Induction of apoptosis in human breast adenocarcinoma MCF-7 cells by pterocarnin A from the bark of *Pterocarya stenoptera* via the Fas-mediated pathway

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Pterocarnin A, isolated from the bark of *Pterocarya stenoptera* (Juylandaceae), was investigated for its antiproliferative activity in human breast adenocarcinoma MCF-7 cells. To identify the anticancer mechanism of pterocarnin A, we assayed its effects on apoptosis, cell cycle distribution, and levels of p53, p21/WAF1, Fas/APO-1 receptor and Fas ligand. The results showed that pterocarnin A induced apoptosis of MCF-7 cells without mediation of p53 and p21/WAF1. We suggest that the Fas/Fas ligand apoptotic system is the main pathway of pterocarnin A-mediated apoptosis of MCF-7 cells. Our study reports here for the first time that the activity of the Fas/Fas ligand apoptotic system may participate in the antiproliferative activity of pterocarnin A in MCF-7 cells. *Anti-Cancer Drugs* 18:555–562 © 2007 Lippincott Williams & Wilkins.

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Introduction

Tannins are naturally occurring water-soluble polyphenols of varying molecular weights and are the most abundant polyphenolic compounds with the ability to precipitate proteins from solutions [1,2]. They are strong antioxidants that occur naturally in foods and can inhibit carcinogenesis [1–3]. *Pterocarya stenoptera* C. DC (Juylandaceae), also known as Chinese Wingnut, is traditionally used as an insecticide, and also in an infusion as a wash to remove scabies, eczema and abscesses. The leaves and bark of this plant are said to be carminative and anthelmintic. Pterocarnin A is a tannin, extracted from the bark of *P. stenoptera* (Juylandaceae) which has been reported to exhibit anti-herpes simplex virus type 2 [4]. To establish the proapoptotic mechanism of pterocarnin A, we assayed the levels of p53, p21/WAF1, Fas/APO-1 receptor and Fas ligand (FasL), which are strongly associated with the signal transduction of apoptosis.

Materials and methods

Test compound

Pterocarnin A (Fig. 1) was isolated from the bark of *P. stenoptera* as described previously [5]. Briefly, the bark of *P. stenoptera* was extracted at room temperature with acetone–water (4 : 1, v/v). The extract was concentrated under reduced pressure and then eluted with acetone through a celite column. The acetone eluate was chromatographed on a Sephadex LH-20 (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA) to give two fractions. Fraction 2 was further chromatographed on Sephadex LH-20 and MCI-gel (Mitsubishi Chemical Industries, Tokyo, Japan) CHP 20P to obtain pterocarnin A. The structure and purity of pterocarnin A were determined by its spectroscopic and physical data as described previously [5]. The stock solution of pterocarnin A was prepared at a concentration of 2 mg/ml of dimethyl sulfoxide (DMSO). It was then stored at −20°C until use. For all experiments, the final concentrations of the test compound were prepared by diluting the stock with Dulbecco's modified Eagle's medium (DMEM). Control cultures received the carrier solvent (0.1% DMSO).

Reagents and materials

Fetal calf serum, penicillin G, streptomycin, amphotericin B, and DMEM were obtained from Gibco BRL (Gaithersburg, Maryland, USA). DMSO, ribonuclease and propidium iodide were purchased from Sigma Chemical (St Louis, Missouri, USA). XTT sodium 3′-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene-sulfonic acid hydrate and p53 pan ELISA kits were obtained from Roche Diagnostics (Mannheim, Germany). Nucleosome ELISA, WAF1 ELISA, FasL,

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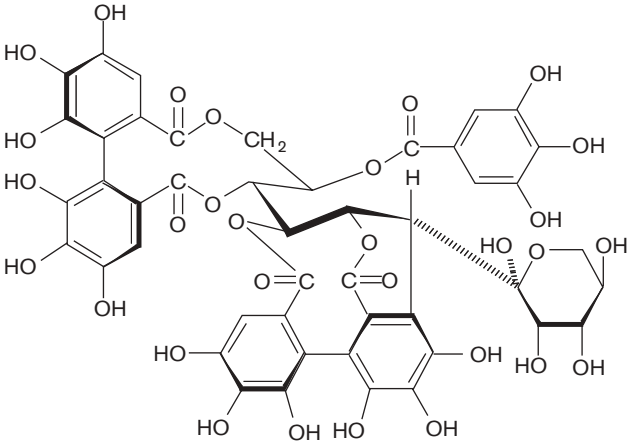
Fig. 1



Chemical structure pterocarnin A isolated from the bark of *Pterocarya stenoptera*.

Fas/APO-1 ELISA, caspase-8 assay kits, caspase-8 inhibitor and benzyloxy-carbonyl-Val-Ala-Asp-fluoromethylketone (Z-IETD-FMK) were purchased fromCalbiochem (Cambridge, Massachusetts, USA). Anti-Fas antibody (ZB4) was obtained from Upstate Biotechnology (Lake Placid, New York, USA). The antibodies to p53 and p21 were obtained from Santa Cruz Biotechnology (Santa Cruz, California, USA). 3T3-L1 nuclear extract was obtained from Active Motif (Carlsbad, California, USA).

Cell culture

Breast cancer cell line MCF-7 was obtained from the American Type Cell Culture Collection (Manassas, Virginia, USA). It was maintained in monolayer culture at 37°C and 5% CO_2 in DMEM supplemented with 10% fetal calf serum, 5 μg/ml insulin, 100 units/ml of penicillin G, 100 μg/ml of streptomycin and 0.25 μg/ml of amphotericin B.

Cell proliferation assay

Inhibition of cell proliferation by pterocarnin A was measured by the XTT assay. Briefly, cells were plated in 96-well culture plates (1×10^4 cells/well). After a 24 h incubation, the cells were treated with 0, 0.5, 2.5, 5 and 10 μmol/l pterocarnin A for 48 h. This concentration range is equivalent to an approximate accumulation of plasma concentration of dietary phenols, polyphenols and tannins by an average adult human [6]. Fifty microliters of XTT test solution, which was prepared by mixing 5 ml of XTT-labeling reagent with 100 μl of electron-coupling reagent, was then added to each well. After 6 h of incubation, the absorbance was measured on an ELISA reader (Multiskan EX; Labsystems, Thermo Electron, Milford, Massachusetts, USA) at a test wavelength of 492 nm and a reference wavelength of 690 nm.

Cell cycle analysis

To determine cell cycle distribution, 5×10^5 cells were plated in 60-mm dishes and treated with pterocarnin A (0, 5 and 10 μmol/l) for 24 h. After treatment, the cells were collected by trypsinization, fixed in 70% ethanol, washed in phosphate-buffered saline, resuspended in 1 ml of phosphate-buffered saline containing 1 mg/ml ribonuclease and 50 μg/ml propidium iodide, incubated in the dark for 30 min at room temperature, and analyzed by an EPICS flow cytometer (Beckman Coulter, Fullerton, California, USA). The data were analyzed using the Multicycle software (Phoenix Flow Systems, San Diego, California, USA).

Measurement of apoptosis by enzyme-linked immunosorbent assay

The induction of apoptosis by pterocarnin A was assayed using the Nucleosome ELISA kit. This kit uses a photometric enzyme immunoassay that quantitatively determines the formation of cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) after apoptotic cell death. MCF-7 cells were treated with 0, 5 and 10 μmol/l pterocarnin A, for 6, 12, 24 and 48 h. The samples of cell lysate were placed in 96-well (1×10^6 per well) microtiter plates. The induction of apoptosis was evaluated by assessing the enrichment of nucleosome in cytoplasm and determined exactly as described in the manufacturer's protocol [7,8].

Assaying the levels of p53, p21, Fas/APO-1 and Fas ligand (mFasL and sFasL)

p53 pan ELISA, WAF1 ELISA, Fas/APO-1 ELISA and FasL ELISA kits were used to detect p53, p21, Fas/APO-1 receptor and soluble (sFasL)/membrane-bound FasL (mFasL). Briefly, MCF-7 cells were treated with 0, 5 and 10 μmol/l of pterocarnin A, for 6, 12, 24 and 48 h. The samples of cell lysate were placed in 96-well (1×10^6 per well) microtiter plates were coated with monoclonal-detective antibodies and incubated for 1 h (Fas/APO-1), 2 h (p53 or p21/WAF1) or 3 h (FasL) at room temperature. It was necessary to determine the FasL in cell culture supernatant by using FasL ELISA kit. After removing the unbound material by washing with washing buffer (50 mmol/l Tris, 200 mmol/l NaCl, and 0.2% Tween 20), the detector antibody that is bound by horseradish peroxidase-conjugated streptavidin was added to bind to the antibodies. Horseradish peroxidase catalyzed the conversion of a chromogenic substrate (tetramethylbenzidine) to a colored solution with the color intensity proportional to the amount of protein present in the sample. The absorbance of each well was measured at 450 nm, and concentrations of p53, p21/WAF1, Fas/APO-1 and FasL were determined by interpolating from standard curves obtained with known concentrations of standard proteins [9,10].

Assay for caspase-8 activity

The assay is based on the ability of the active enzyme to cleave the chromophore from the enzyme substrate, Ac-IETD-pNA. The cell lysates were incubated with peptide substrate in assay buffer (100 mmol/l NaCl, 50 mmol/l *N*-2-hydroxyl piperazine-*N*′-2-ethane sulfonic acid, 10 mmol/l dithiothreitol, 1 mmol/l ethylenediaminetetraacetic acid, 10% glycerol, 0.1% CHAPS, pH 7.4) for 3 h at 37°C. The release of *p*-nitroaniline was monitored at 405 nm. Results are represented as the percentage change of the activity compared with the untreated control [9,10].

Western blotting

Cells were treated with 10 μmol/l of pterocarnin A for the indicate times. The cells were lysed on ice for 40 min in a solution containing 50 mmol/l Tris, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 150 mmol/l NaCl, 2 mmol/l Na_3VO_4, 2 mmol/l ethylene glycol-bis (*b*-aminoethyl ether), 12 mmol/l β-glycerolphosphate, 10 mmol/l NaF, 16 μg/ml benzamidine hydrochloride, 10 μg/ml phenanthroline, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin and 1 mmol/l phenylmethylsulfonyl fluoride. The cell lysate was centrifuged at 14 000*g* for 15 min and the supernatant fraction was collected for immunoblotting. Equivalent amounts of protein were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10–12%) and transferred to polyvinylidine diflouride membranes. After blocking for 1 h in 5% nonfat dry milk in Tris-buffered saline, the membrane was incubated with the desired primary antibody for 1–16 h. The membrane was then treated with appropriate peroxidase-conjugated secondary antibody and the immunoreactive proteins were detected using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Statistical analysis

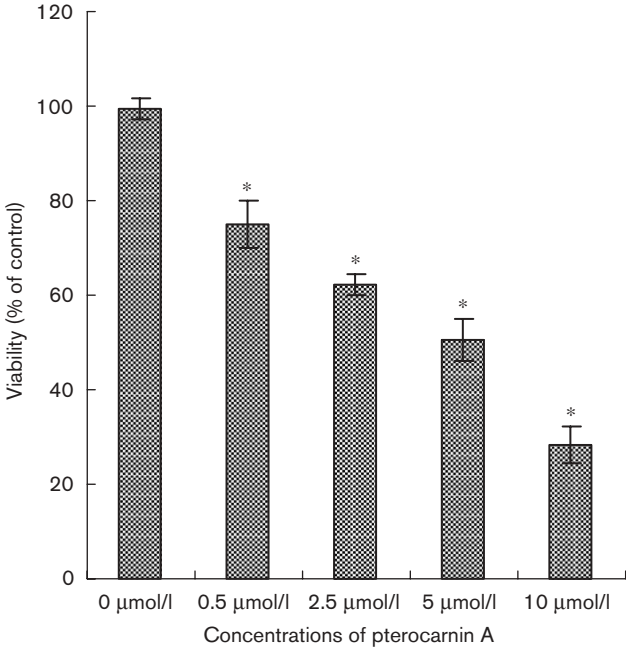
Data are expressed as means ± SD. Statistical comparisons of the results were made using analysis of variance. Significant differences ($P < 0.05$) between the means of control and pterocarnin A-treated cells were analyzed by Dunnett's test.

Results

Effect of pterocarnin A on MCF-7 cell proliferation

We first tested the antiproliferative effect of pterocarnin A in the breast cancer cell line, MCF-7. As shown in Fig. 2, the proliferative inhibitory effect of pterocarnin A was observed in a dose-dependent manner. At 48 h, the maximal effect on proliferation inhibition was observed with 10 μmol/l pterocarnin A, which inhibited proliferation in 71.8% of MCF-7 cells. The IC_{50} value was 5.3 μmol/l.

Fig. 2



The antiproliferative effect of pterocarnin A in MCF-7 cells. Adherent cells plated in 96-well plates (10^4 cells/well) were incubated with different concentrations of pterocarnin A at 48 h. Cell proliferation was determined by sodium 3′-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene-sulfonic acid hydrate assay. Results are expressed as the percentage of the cell proliferation of control at 0 h. The data shown are the mean from three independent experiments. Standard deviations were less than 10%. The asterisk indicates a significant difference between control and test-treated cells, as analyzed by Dunnett's test ($P<0.05$).

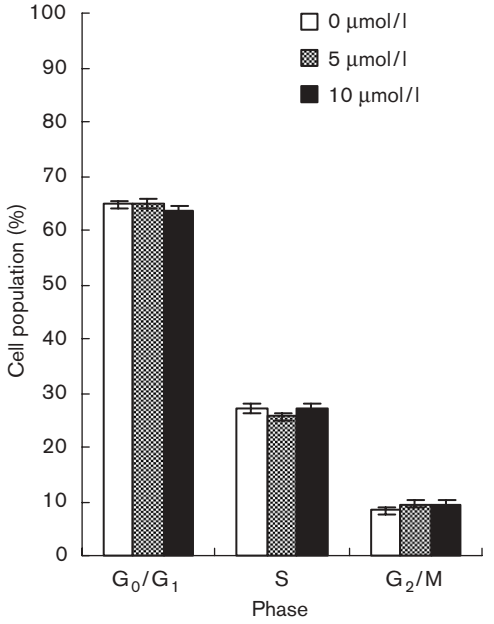
Pterocarnin A induced apoptosis in MCF-7 cells, without affecting the cell cycle distribution

To clarify the mechanism of antiproliferative effect, EPICS flow cytometry and apoptotic ELISA kits were used to analyze cell cycle distribution and apoptosis, respectively. In cell cycle distribution, our results did not show any significant change between the control group and the pterocarnin A-treated group for up to 10 μmol/l at 24 h (Fig. 3). By using the Nucleosome ELISA kit, we demonstrated that pterocarnin A induced apoptosis of MCF-7 cells in a dose- and time-dependent manner (Fig. 4). In contrast to the controls, when cells were treated with pterocarnin A, the number of cells undergoing apoptosis increased from about 4.6-fold at 5 μmol/l pterocarnin A to 8.1-fold at 10 μmol/l pterocarnin A at 48 h.

p53 and p21/WAF1 were not involved in pterocarnin A-mediated cell proliferation inhibition

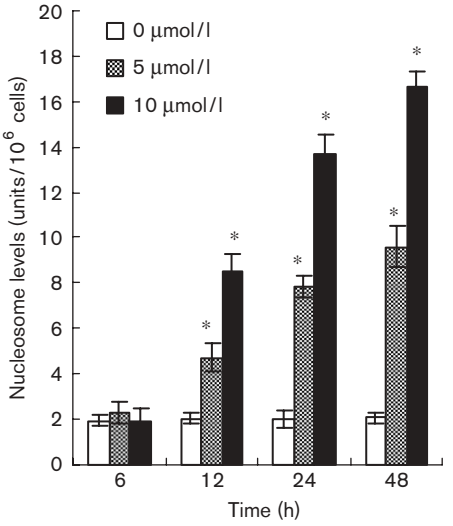
To understand the molecular mechanism of how pterocarnin A works to induce apoptosis, the ELISA and Western blotting results were used to analyze p53 and its downstream molecule p21/WAF1. Treatment of pterocarnin A for up to 10 μmol/l at 48 h did not affect the protein expression of p53 and p21/WAF1 (Fig. 5). Therefore, pterocarnin A-induced apoptosis might not be regulated by p53 and p21/WAF1.

Fig. 3



The effects of pterocarnin A on cell cycle distribution in MCF-7 cells. MCF-7 cells after treatment with 0, 5 and 10 μmol/l pterocarnin A for 24 h were fixed and stained with propidium iodide, and the cell cycle distribution was then analyzed by flow cytometry. The data indicate the percentage of cells in G_0/G_1, S and G_2/M phases of the cell cycle ($P > 0.05$). The results represent the mean values + SD from three individual experiments.

Fig. 4



Induction of apoptosis in MCF-7 cells by pterocarnin A. MCF-7 cells were cultured with 0, 5 and 10 μmol/l of pterocarnin A for 6, 12, 24 and 48 h. Cells were harvested and lysed with lysis buffer. Cell lysates containing cytoplasmic oligonucleosomes of apoptotic cells were analyzed by means of Nucleosome ELISA. Each value is the mean ± SD of three determinations. The data shown are the mean from three independent experiments. The asterisk indicates a significant difference between control and pterocarnin A-treated cells, as analyzed by Dunnett's test ($P < 0.05$).

The Fas/Fas Ligand apoptotic system might be a possible pathway of pterocarnin A-mediated apoptosis

By using Fas/APO-1 ELISA and FasL ELISA kits, we found that pterocarnin A increased expression of Fas/APO-1 receptor and s/mFasL in MCF-7 cells as early as 6 h after treatment in a dose-dependent fashion (Fig. 6). The maximum effect was observed after 24 h of treatment. The time relationship between the expression of Fas/FasL at 6 h of treatment and the occurrence of apoptosis at 12 h of treatment could support the idea that the Fas/FasL system might mediate pterocarnin A-induced apoptosis of MCF-7 cells.

When MCF-7 cells were pretreated with an antagonistic anti-Fas antibody, ZB4, the antiproliferative and proapoptotic effects of pterocarnin A were effectively prevented. At 10 μmol/l of pterocarnin A, cell proliferation inhibition decreased from 72.2 to 19.2% (Fig. 7a). Compared with the control, the oligonucleosome DNA fragmentation of apoptosis induced by 10 μmol/l of pterocarnin A decreased from about 8.1- to 2.8-fold at 48 h in ZB4-pretreated MCF-7 cells (Fig. 7b).

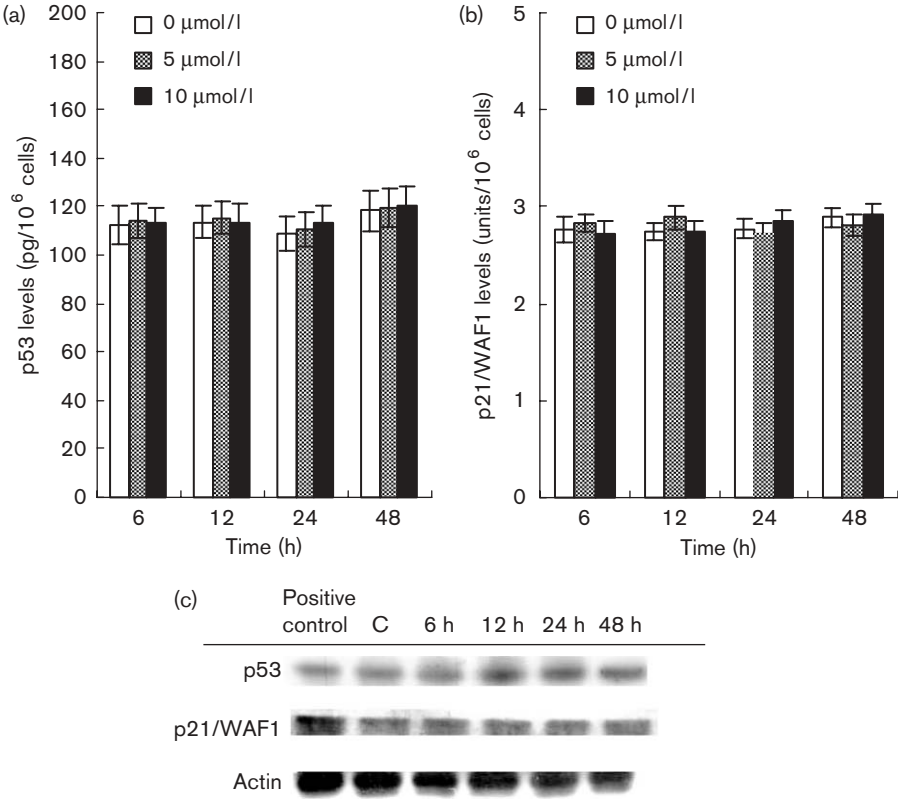
We next measured the downstream caspase of the Fas/FasL system. The results showed that caspase-8 activity increased at 12 h and reached maximum induction at 48 h in 10 μmol/l pterocarnin A-treated MCF-7 cells (Fig. 8a). Furthermore, our results showed that the antiproliferative activity and induction of apoptosis by pterocarnin A were significantly decreased in the presence of inhibitor of caspase-8 (Z-IETD-FMK) (Fig. 8b and c).

Discussion

Normal p53 gene is well known to play a crucial role in inducing apoptosis and acting as cell cycle checkpoints in human and murine cells following DNA damage [11]. p21/WAF1 protein blocks the activities of various cyclin-dependent kinase [12,13] and inhibits the phosphorylation of retinoblastoma protein, thereby preventing the G_1–S phase transition [12,14]. Previous studies have shown that p21/WAF1 is transcriptionally regulated by p53-dependent and p53-independent pathways [15–17]. Our results did not show any significant change between the control group and the pterocarnin A-treated group for up to 10 μmol/l at 48 h when assayed for protein expression of p53 and p21/WAF1 (Fig. 5), so it is clear that p53 and p21/WAF1 may not participate in pterocarnin A-affected cell cycle distribution and pterocarnin A-induced apoptosis in MCF-7 cells.

The Fas/FasL system is a key signaling transduction pathway of apoptosis in cells and tissues [18]. Ligation of

Fig. 5

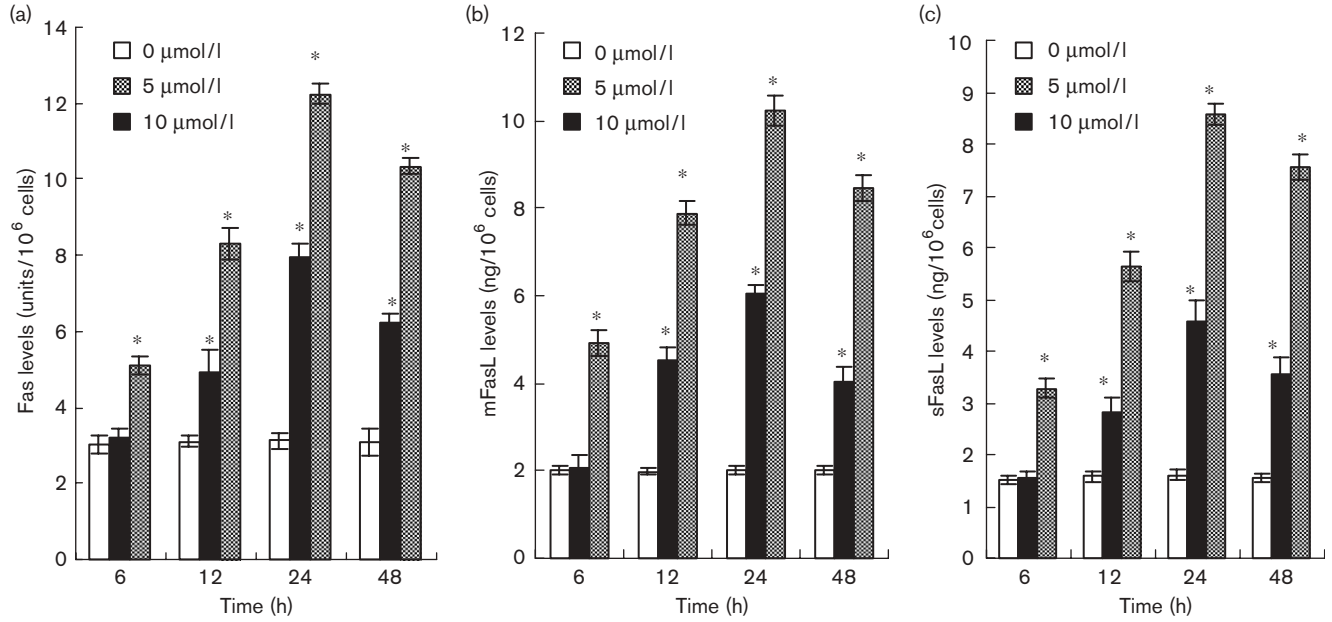


Effects of pterocarnin A on protein expression of p53 and p21/WAF1. (a) The level of p53 protein in MCF-7 cells. (b) The level of p21/WAF1 in MCF-7 cells. (c) The levels of p53 and p21/WAF1 were determined by Western blotting assay. Human breast adenocarcinoma MCF-7 cells were treated with 0, 5 and 10 μmol/l of pterocarnin A. p53 and p21/WAF1 levels were determined by ELISA and Western blotting assay. Positive control: 3T3-L1 nuclear extract for p53 and MDA-MB-231 cell extract for p21/WAF1. The detailed protocol is described in Materials and methods. The data shown are the means from three independent experiments. Each value is the mean ± SD of three determinations.

Fas by agonistic antibody or its mature ligand induces receptor oligomerization and formation of death-inducing signaling complex, followed by activation of caspase-8, then further activating a series of caspase cascades resulting in cell apoptotic death [18,19]. Although p53 has been demonstrated to modulate the expression of Fas, other factors also clearly regulate the transcriptional activity of the Fas gene because other studies have shown abundant Fas protein expression in the absence of the wild-type p53 protein expression [20,21]. FasL is a tumor necrosis factor-related type II membrane protein [22]. Cleavage of mFasL by a metalloprotease-like enzyme results in the formation of sFasL [23]. Defects in the Fas/FasL apoptotic signaling pathway provide a survival advantage to cancer cells and may be implicated in tumorigenesis. Indeed, expression of FasL by breast cancer cells is associated with the loss of Fas expression, thus eliminating the possibility of self-induced apoptosis, and is involved in drug resistance [24,25] and MCF-7 cell line, which has been described to be Fas-sensitive [26]. Reimer *et al.* [27] have reported that the selection process leading to highly aggressive breast tumor variants might be enhanced by FasL-mediated tumor fratricide,eventually a possible target for novel therapeutic strategies. Our study indicated that Fas ligands (mFasL and sFasL) increased in pterocarnin A-treated MCF-7 cells. Moreover, levels of Fas/APO-1 and the activity of caspase-8 were simultaneously enhanced in FasL-upregulating MCF-7 cells. Furthermore, when the Fas/Fas ligand system was blocked by ZB4, a decrease in both cell proliferative inhibition and the proapoptotic effect of pterocarnin A was noted. Similarly, cell proliferative inhibition and apoptotic induction of pterocarnin A decreased in MCF-7 cells treated with caspase-8 inhibitor. These findings are novel to show that the Fas/FasL system plays an important role in pterocarnin A-mediated MCF-7 cellular apoptosis.

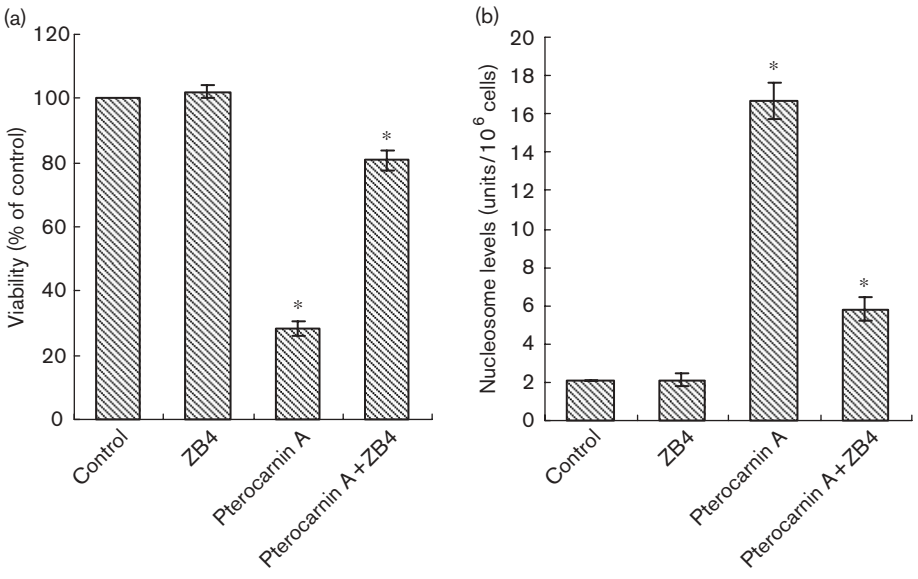
Overall, our results have demonstrated that pterocarnin A inhibits cell proliferation in a p53-independent manner, and that enhanced Fas-mediated apoptosis may present interesting therapeutic prospects for the compound in the treatment of human breast cancer. As down-regulation of Fas is associated with a poor prognosis in breast

Fig. 6



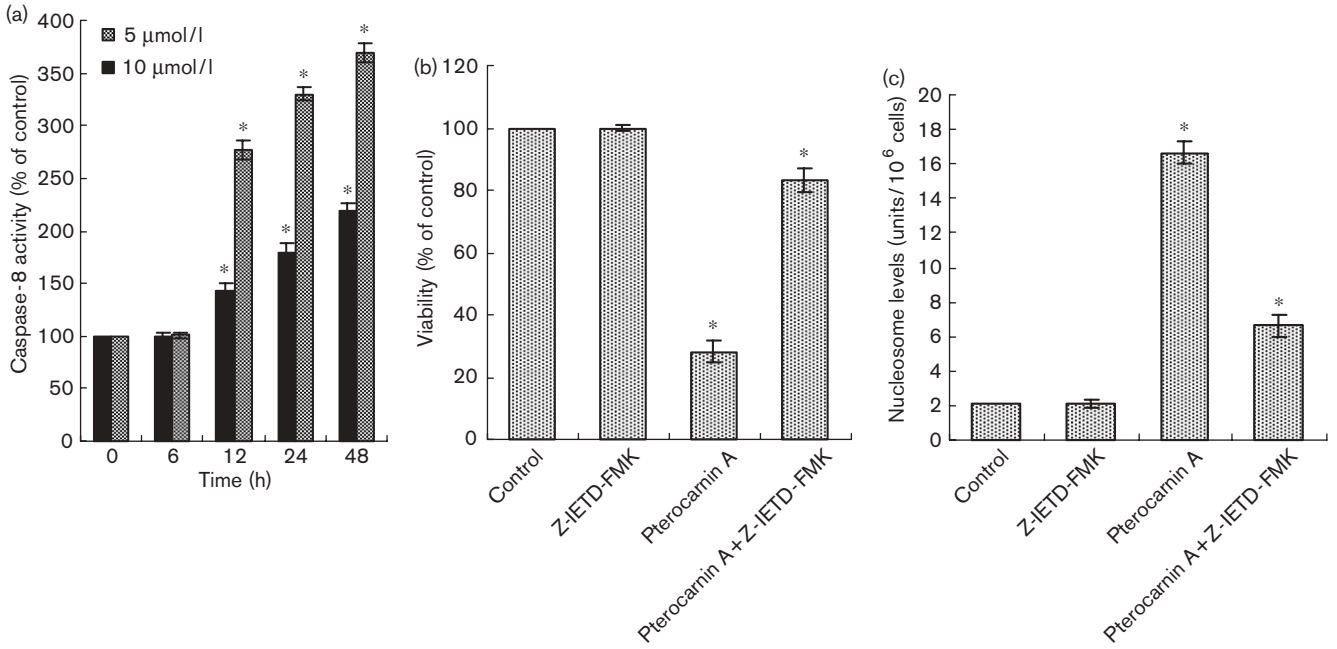
The Fas/FasL apoptotic system was involved in pterocarnin A-mediated apoptosis. MCF-7 cells were incubated with 0, 5 and 10 μmol/l of pterocarnin A for 6, 12, 24 and 48 h. (a) The level of Fas/APO-1 receptor in MCF-7 cells. (b) The amount of membrane-bound Fas ligand (mFasL) in MCF-7 cells. (c) The amount of soluble Fas ligand (sFasL) in MCF-7 cells. Each value is the mean ± SD of three determinations. The data shown are the means from three independent experiments. The asterisk indicates a significant difference between control and pterocarnin A-treated cells, as analyzed by Dunnett's test ($P<0.05$).

Fig. 7



Effect of antagonistic anti-Fas antibody (ZB4) on pterocarnin A in MCF-7 cells. (a) The antiproliferative and (b) proapoptotic effect of pterocarnin A was decreased by Fas antagonist ZB4. For blocking experiments, cells were preincubated with 250 ng/ml ZB4 for 1 h and then treated with 10 μmol/l of pterocarnin A for 48 h. Cell viability and apoptosis induction were examined by sodium 3′-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene-sulfonic acid hydrate and Nucleosome ELISA kit. The data shown are the means from three independent experiments. The data shown are the mean ± SD of three determinations. The asterisk indicates a significant difference between control and pterocarnin A-treated cells, as analyzed by Dunnett's test ($P<0.05$).

Fig. 8



(a) The activation of caspase-8 in MCF-7 cells by pterocarnin A. (b) Effect of caspase-8 inhibitor on pterocarnin A-mediated antiproliferation. (c) Effect of caspase-8 inhibitor on pterocarnin A-induced apoptosis. MCF-7 cells were incubated with various concentrations of pterocarnin A for the indicated times. For blocking experiments, cells were preincubated with Z-IETD-FMK (10 μmol/l) for 1 h before the addition of 10 μmol/l pterocarnin A. After 48 h of treatment, cell viability and induction of apoptosis were measured by sodium 3′-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene-sulfonic acid hydrate and Nucleosome ELISA kit. The data shown are the mean from three independent experiments. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between control and pterocarnin A-treated cells, as analyzed by Dunnett's test ($P < 0.05$).

cancer [27], it remains to be determined whether pterocarnin A treatment will prove useful in the fight against advanced breast cancer.

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