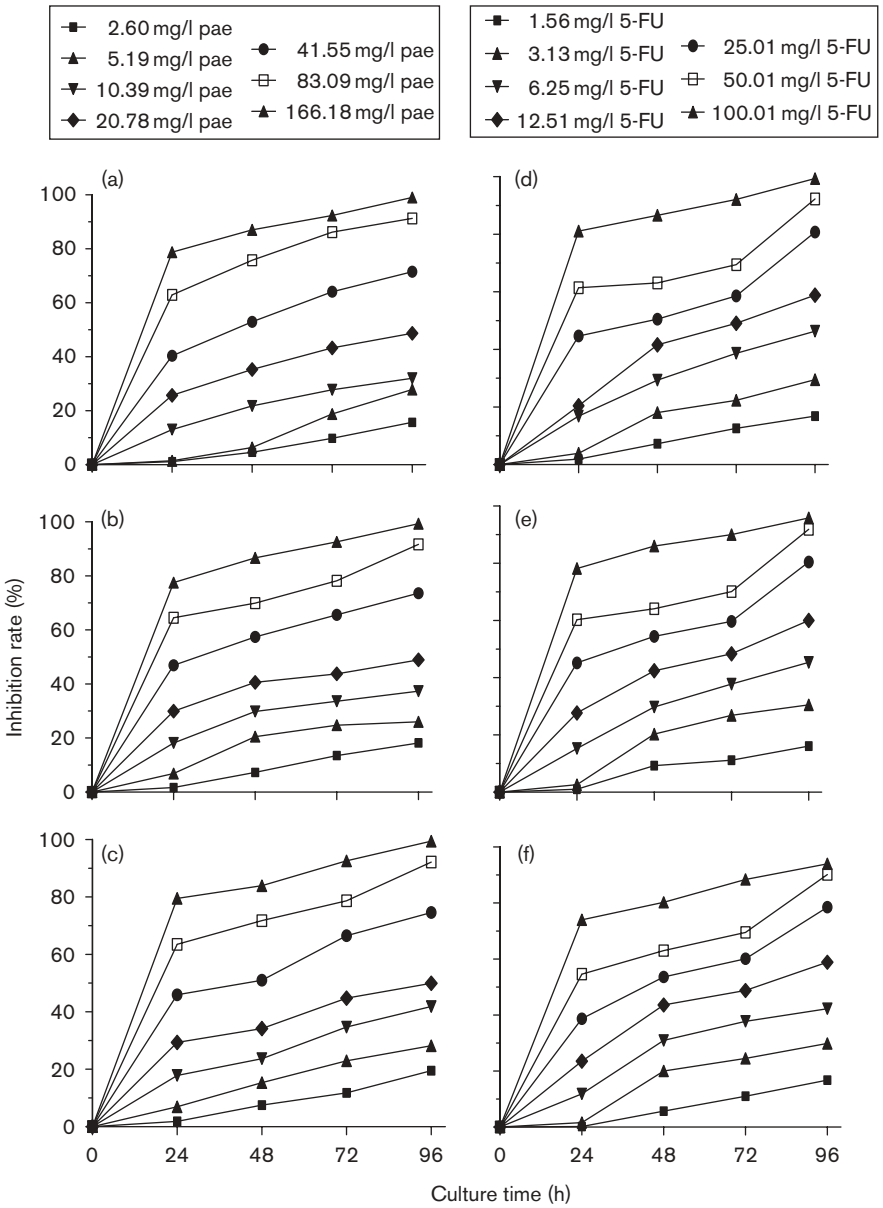
Traditional antineoplastic therapy is based on the use of chemotherapeutic compounds, which exert a cytotoxic effect on proliferating cells and promote the destruction of sensitive tumors. As a preliminary screening of its antiproliferative activity, the in-vitro cytotoxic effect of paeonol was evaluated on a panel of HCC cell lines by MTT assay. For comparison, the cytotoxic activity of 5-FU was evaluated under the same experimental conditions. Exposure of HCC cell lines to paeonol in increasing concentrations resulted in a strong dose-dependent, clonogenic growth inhibition (Fig. 2a–c). Paeonol and 5-FU (Fig. 2d–f) each significantly inhibited the growth of the HCC cell lines cells. The MTT assays showed that paeonol inhibited the growth of HCC cells in a dose/time-dependent manner.

Paeonol enhances the cytotoxicity of chemotherapeutic drugs on three human hepatocellular carcinoma cell lines

Growth inhibition assays were carried out to investigate whether paeonol could enhance the antiproliferative effects of a chemotherapeutic agent (5-FU) on the three HCC cell lines. Seven doses of paeonol were combined with different concentrations of 5-FU. For each experiment, a dose–response curve of each single chemotherapeutic agent and its combination with paeonol was determined. The curves demonstrated that paeonol increased the cytotoxicity of 5-FU on the three HCC cell lines (Fig. 3). The IC_{50} value of the combination of paeonol and 5-FU was decreased to varying extents amongst the three HCC cell lines when compared with the IC_{50} value of either drug by itself (Fig. 4).

We analyzed the nature of the interaction between paeonol and 5-FU using CDI, which quantitatively measures the interaction of two drugs. Paeonol and

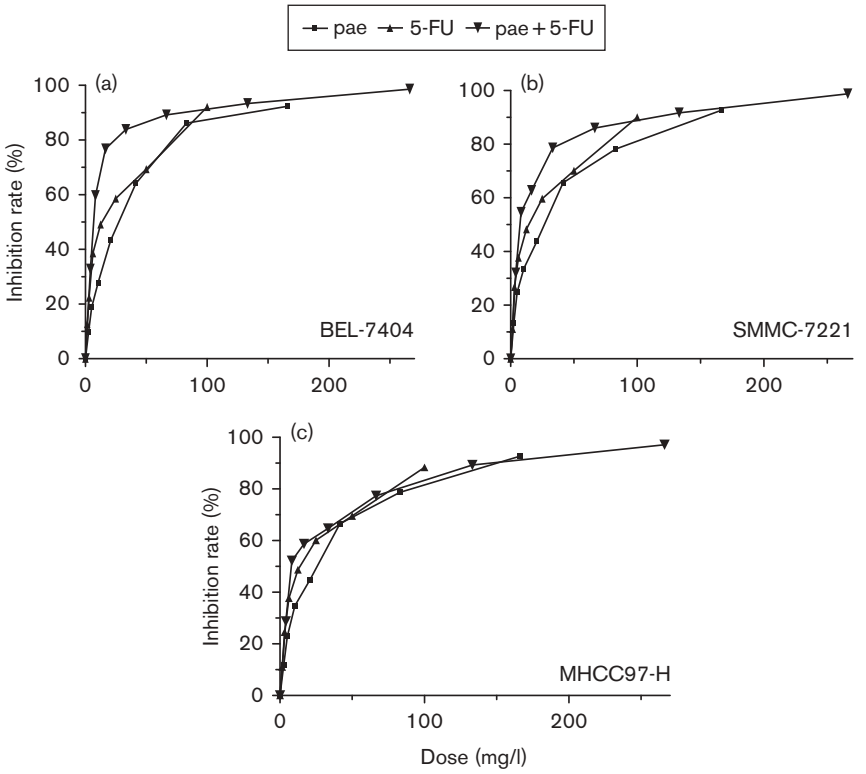
Fig. 2



Paeonol (pae) and 5-fluorouracil (5-FU) inhibited the proliferation of three human hepatocellular carcinoma (HCC) cell lines. HCC cell lines (BEL-7404, SMMC-7721, and MHCC97-H) were treated with pae (2.60–166.18 mg/l) and 5-FU (1.56–100.018 mg/l) for varying times and cytotoxicity was analyzed by MTT assay. (a) BEL-7404, (b) SMMC-7721, and (c) MHCC97-H cells treated with pae. (d) BEL-7404, (e) SMMC-7721, and (f) MHCC97-H cells treated with 5-FU. Each data point represents the mean fractional death rate.

5-FU yielded synergistic interactions across a wide concentration range. The synergistic effect in BEL-7404 cells most prominent when 5.19, 10.39, and 20.78 mg/l paeonol was combined with 3.13, 6.25, and 12.51 mg/l 5-FU, respectively (Fig. 5a). The CDI was 0.6358, 0.5209, and 0.5537, respectively, for the previously mentioned drug combinations. An antagonistic effect was obtained when paeonol reached 83.09 and 168.18 mg/l in combination with 50.01 and 100.01 mg/l 5-FU, respectively. In SMMC-7721 cells, the synergistic effect was observed over a wide concentration range of the two drugs and the CDI was between 0.7473 and 0.9765. An antagonistic effect was obtained when paeonol reached 83.09 and 168.18 mg/l in combination with 50.01 and 100.01 mg/l 5-FU (Fig. 5b). In MHCC97-H cells, a significant synergistic effect was only obtained when 2.6 and 5.19 mg/l paeonol was combined with 1.56 and 3.13 mg/l 5-FU. The CDI were 0.9084 and 0.9922, respectively. An

Fig. 3



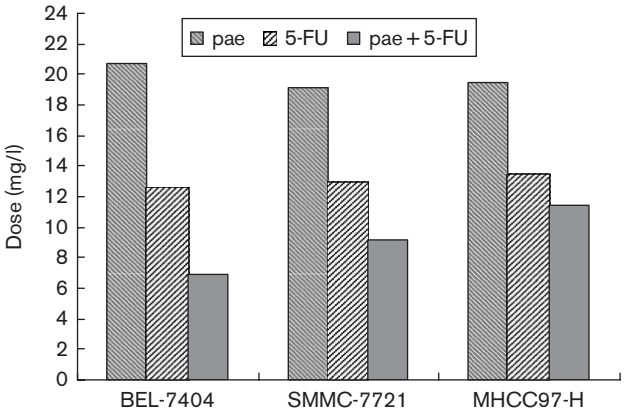
Inhibition rate of paeonol (pae), 5-fluorouracil (5-FU), and pae combined with 5-FU on three human hepatoma cell lines for 72 h.

antagonistic effect was obtained with other concentrations of paeonol combined with 5-FU (Fig. 5c).

Paeonol, 5-fluorouracil and paeonol combined with 5-fluorouracil induce apoptosis and DNA fragmentation in the BEL-7404 cell lines

Apoptotic DNA was detected over time after exposure to paeonol or 5-FU individually or in combination at the IC_{50} concentration (the actual IC_{50} dosages used are shown in Fig. 6). With increased exposure time, enhanced DNA fragmentation was observed in the BEL-7404 cell line (Fig. 7). The drugs induced apoptosis at 12 h and apoptosis progressed over time. At 72 h, no DNA fragmentation could be detected because of vast cell death. These results suggest that paeonol's action on HCC cell lines includes inhibition of cell growth and induction of cell apoptosis.

Fig. 4

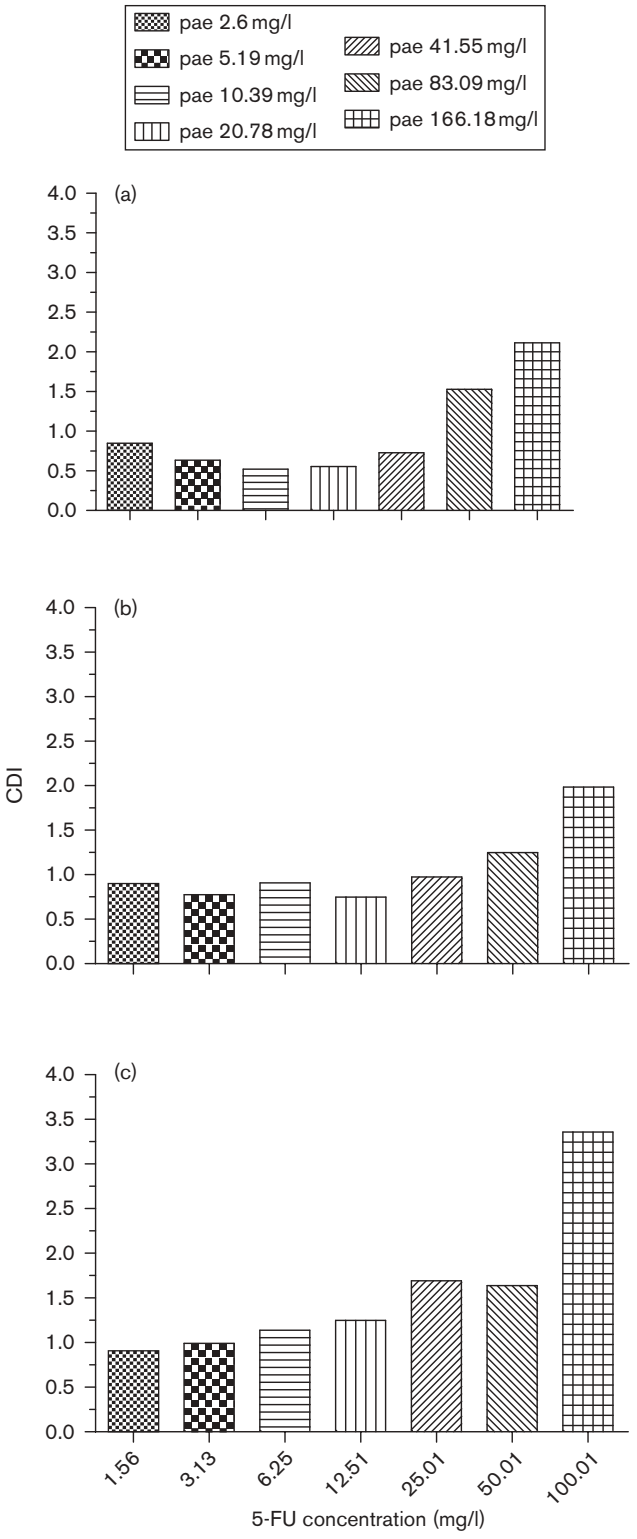


Determination of the 72 h IC_{50} of paeonol (pae), 5-fluorouracil (5-FU), and pae + 5-FU on three human hepatocellular carcinoma cell lines.

Effects of paeonol on mRNA of PTEN, AKT1/2 of BEL-7404

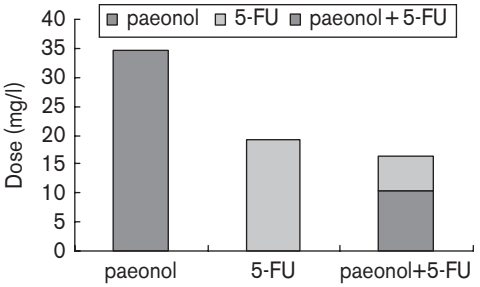
PTEN is a tumor suppressor gene [19] that inhibits the activation of the oncogene AKT [20]. AKT has three main isoforms, AKT1, AKT2, and AKT3, of which AKT1 and AKT2 have both been associated with tumorigenesis [21]. To determine the basal levels of PTEN and AKT1/2 mRNA in control or treated BEL-7404 cells, quantitative real-time RT-PCR studies were carried out using specific primers chosen from human DNA sequences. We generated 112-bp human AKT1, 145-bp AKT2, and

Fig. 5



The synergistically antiproliferative effect of paeonol (pae) combined with 5-fluorouracil (5-FU) on three hepatocarcinoma cell lines, BEL-7404 (a), SMMC-7721 (b), and MHCC97-H (c). The coefficient of drug interaction (CDI) for the combination treatment is shown on the *y*-axis.

Fig. 6



The concentration of the 48-h IC_{50} of paeonol, 5-fluorouracil (5-FU), and paeonol + 5-FU on the BEl-7404 cell line, which was used to cultured the cells for varying times to induce apoptosis.

723-bp PTEN amplicons from the total RNA samples isolated form HCC cell lines. Paeonol treatment reduced the expression of AKT1 and AKT2 mRNA. PTEN mRNA was markedly increased in all three paeonol treatment groups compared with the control group ($P < 0.05$, $n = 6$; Fig. 8).

Effects of paeonol on protein expression of PTEN, p-PTEN, AKT, and p-AKT

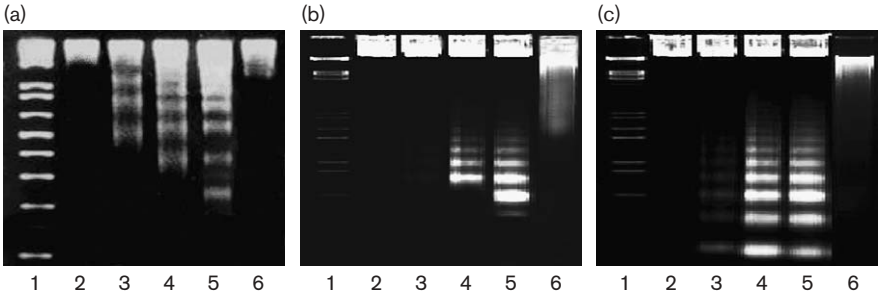
To confirm that the results obtained at the mRNA level corresponded at the protein level, western blot analysis was carried out. We detected the 60-kDa PTEN, 60-kDa AKT1/2, 60-kDa p-PTEN, and 60-kDa $pThr^{308}$-AKT proteins from BEL-7404 cells (Fig. 9a). Digital image analysis of the western blot signals demonstrated significantly higher levels of PTEN and lower levels of AKT1/2 proteins in the paeonol-treated group relative to control (Fig. 9b).

PTEN is inactive when it is phosphorylated [22,23]. Inversely, AKT is activated when phosphorylated [24]. To determine the effect of paeonol on the activation status of AKT and PTEN, we evaluated their phosphorylation state in BEL-7404 cells treated with paeonol. In all of the paeonol-treated groups, phosphorylated PTEN (p-PTEN) was significantly lower than in the control group. Similarly, AKT phosphorylation (Thr^{308} phosphorylation) was markedly decreased in BEL-7404 cells treated with paeonol.

Discussion

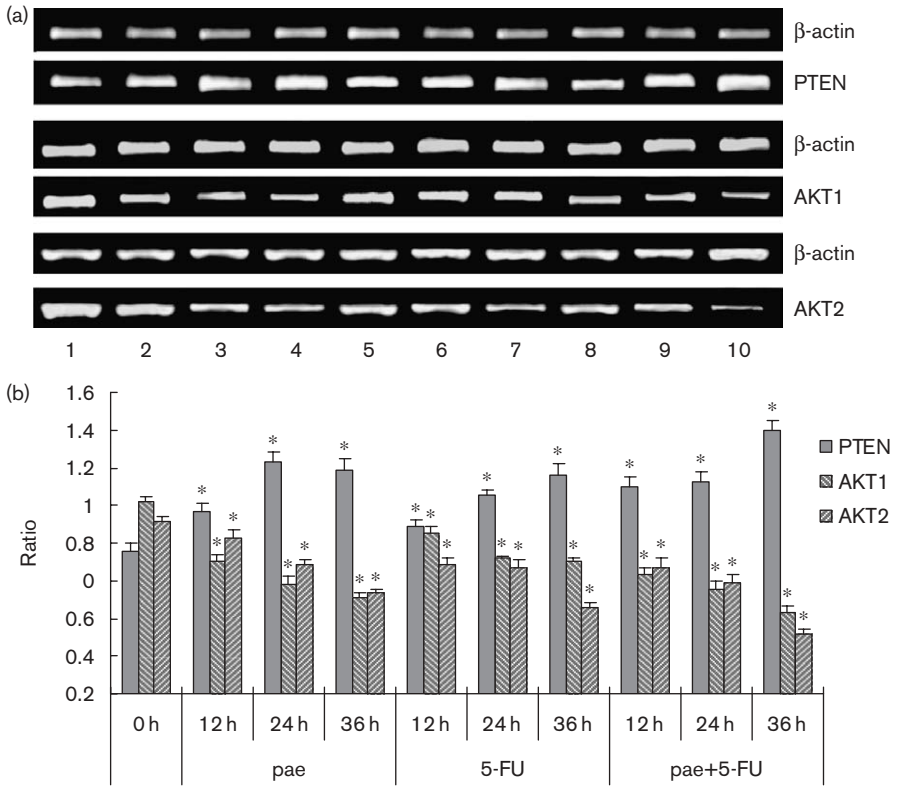
Currently, a variety of cytotoxic and antiproliferative agents have been tested in HCC treatment, which are used alone or in combination with other drugs or other treatment modalities. Agents with partial response rates near or above 10% include 5-FU. High doses of these drugs, however, lead to severe toxicities, which have a negative effect on patients' survival. The use of less toxic doses in combination with other antiproliferative agents would be desirable [25,26].

Fig. 7



DNA fragmentation induced by paeonol. Agarose gel electrophoresis showing DNA fragmentation indicative of cell apoptosis induced by paeonol (a), 5-fluorouracil (5-FU) (b), and paeonol combined with 5-FU (c) in BEL-7404 cells. The concentration of the drugs used as Fig. 6. Lane 1, marker; lane 2, 0 h; lane 3, 12 h; lane 4, 24 h; lane 5, 36 h; lane 6, 72 h.

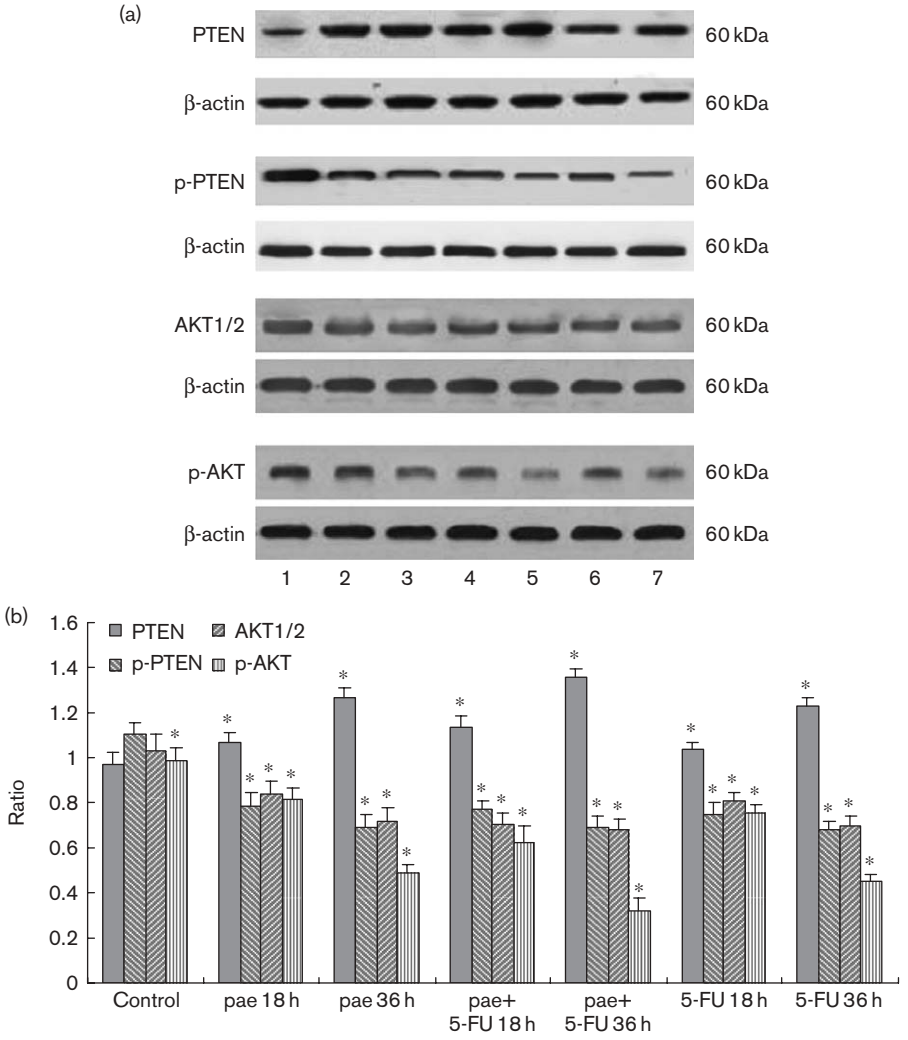
Fig. 8



Paeonol (pae) and 5-fluorouracil (5-FU) altered expression levels of PTEN, AKT1, and AKT2 mRNA. (a) Cells were treated with 72-h IC_{50} concentration of pae, 5-FU and pae combined with 5-FU at 0, 12, 24, and 36 h. Quantitative real-time reverse transcription-PCR analyses of mRNA expression of PTEN, AKT1, and AKT1 in BEL-7404 was carried out. Lane 1, control 0 h; lane 2, pae 12 h; lane 3, pae 24 h; lane 4, pae 36 h; lane 5, 5-FU 12 h; lane 6, 5-FU 24 h; lane 7, 5-FU 36 h; lane 8, pae + 5-FU 12 h; lane 9, pae + 5-FU 24 h; lane 10, pae + 5-FU 36 h. (b) Results represent mean ± SD of six independent experiments. Columns with different superscripts are significantly different (*$P < 0.05$).

In this study, paeonol exhibited an antiproliferative effect on three HCC cell lines in a dose-dependent manner. Although the mechanism of this antiproliferative effect is not known, some reports support that the induction of apoptosis is involved [27]. Induction of apoptosis is an effective strategy for cancer therapy. In this study, the BEL-7404 HCC cell line treated with paeonol showed typical characteristics of apoptosis. Additionally, our results support that PTEN may modulate the PI3K pathway and its potential proliferative signals in these HCC cell lines. In this study, paeonol enhanced PTEN mRNA and protein expression, and reduced the total

Fig. 9



Paeonol (pae), 5-fluorouracil (5-FU), and pae combined with 5-FU altered expression levels of some apoptotic proteins. (a) BEL-7404 cells were treated with pae, 5-FU, paeonol combined 5-FU at 0, 18, and 36 h. The 72-h IC_{50} was used. Equal amounts (80 μg/lane) of cellular protein were isolated and separated on a 10% polyacrylamide denaturing gel. Then, the protein was transferred to a polyvinylidene fluoride membrane and western blot analysis was carried out. Lane1, 0 h; lane 2, pae 12 h; lane 3, pae 18 h; lane 4, pae + 5-FU18 h; lane 5, pae + 5-FU 36 h; lane 6, 5-FU 18 h; lane 7, 5-FU 36 h. (b) Representative figure shows analysis of PTEN, AKT1/2, p-PTEN, and p-AKT expression in BEL-7404. ($n=6$, mean number of ratio ± SD; *$P<0.05$; bars indicate SD).

level of phosphorylated PTEN in HCC cell lines. This would suggest an activation of PTEN activity. PTEN is a negative regulator of the PI3K pathway that blocks the precancer activity of the PI3K pathway [28–30] and, therefore, our observations suggest that paeonol may inhibit the PI3K pathway and its proproliferative effects through the activation of PTEN. In addition, our results suggest that paeonol inhibits the activity of another component of the PI3K pathway. Paeonol inhibited both AKT1 and AKT2 mRNAs and protein, and led to decreased levels of phosphorylated AKT, suggesting that paeonol administration decreases overall levels of AKT activity in the HCC *in vitro*. This would also favor a possible negative regulation of the PI3K pathway in response to paeonol. It is not clear from this study whether paeonol mediates its effects on proteins in the PI3K pathway directly or indirectly through activation of PTEN. Additional studies will need to be carried out to address this issue. Regardless, the changes in overall phosphorylation levels of PTEN and AKT suggesting an activation of PTEN and associated inhibition of AKT activity were associated with the inhibition of HCC cell growth *in vitro*. This observation is consistent with the possibility that paeonol mediates inhibition of tumor growth through inhibition of the PI3K pathway.

The HCC cell lines were also treated with the combination of paeonol and the chemotherapeutic agent 5-FU. The results indicated that the antiproliferative effect of 5-FU was enhanced significantly by paeonol. Among the three cell lines, BEL-7404 and SMMC-7721 showed the greatest synergistic effects. The synergistic effect was most prominent in the BEL-7404 cell line (CDI < 0.7) when 5.19–20.78 mg/l paeonol was combined with 3.13–12.51 mg/l 5-FU. This suggests that the combination of paeonol and 5-FU at certain concentrations might help reduce the side effects of 5-FU. The results also demonstrated that paeonol and 5-FU are synergistic but only at low concentrations. It is possible that a greater antiproliferative effect was not observed at higher concentrations due to an activation of cellular defense mechanisms that modulate and reduce the cytotoxicity of the compounds. This phenomenon of high drug concentrations leading to lesser antiproliferative action has been proposed because of certain effects observed at low toxicant concentrations, such as hormesis [31]. Additonal studies are required to investigate the mechanisms of these synergisms and antagonisms, which favor the reasonable application of paeonol to HCC treatment.

In summary, paeonol, an extract of the Chinese herb medicine Moutan Cortex, alters PTEN/AKT expression in HCC cell lines, creating a potential cellular environment where the tumor suppressor activity of PTEN is high and the oncogenic activity of AKT is inhibited, which ultimately could inhibit tumor growth. These promising data along with the observed lack of toxicity provide a rationale for further exploration of paeonol as a therapeutic agent for hepatoma.

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