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Perifosine induces cell cycle arrest and apoptosis in human hepatocellular carcinoma cell lines by blockade of Akt phosphorylation

Hong-rong Fei · Geng Chen · Jian-mei Wang · Feng-ze Wang

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Abstract Hepatocellular carcinoma (HCC) is one of the most common solid cancers, representing the third cause of cancer-related death among cirrhotic patients. Treatment of advanced HCC has become a very active area of research. Perifosine, a new synthetic alkylphospholipid Akt inhibitor, has shown anti-tumor activity by inhibition of Akt phosphorylation. In this study, the effect of perifosine on the cell proliferation and apoptosis in hepatoma cells has been investigated. Cell growth inhibition was detected by MTT assay, cell cycle was analyzed by flow cytometry, AnnexinV-FITC apoptosis detection kit was used to detect cell apoptosis, and protein expression was examined by Western blotting analysis. Our present studies showed that Akt phosphorylation was inhibited by perifosine in HepG2 and Bel-7402 human hepatocellular carcinoma cells. Perifosine inhibited the growth of HepG2 cells and Bel-7402 cells in a dose-dependent manner, and arrested cell cycle progression at the G_2 phase. Apoptosis induction became more effective with increasing perifosine concentration. The caspase cascade and its downstream effectors, Poly (ADP-ribose) polymerase (PARP), were also activated simultaneously upon perifosine treatment. The proapoptotic effect of perifosine was in part depending on regulation of the phosphorylation level of ERK and JNK. Perifosine cotreatment substantially increased cytotoxic effects of cisplatin in HepG2 cells. Down-regulating the expression of Bcl-2 and up-regulating the level of Bax may be the potential mechanism for this synergistic effect. Our findings suggest that the small molecule Akt inhibitor perifosine shows substantial anti-tumor activity in human hepatoma cancer cell lines, and is a good candidate for treatment combinations with classical cytostatic compounds in hepatocellular carcinoma.

Keywords Hepatocellular carcinoma · Perifosine · PI3 K/Akt · Apoptosis · Caspase · Bcl-2

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common and malignant disease worldwide. It is a major health problem and the prognosis for HCC patients is still very poor. From World Health Organization (WHO) statistics in 2000, it has been estimated that there were at least 5,64,000 new cases of HCC per year around the world (Yuen et al. 2009). More than 70% of all newly diagnosed liver cancers

occur in Asia, and China alone accounts for 55% cases of HCC worldwide (Parkin et al. 2005). Extensive epidemiological evidence suggests that chronic alcohol intake, aflatoxin exposure, and cirrhosis of any etiology are clear risk factors for HCC development besides of viral infections (HBV or HCV) (Mínguez et al. 2009). Nowadays, the molecular pathogenesis of HCC is under intense investigation in order to identify new means to prevent or treat this global health problem. Like most cancers, HCC is also a heterogeneous tumor with a complex variety of genetic changes, viral infection, and environmental factors, regulated by different signaling pathways (Boucher et al. 2009).

Phosphatidylinositol-3-kinase (PI3 K)/Akt pathway plays an important role in the development and growth of various types of cancer, including hepatocellular carcinoma. Akt, also known as protein kinase B (PKB), plays a key role in protecting the cells from various types of apoptotic stimuli and regulating cell proliferation and cell cycle by interacting, either directly or indirectly, with numerous other regulatory proteins such as p53, cyclin D_1, p21Cip1, glycogen synthase kinase-3, and mammalian target of rapamycin (Franke 2008; Manning and Cantley 2007). Alterations of the Akt pathway have been detected in a variety of human malignancies cancer, and blockage of Akt signaling results in programmed cell death and growth inhibition of tumor cells (Fulda 2009). For this reason, Akt has become a promising drug target for the cancer therapy.

Many inhibitors of PI3 K/Akt are being developed. The first-generation PI3 K inhibitors include LY294002 and wortmannin, both targeting the catalytic site of p110, which have been used as research tools to elucidate the value of PI3 K as therapeutic target (Vlahos et al. 1994). For the unfavorable pharmaceutical properties, toxicity, and crossover inhibition of other lipid and protein kinases, they were not extensively used in clinical trials (Carnero et al. 2008). Due to feed back activation of Akt that results from mTOR inhibition, inhibiting Akt directly may have advantages over targeting more distal components of the pathway. Perifosine, a synthetic alkylphosphocholine anti-tumour agent, is orally active and inhibits Akt activation by targeting the pleckstrin homology domain of Akt (Patel et al. 2002; Gills and Dennis 2010). The clinical efficacy of the drug is currently evaluated in a phase I/II clinical trial in patients with advanced solid tumors, which has shown anti-tumor activity in a variety of cancers such as breast and prostate cancer in vitro and in vivo (Chiarini et al. 2008; Kondapaka et al. 2003).

The current study investigates a potential anti-tumor activity of perifosine, in vitro models of hepatocellular carcinoma, using perifosine either on its own or in combination with an established chemotherapeutic drug. We demonstrate that perifosine inhibits the proliferation and induces the apoptosis of hepatoma cell lines by regulating the phosphorylation level of ERK and JNK. Cotreatment with perifosine substantially increases cytotoxic effect of cisplatin in hepatocellular carcinoma cells. Our results indicate that perifosine is a promising drug for treatment of hepatocellular carcinoma characterized by PI3 K/Akt signaling downregulation.

Materials and methods

Reagent

Perifosine was obstained from Selleck Chemicals (Houston, TX, USA), and dissolved with 100% DMSO. Cisplatin was purchased from the Ge Jiu Biological Pharmacy (Gejiu, P.R. China). JNK Inhibitor SP600125 was purchased from Merck-chemicals (Darmstadt, Germany); RPMI 1640 medium was purchased from GIBCO (BRL, USA); fetal calf serum was purchased from Lanzhou National HyClone Bio-Engineering (Lanzhou, Germany); 3-[4, 5-dimethylthiazol-2-y-l]-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Genview (Houston, TX, USA); propidium iodine (PI) and 4′, 6-Diamidino-2-phenylindole dihydrochloride hydrate (DAPI) were purchased from Sigma Chemical Co (St Louis, MO, USA); Annexin V-FITC apoptosis detection kit was purchased from KeyGEN Biotech (Nanjing, P.R.China); ECL Western Blotting Detection Reagents was purchased from Thermo Scientific Pierce (Rockford, IL, USA).

Cells and cell culture

HepG2 cell line was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), and Bel-7402 cell line was obtained from the KeyGEN Biotech. Cells were

cultured in RPMI 1640 medium containing 10% heat-inactivated in a humidified atmosphere (37.0 °C; 5% CO_2).

MTT assay

Cell proliferation was measured by using MTT method. Briefly, an amount of 200 μL cell suspensions (5×10^3 cells/mL) was added to 96-well plates and incubated for different time. An amount of 20 μL MTT (5 mg/mL) was added for 4 h. After removing supernatant, 150 μL dimethylsulfoxide (DMSO) was added to resolve formazan crystals, and the value of optical density (O.D) was detected at 490 nm. The coefficient of drug interaction (CDI) was used to analyze the synergistically inhibitory effect of drug combinations. CDI is calculated as follows: CDI = AB/(AXB). 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 of less than, equal to or greater than 1 indicates that the drugs are synergistic, additive or antagonistic, respectively. CDI less than 0.7 indicate that the drugs are significantly synergistic (Wang et al. 2008).

Flow cytometry analysis

The DNA contents of cells were measured by the PI staining method. Cells (1×10^6) were harvested by trypsinization and washed twice with PBS. Washed cells were resuspended in 0.5 mL PBS (pH 7.4) and fixed by addition of 1.5 mL 100% ethanol for at least 18 h at 4 °C. Fixed cells were rinsed twice with PBS, and re-suspended in PBS without calcium and magnesium containing 50 μg/mL RNase A (Sigma), then incubated in 37 °C for 30 min. PI was added to the cells suspension (50 μg/mL) and incubated in the dark for 20 min. Stained cells were analyzed by a FAC Scan flow cytometer and Cell Quest analysis software (Becton–Dickinson, CA). Annexin V-FITC apoptosis detection kit was used for detection of cell apoptosis. Annexin V staining was performed according to the manufacturer's protocols. In briefly, after two treatments with washing buffer, cells were resuspended in 400 μL of Dulbecco's PBS and incubated with 10 μL PI and 10 μL of Annexin V-FITC for 15 min at room temperature in the dark. Cells were analyzed by flow cytometry.

Morphological evaluation of apoptotic cells

The detailed procedures were followed accordingly (Duan et al. 2009). In brief, hepatoma cells at 70% confluence were treated for 48 h with perifosine at concentrations of 0 (DMSO, vehicle as control) and 10 μmol/L, respectively. The treated cells were fixed with 95% ethanol for 30 min at 4 °C, washed in PBS, and stained with 1 mmol/L DAPI for 30 min at room temperature. The morphological changes in the nuclear chromatin were observed under a fluorescent microscope (Olympus, Japan).

Western blotting assay

Western blotting analysis was performed after treatment with different doses of perifosine. Cells were washed twice in cold PBS and lysed with ice-cold lysis buffer (150 mmol/L NaCl; 20 mmol/L Tris–HCl, pH 7.4, 0.1% SDS, 1% NP-40, 0.5% Na-DOC, 0.2 mmol/L PMSF, and protease inhibitor cocktails) for 30 min on ice. Lysates were centrifuged at 13,000 g with 20 min and the supernatants were used as total cell lysates. Protein concentration was determined by Bradford protein assay (Bio-Rad, Hercules, CA, USA). A quantity of 30–40 μg total protein per lane was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, USA). Membranes were blocked with 5% milk powder in 0.05% Tween-TBS, incubated with the specific antibodies such as mouse anti-β-actin (Sigma, 1:10000 dilution), rabbit anti-Akt (Cell signaling, Dallas, TX, USA, 1:1000 dilution), rabbit anti-p-Akt (Cell signaling, 1:300 dilution), rabbit anti-ERK (Cell signaling, 1:800 dilution), rabbit anti-caspase-9 (Cell signaling, 1:600 dilution), rabbit anti-caspase-3 (Cell signaling, 1:500 dilution), rabbit anti-Cleaved caspase-3(Cell signaling, 1:300 dilution), rabbit anti-PARP (Cell signaling, 1:800 dilution), anti-cyto c (Santa Cruz, CA, USA, 1:400 dilution), mouse anti-p-ERK (Cell signaling, 1:600 dilution), mouse anti-JNK1/2 (SAB, Pearland, TX, USA, 1:800 dilution), mouse anti-p-JNK1/2 (Santa Cruz, 1:600 dilution), mouse anti-p53 (Santa Cruz, 1:800 dilution), mouse anti p-p38 (Santa Cruz, 1:600 dilution), rabbit anti-cyclinB_1 (Santa Cruz, 1:800 dilution), rabbit anti-cyclinD_1 (Santa Cruz, 1:500 dilution), mouse

anti-p21 (NeoMarkers, CA, USA, 1:500 dilution), rabbit anti-Bcl-2 (Bioworld, USA, 1:600 dilution), rabbit anti-Bax (Santa Cruz, 1:400 dilution), and the horseradish peroxidase-conjugated secondary antibodies such as rabbit anti-mouse IgG (Zhong shan, Beijing, P.R.China, 1:5000 dilution), goat anti-rabbit IgG (Zhong shan, 1:5000 dilution), rabbit anti-goat IgG (Santa Cruz, 1:6000 dilution) were diluted in 1%BSA/Tween-TBS, respectively. Detection of the target proteins on the membranes was performed using the ECL Western Blotting Detection Reagents.

Statistical analysis

All the data were expressed as mean ± standard deviation (SD). Statistical analysis was performed by the Student's t test, $P < 0.05$ was indicated to be statistical significance.

Results

Perifosine specifically blocks the PI3 K/Akt pathway in hepatoma cells

Since perifosine is claimed to be an Akt inhibitor, we performed Western blotting assay to show the inhibitory effect of it on the modulation of the PI3 K/Akt pathway in hepatoma cells. When exposed to the HepG2 cell line, perifosine reduced the levels of S473p-Akt in a dose-dependent manner (Fig. 1). A similar pattern of inhibition was also observed in Bel-7402 cells. However, no significant changes on total Akt protein levels were observed, showing that the perifosine has no effect on total Akt protein stability.

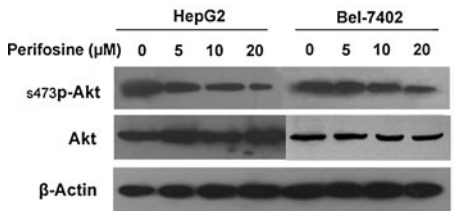


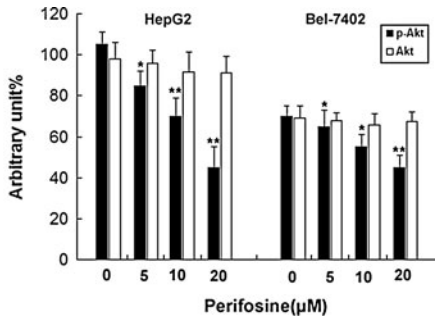
Fig. 1 Western blotting analysis demonstrating ^{Ser473}p-Akt and total Akt levels in HepG2 cells and Bel-7402 cells treated with increasing concentrations of perifosine for 24 h. Beta-actin served as loading control. Bands were analyzed by Glyco Band-Scan software. Each bar corresponds to the mean ± SD for at least three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. control, Student's t test

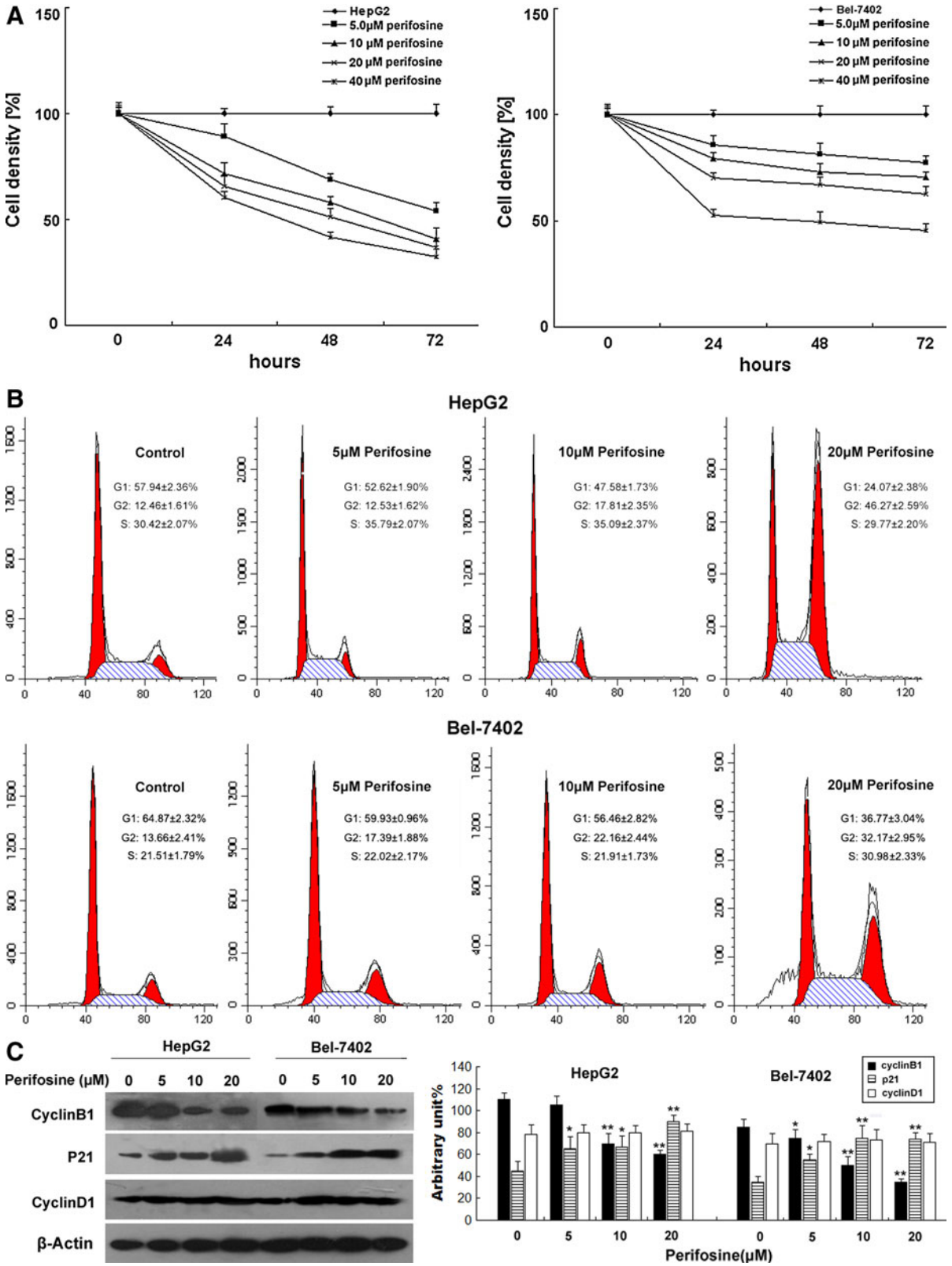
Perifosine inhibits cell growth and induces apoptosis in hepatoma cells

Both HepG2 cells and Bel-7402 cells were incubated with increasing concentrations of perifosine for 72 h. Cell growth was determined every 24 h using MTT assay. As indicated in Fig. 2a, hepatoma cells showed a time- and a dose-dependent reduction in cell proliferation when treated with perifosine.

As perifosine blocks hepatoma cells proliferation, we assume a cell cycle arrest upon Akt inhibition with perifosine. Dose–response analysis was performed that HepG2 cells and Bel-7402 cells were incubated with increasing concentrations of perifosine. As illustrated in Fig. 2b, perifosine treatment resulted in the accumulation of hepatoma cells in the G_2/M phase. Proliferation control HepG2 cells yielded 57.94 ± 2.36% cells in the G_0/G_1 phase,

Fig. 2 Perifosine inhibits cell growth in hepatoma cell lines HepG2 and Bel-7402. **a** HepG2 and Bel-7402 human hepatoma cells were incubated with none, 5, 10, 20 or 40 μM of perifosine for 72 h and cell growth was determined by the MTT assay every 24 h. **b** For the cell cycle analysis, cells were seeded at 3×10^5 per well in six-well paltes in triplicates and incubated with perifosine (5, 10, and 20 μM). After treated with perifosine for 24 h, cells were fixed, permeabilized, stained with PI and analyzed by flow cytometry. **c** Perifosine down-regulates cyclinB_1 level and up-regulates p21 expression in hepatoma cancer cells. Cells were cultured for 24 h in the presence of increasing doses perifosine (5, 10, and 20 μM). Bands were analyzed by Glyco Band-Scan software. * $P < 0.05$, ** $P < 0.01$ vs. control, Student's t test




A
HepG2
5.0μM perifosine
10 μM perifosine
20 μM perifosine
40 μM perifosine
Cell density [%]
hours
Bel-7402
5.0μM perifosine
10 μM perifosine
20 μM perifosine
40 μM perifosine
Cell density [%]
hours
B
HepG2
Control
G1: 57.94±2.36%
G2: 12.46±1.61%
S: 30.42±2.07%
5μM Perifosine
G1: 52.62±1.90%
G2: 12.53±1.62%
S: 35.79±2.07%
10μM Perifosine
G1: 47.58±1.73%
G2: 17.81±2.35%
S: 35.09±2.37%
20μM Perifosine
G1: 24.07±2.38%
G2: 46.27±2.59%
S: 29.77±2.20%
Bel-7402
Control
G1: 64.87±2.32%
G2: 13.66±2.41%
S: 21.51±1.79%
5μM Perifosine
G1: 59.93±0.96%
G2: 17.39±1.88%
S: 22.02±2.17%
10μM Perifosine
G1: 56.46±2.82%
G2: 22.16±2.44%
S: 21.91±1.73%
20μM Perifosine
G1: 36.77±3.04%
G2: 32.17±2.95%
S: 30.98±2.33%
C
HepG2
Bel-7402
Perifosine (μM) 0 5 10 20 0 5 10 20
CyclinB1
P21
CyclinD1
β-Actin
HepG2
Bel-7402
cyclinB1
p21
cyclinD1
Arbitrary unit%
Perifosine(μM)

30.42 ± 2.07% cells in the S phase, and 12.46 ± 1.61% cells in G_2/M phase of cell cycle 24 h after plating. Treatment of HepG2 cells with 20 μM perifosine increased the percentage of cells in the G_2/M phase to 46.27 ± 2.59% and reduced the percentage of the cells in the G_1 phase. Similar observations were made for Bel-7402 cells. As shown in Fig. 2b, when Bel-7402 cells were treated with 20 μM perifosine, the percentage of cells in the G_2/M phase increased significantly.

Previous studies have shown that G_2/M phase arrest on exposure of HN12 cells and PC-3 cells to perifosine is associated with increase of endogenous p21 (Floryk and Thompson 2008), we asked whether perifosine also up-regulated expression of p21 in hepatoma cells. Western blotting results indicated that perifosine increased p21 level in both HepG2 cells and Bel-7402 cells (Fig. 2c). Moreover, cells treated with perifosine exhibited a dose-dependent decrease in the levels of cyclin B_1, whereas cyclin D_1 levels remained unchanged through the course of treatment.

Perifosine induces cell apoptosis in hepatoma cells

Asymmetry and permeability of the cell membrane were analyzed by Annexin V-FITC and PI staining in hepatoma cells. Cells were incubated with increasing concentrations of perifosine for 24 h. After the incubation period, cells were harvested and further processed according to the protocol of manufacture for flow cytometry analysis. As shown in Fig. 3, induction of apoptosis occurred in cancer cells. Time course experiments revealed that long-term exposure to perifosine resulted in significant apoptosis of both HepG2 cells and Bel-7402 cells (Fig. 3a). DAPI staining showed that the typical morphological changes, such as formation of apoptotic bodies appeared after the cells were treated for 48 h with 10 μM perifosine, whereas the control cells did not show the evident apoptotic morphological changes (Fig. 3b).

Caspase cascade is a key intracellular signaling pathway to mediate cell apoptosis. Caspase-3 and caspase-9 have been proven to be the important components of caspase family. Therefore, we determined activities of caspase-3 and caspase-9 in perifosine-treated cells by Western blotting assay. As shown in Fig. 3c, there is a gradual increase in caspase-3 activities in the perifosine-treated cancer cells. Cleavage of procaspase-9 into the characteristic 37/35-kDa fragments was already evident after treatment with 10 μM perifosine. As caspase dependant cleavage of PARP is the prime hallmark for caspase dependent apoptosis, so we also examine the effect of perifosine on PARP. As expected, perifosine induced the cleavage of PARP in a dose dependent manner, but the level of p53 and Bcl-2 was not affected in the HepG2 cells.These results indicate that perifosine treatment induces apoptotic death in hepatoma cells, at least in part through a caspase-dependent pathway.

Perifosine triggers the activation of JNK and inhibition of ERK1/2

Based on these findings and previous reports, we sought to examine whether perifosine reduced hepatoma cells proliferation and induced apoptosis was accompanied by the regulation MAPK signaling pathways. Perifosine was found to affect JNK and ERK, which mediated apoptosis in different experimental models. The phosphorylation of JNK was dose-dependently induced by perifosine (Fig. 4a), and the phosphorylation of ERK was inhibited by the perifosine, at variance with other reports. The total levels of JNK and ERK were unaffected by perifosine treatment. At the same time, the phosphorylation of p38 MAPK was not altered.

Next, we used the JNK inhibitor SP600125 to detect whethere JNK activity is essential in the perifosine-induced cell apoptosis. HepG2 cells were incubated with 10 μM perifosine for 24 h with or without pretreatment of 20 μM SP600125. The percentage of apoptosis was analyzed by AnnexinV-FITC/PI assay. As shown in Fig. 4b, JNK inhibition reduced the cell apoptosis caused by perifosine treatment. Therefore, the activation of JNK pathway is suggested to be involved in the perifosine-induced hepatoma cell apoptosis.

Effect of perifosine and cisplatin on hepatoma cells survival

Previous findings indicated that perifosine was a useful agent for the treatment of human endometrial cancer, we finally wanted to know whether perifosine could enhance the effect of current chemotherapeutical protocols. HepG2 cells were cultured with serial concentrations of perifosine (range 0.5–6.0 μmol/L)

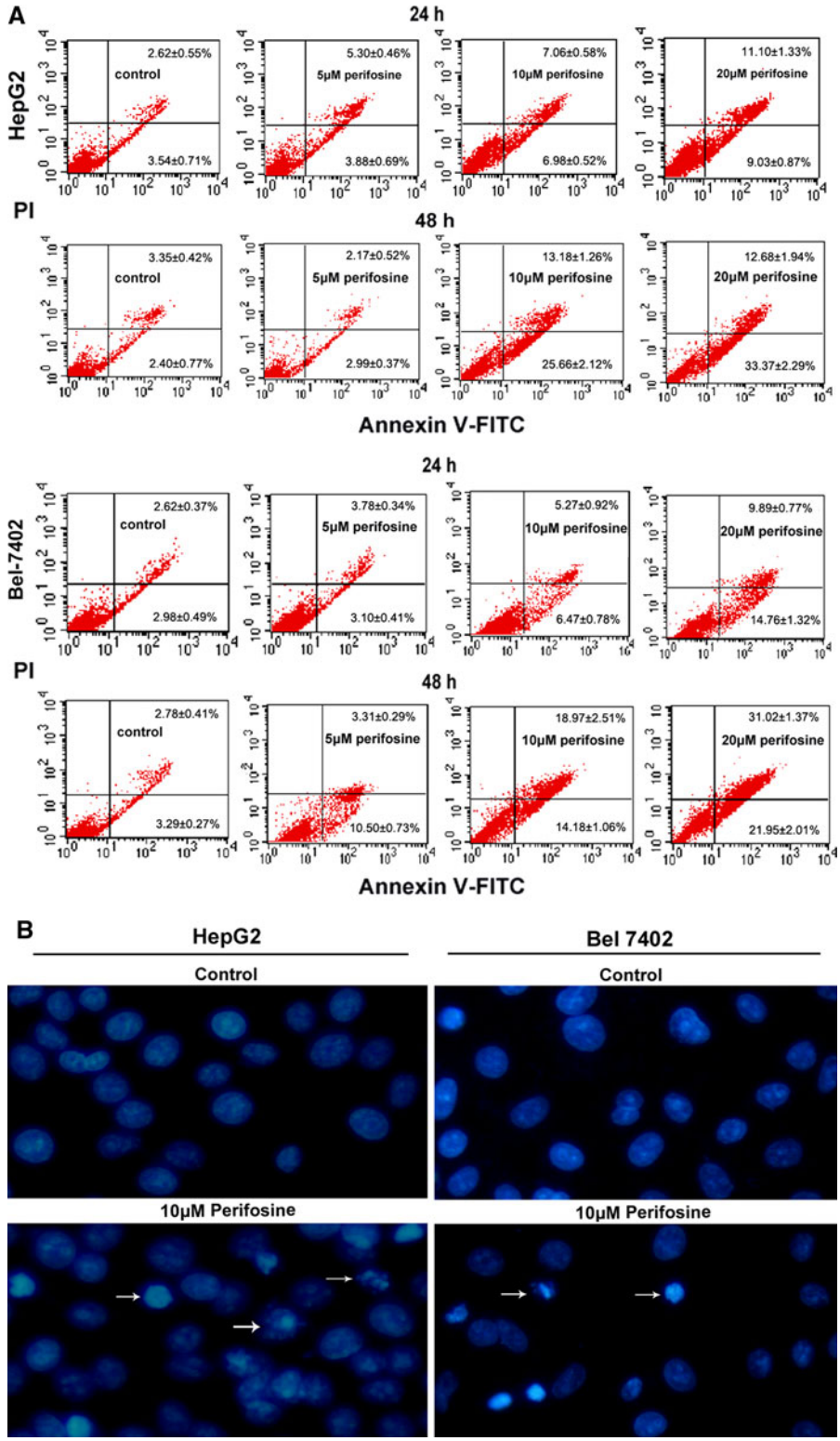
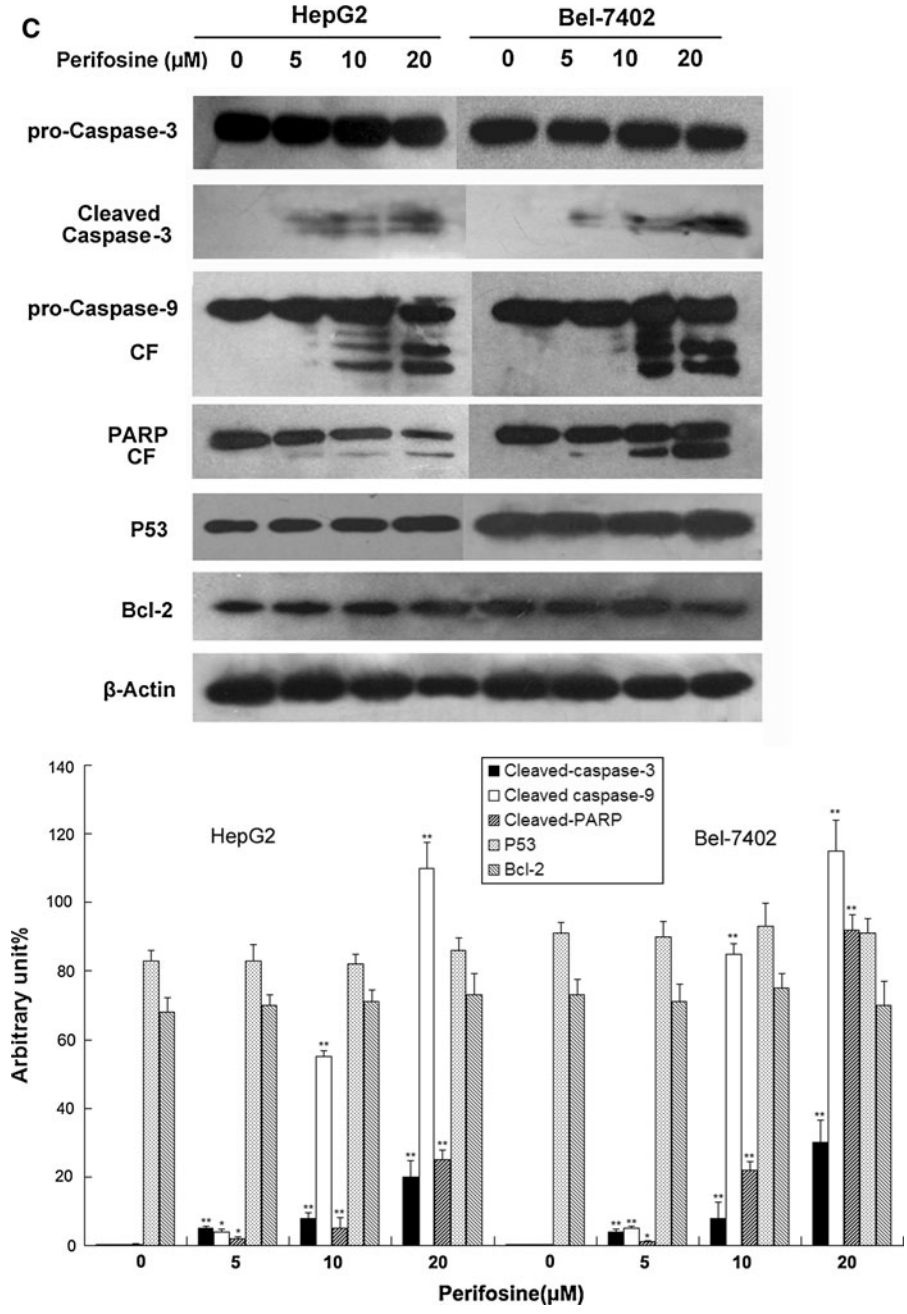


Fig. 3 Perifosine induces HepG2 cells and Bel-7402 cells apoptosis. **a** Annexin V-FITC/PI staining analysis of human hepatocellular carcinoma cell lines treated with perifosine. **b** Induction of apoptosis in hepatoma cells by treatment for 48 h with 10 μM perifosine. The treated cells were stained with DAPI and the apoptotic morphological changes in the nuclear chromatin were observed under a fluorescent microscope. **c** Western blotting assay showing cleavage of caspase-3, caspase-9 and PARP in response to perifosine treatment in hepatoma cells. Each bar corresponds to the mean ± SD for at least three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. control, Student's *t* test

Fig. 3 continued



and cisplatin (range 0.05–0.6 μg/mL) at a constant ratio for 24 h. As evaluated by MTT assays (Fig. 5a), the combined treatment was much more cytotoxic than either of the single treatments. All the combinations gave an effect which ranged from synergistic (CDI < 1.0) to highly synergistic (CDI < 0.7; Fig. 5b) except the lowest group (perifosine $=$ 0.5 μmol/L; cisplatin $=$ 0.05 μg/mL). The results suggest that perifosine can act synergistically with cisplatin in treating hepatoma cancer. To determine whether decreased cell survival was related to apoptosis, Annexin V-FITC/PI analysis was performed. Results of flow cytometry indicated that the combination of perifosine and cisplatin induced higher apoptotic cell death of hepatoma cell than the single drugs using (Fig. 5c).

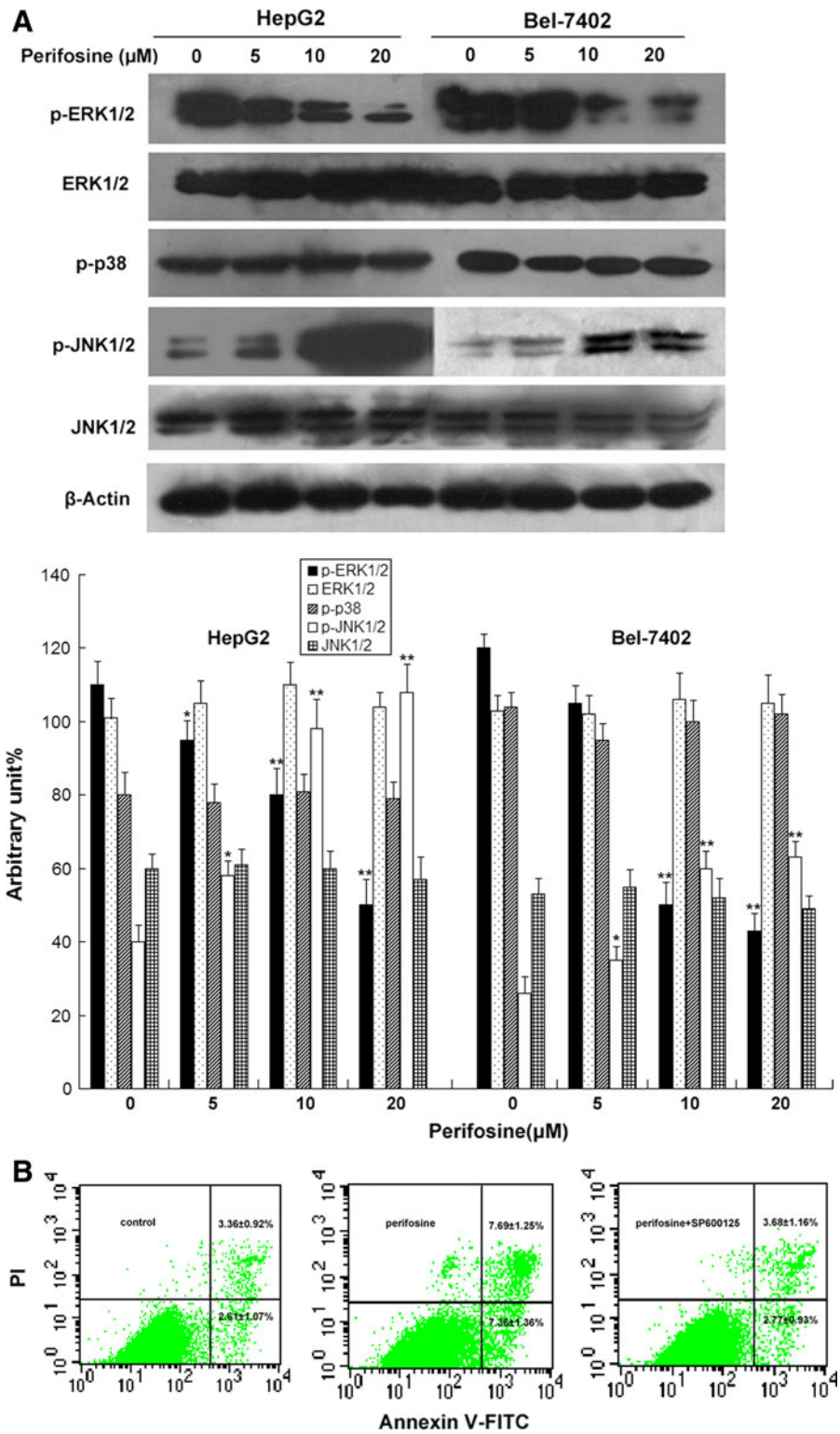


Fig. 4 **a** Perifosine regulates the phosphorylation of ERK and JNK. The antibody to beta-actin demonstrated equal loading in the lanes. Bands were analyzed by Glyco Band-Scan software. Each bar corresponds to the mean ± SD for at least three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. control, Student's t test. **b** Effects of SP600125 on perifosine-induced apoptosis of HepG2 cells, determined by Annexin V-FITC/PI apoptotic kit. HepG2 cells were pre-treated with or without 20 μmol/L JNK inhibitor SP600125 for 60 min. Cells were then exposed or not exposed to 10 μmol/L perifosine for 24 h. Experiment was repeated three times

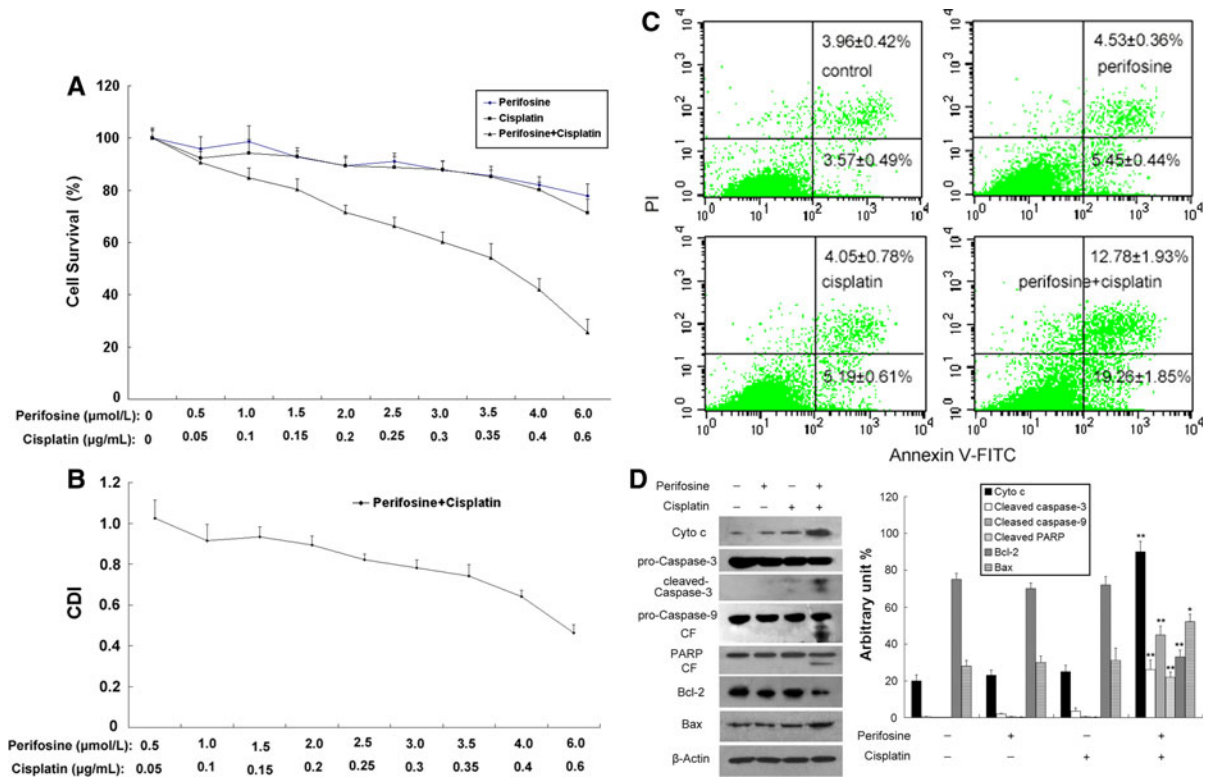


Fig. 5 Perifosine synergistically enhances cisplatin-induced cell death in HepG2 cells. **a** HepG2 cells were treated for 24 h with either perifosine or cisplatin alone and in combination at the indicated concentrations. **b** CDI was calculated from experiments reported in A. CDI less than 0.7 indicate that the drugs are significantly synergistic. The results showed that perifosine and cisplatin synergistically influenced apoptosis of HepG2 cells. **c** Annexin V-FITC/PI staining analysis of HepG2 cells treated with either perifosine (3.5 μM) or cisplatin (0.35 μg/mL) alone and with two drugs together for 24 h. **d** Western blotting analysis of expression of caspase and Bcl-2 family members in HepG2 cells treated with perifosine or cisplatin and with two drugs together. Each bar corresponds to the mean ± SD for three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. control, Student's t test

We next investigated the levels of cytochrome c in the soluble cytosolic fractions of HepG2 cells after perifosine or cisplatin treatment for 24 h. Cotreatment with perifosine and cisplatin increased much more cytochrome c to be released into the cytosol than either drug alone (Fig. 5d). Furthermore, we examined the cleavage of procaspase-3 and procaspase-9 in cells by Western blotting assay. As shown in Fig. 5d, both caspases were cleaved into the characteristic active fragments by cotreatment with perifosine and cisplatin in the HepG2 cells. The cytochrome c-initiated caspase cascade has been shown to be regulated by the Bcl-2 family proteins. Therefore, we were to test if perifosine promoted cisplatin-induced cell death through regulation of Bcl-2 family members. We analyzed Bcl-2 and Bax expression levels in cells treatment with perifosine and cisplatin. Western blotting results showed that combined-treatment caused a significant downregulation of Bcl-2 as well as upregulation of Bax than in cells treated with each reagent alone.

Discussion

PI3 K/Akt signaling pathway plays a central role in diverse cellular functions, including proliferation, apoptosis, survival and metabolism. All of these cellular processes are being considered as crucial features for the establishment of the tumorigenic phenotype. The prevalence of PI3 K/Akt signaling abnormalities in human cancer cells has suggested the potential use of Akt pathway modulators as novel targeted therapeutic agents. Perifosine is structurally

related to miltefosine (Zeisig et al. 1998), approved for use in Europe for topical treatment of cutaneous lymphomas and cutaneous metastases from breast cancer (Leonard et al. 2001). In phase-I/II studies for advanced solid tumors, perifosine did not cause significant hematological toxicity.

In this study, we first detected the phosphorylation of Akt and total Akt protein levels in hepatocellular carcinoma cells by Western blotting assay. Decreased level of S473P-Akt was observed during the course of growth inhibition by perifosine treatment (Fig. 1), and inhibition of cell growth by perifosine was both dose- and time-dependent (Fig. 2a). To examine the antiproliferative effect of perifosine in more detail, cell cycle progression of hepatoma cells exposed to perifosine was determined. Accumulation of cells with G_2/M DNA content was observed both in HepG2 cells and Bel-7402 cells (Fig. 2b). Western blotting results suggested that perifosine up-regulated the levels of p21 and inhibited the expression of cyclin B_1 in hepatoma cells (Fig. 2c).

Next, we examined the effect of perifosine on the cell apoptosis. After the cells were treated with perifosine for 24 or 48 h, significant induction of apoptosis occurred in HepG2 cells and Bel-7402 cells (Fig. 3a). Compared with control, a large number of cells displayed morphological changes with typical characteristics of apoptotic cell death (Fig. 3b). In mammalian cells, apoptosis is mediated by cysteine proteases, which are divided into initiators (e.g., caspase-8, -9 and -12) and executors (e.g., caspase-3, -6 and -7). Caspases are synthesized as relatively inactive zymogens, which become activated upon cleavage, a common mechanism for most protease zymogens. We showed that perifosine activated caspase-3 and -9 in hepatoma cells; in addition, PARP, downstream effector of caspase, was also activated upon perifosine treatment (Fig. 3c). In addition, no significant changes in the levels of p53 and Bcl-2 were detected from Western blotting assay.

The family of serine-threonine protein kinases functions in multiple cell apoptosis and survival signaling pathways. Especially the MAPK family, which includes extracellular signal regulating kinase1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38 mitogen activated protein kinase (MAPK), plays an important role in cell survival/death in many physiological and pathological settings (Brown and Sacks 2009; Boutros et al. 2008; Anjum and Blenis 2008). ERK is preferentially activated by mitogen through the Ras/Raf/MEK (MAP kinase kinase) signaling pathways, thus leading to cell growth and survival (Khavari and Rinn 2007). On the other hand, JNK and p38 MAPK signaling molecules are predominantly activated by the inflammatory cytokines and environmental stress, which are leading to cell differentiation and apoptosis (Wagner and Nebreda 2009). Another report, however, has shown that perifosine inhibited cell proliferation and induced apoptosis *via* MAPK pathway in human T-ALL CEM cell lines, so the activity of ERK, JNK and p38 was detected by Western blotting assay. The phosphorylation of JNK was induced by perifosine and phosphorylation of p38 MAPK was unaffected (Fig. 4a). Contrary to a previous report, phosphorylation of ERK was inhibited by the perifosine in a dose-dependent manner. This discrepancy may be due, at least in part, to cell type's variation.

Among the current chemotherapy drug regimens, cisplatin represents one of the clinically most important antineoplastic agents with anticancer activity against a wide variety of solid tumors (Arafa et al. 2009), which is an effective DNA-damaging antitumor agent and employed for the treatment of various human cancers. Resistance to cisplatin-based chemotherapy is one of the causes of treatment failure in human hepatocellular carcinoma. Previous studies have indicated that Akt activation contributes to cisplatin resistance (Liu et al. 2007; Yang et al. 2006), so we examined whether combination of perifosine with cisplatin could increase anti-tumorigenic effects. As far as we know, there is no report indicating the combined effects with perifosine and cisplatin in cells. Indeed, when HepG2 cells were cotreated with perifosine and cisplatin for 24 h, the proportion of dead cells increased remarkably and was much greater than that observed in the cells treated with each reagent alone (Fig. 5c). As shown in Western blotting results (Fig. 5d), the release of cytochrome c in the cytosolic fraction increased remarkably by cotreatment with the perifosine and cisplatin. Moreover, perifosine and cisplatin combination significantly increased the cleavation caspase-3, caspase-9 and PARP in HepG2 cells. Bcl-2 protein is one of the key factors in the common final pathways involved in the regulation of apoptosis, it also plays an important role in the development of cisplatin resistance in cancer cells, so we detected the

expression of Bcl-2 and Bax after cotreatment with perifosine and cisplatin. Based on Western blotting results, we concluded that down-regulation of the expression of Bcl-2 and up-regulation of the level of Bax were the potential mechanism for this synergistic effect.

In summary, we have demonstrated here that perifosine, which currently is tested in a phase I/II study, inhibits Akt phosphorylation and activation in hepatoma cells. Further exploitation of these findings in the design of novel agents may lead to improved approaches to hepatocellular carcinoma treatment. Our observations offer a valid therapeutic alternative, alone or in combination with cisplatin, for the treatment of hepatocellular carcinoma.

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