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Induction of apoptosis in human breast adenocarcinoma MCF-7 cells by prodelphinidin B-2 3,3′-di-*O*-gallate from *Myrica rubra* via Fas-mediated pathway

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Abstract

Myrica rubra Sieb et Zucc. (Myricaceae) is well known as a rich source of tannins. Prodelphinidin B-2 3,3′-di-*O*-gallate (PB233′OG) is a proanthocyanidin gallate that has been reported to exhibit antioxidant and antiviral activity. In this study, we evaluated the anti-proliferative activity of PB233′OG isolated from the bark of *M. rubra* in human breast adenocarcinoma MCF-7 cells. To identity the anticancer mechanism of PB233′OG, we assayed its effect on apoptosis, cell cycle distribution, and levels of p53, p21/WAF1, Fas/APO-1 receptor and Fas ligand. The results showed that PB233′OG induced apoptosis of MCF-7 cells without mediation of p53 and p21/WAF1. We suggest that Fas/Fas ligand apoptotic system is the main pathway of PB233′OG-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 anti-proliferative activity of PB233′OG in MCF-7 cells.

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Introduction

Apoptosis plays an important role in homeostasis and development of tissue in organisms (Igney & Krammer 2002). Imbalance between cell proliferation and apoptotic cell death will result in serious disease such as cancer. Many studies have demonstrated that cancer treatment by chemotherapy and γ-irradiation kills target cells primarily by the induction of apoptosis (Evan & Vousden 2001; Igney & Krammer 2002).

Tumour suppressor gene p53 is activated in response to various genotoxic stresses, resulting in cell cycle arrest or apoptosis (May & May 1999; Kemp et al 2001; Sharpless & DePinho 2002). Functional p53 protein is a transcription factor with sequence-specific DNA binding activity (Qian et al 2002). The induction of p21/WAF1 causes subsequent arrest in the G0/G1 or G2/M phase of the cell cycle by binding of cyclin–cdk complex (May & May 1999; Sharpless & DePinho 2002; Qian et al 2002). How p53 triggers apoptosis is yet to be elucidated, but it seems to involve both transcription-dependent and -independent mechanisms (Sionov & Haupt 1999; Sheikh & Fornace 2000; Chopin et al 2002).

Several previous publications have reported that anti-cancer drugs may induce apoptosis via the Fas/FasL system (Glick et al 1999; Yu et al 1999; Kamsteeg et al 2003; Syed & Ho 2003). Fas is a cell surface receptor comprising a type I integral membrane protein that expresses a cytoplasmic death domain and belongs to the tumour necrosis factor receptor superfamily (Nagata & Golstein 1995; Chopin et al 2002). Activation of Fas by its ligand (FasL) results in the oligomerization of its intracellular death domain and the recruitment of the intracellular adaptor FADD (Fas-associated death domain). Once bound, FADD is able to activate procaspases-8 and -10 in a death-inducing signalling complex. In turn, caspases-8 and -10 activate downstream caspases, resulting in apoptotic cell death (Juo et al 1998; Vergote et al 2002).

Myrica rubra Sieb et Zucc. (Myricaceae) is well known as a rich source of tannins. Its bark was traditionally used as an astringent, an anti-diarrhoeal and also as a dyeing and tanning agent in Japan and China (Nonaka et al 1983). Recent studies have shown that *M. rubra* exhibits a variety of biological effects (Matsuda et al 2001, 2002; Plumb et al 2002; Tao et al 2002; Yang et al 2003). Prodelphinidin B-2 3,3′-di-*O*-gallate (PB233′OG) (Figure 1), extracted from *M. rubra*, is a proanthocyanidin gallate that

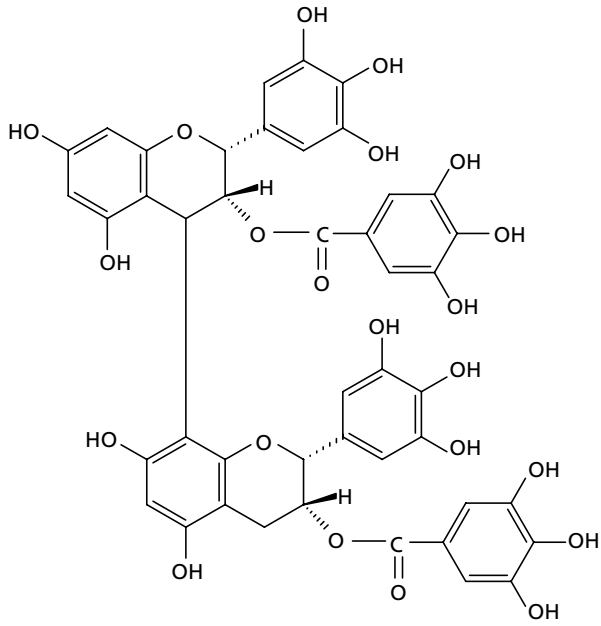

Figure 1 Chemical structure of PB233′OG.

has been reported to exhibit antioxidant and antiviral activity (Takechi et al 1985; Ohta et al 1992; Cheng et al 2003). In this study, we determined the anti-proliferative activity of PB233′OG and examined its effect on cell cycle distribution and apoptosis in MCF-7 cells. Furthermore, to establish the anti-cancer mechanism of PB233′OG, 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 and affect the chemosensitivity of tumour cells to anti-cancer agents.

Materials and Methods

Reagents and materials

Fetal calf serum (FCS), penicillin G, streptomycin, amphotericin B and Dulbecco's modified Eagle's medium (DMEM) were obtained from GIBCO BRL (Gaithersburg, MD). Dimethyl sulfoxide (DMSO), ribonuclease (RNase), and propidium iodide (PI) were purchased from Sigma Chemical (St Louis, MO). 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 GmbH (Mannheim, Germany). Nucleosome ELISA, WAF1 ELISA, Fas Ligand, Fas/APO-1 ELISA, caspase-8 assay kits and caspase-8 inhibitor, benzyloxy-carbonyl-Val-Ala-Asp-fluoromethylketone (Z-IETD-FMK), were purchased from Calbiochem (Cambridge, MA). Anti-Fas Ab (ZB4) was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY).

Test compound

PB233′OG (purity > 95%) was isolated from the bark of *Myrica rubra* (Myricaceae) and identified for its structure as previously described (Nonaka et al 1983). The stock solution of PB233′OG was prepared at a concentration of 2 mg mL^{-1} of 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 DMEM. Control cultures received the carrier solvent (0.1% DMSO).

Cell culture

Breast cancer cell line MCF-7 was obtained from the American Type Cell Culture Collection (Manassas, VA). It was maintained in monolayer culture at 37°C and 5% CO_2 in DMEM supplemented with 10% FCS, 5 μg mL^{-1} insulin, 100 U mL^{-1} of penicillin G, 100 μg mL^{-1} of streptomycin and 0.25 μg mL^{-1} of amphotericin B.

Cell proliferation assay

Inhibition of cell proliferation by PB233′OG was measured by XTT assay. Briefly, cells were plated in 96-well culture plates (1×10^4 cells/well). After 24 h incubation, the cells were treated with 0, 0.5, 2.5, 5, and 10 μM PB233′OG for 12, 24, 48 and 72 h. Fifty microlitres of XTT test solution, which was prepared by mixing 5 mL of XTT-labelling reagent with 100 μL of electron coupling reagent, was then added to each well. After 6 h incubation, the absorbance was measured on an ELISA reader (Multiskan EX; Labsystems. Thermo Electron Corporation, Milford, 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 PB233′OG (0, 5, and 10 μM) for 24 h. After treatment, the cells were collected by trypsinization, fixed in 70% ethanol, washed in PBS, re-suspended in 1 mL of PBS containing 1 mg mL^{-1} RNase and 50 μg mL^{-1} propidium iodide, incubated in the dark for 30 min at room temperature, and analysed by EPICS flow cytometer. The data were analysed using the Multicycle software (Phoenix Flow Systems, San Diego, CA).

Measurement of apoptosis by ELISA

The induction of apoptosis by PB233′OG 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 (mono- and oligonucleosomes) after apoptotic cell death. MCF-7 cells were treated with 0, 5, and 10 μM PB233′OG for 6, 12, 24 and 48 h. The samples of cell lysate were placed in 96-well (1×10^6 per well) microtitre 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.

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 Fas Ligand ELISA kits were used to detect p53, p21, Fas/APO-1

receptor and soluble (sFasL)/ membrane-bound Fas ligand (mFasL), respectively. Briefly, MCF-7 cells were treated with 0, 5 and 10 μM PB233′OG for 6, 12, 24 and 48 h. The samples of cell lysate were placed in 96-well (1×10^6 per well) microtitre plates 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 soluble Fas ligand in cell culture supernatant by using Fas Ligand ELISA kit. After removing the unbound material by washing with washing buffer (50 mM Tris, 200 mM 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 catalysed the conversion of a chromogenic substrate (tetramethylbenzidine) to a coloured solution with colour 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.

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 mM NaCl, 50 mM HEPES, 10 mM dithiothreitol, 1 mM EDTA, 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 percent change of the activity compared with the untreated control.

Statistical analysis

Data were expressed as means ± s.d. Statistical comparisons of the results were made using analysis of variance. Significant differences ($P < 0.05$) between the means of control and PB233′OG-treated cells were analysed by Dunnett's test.

Results

Effect of PB233′OG on MCF-7 cell proliferation

We first tested the anti-proliferative effect of PB233′OG in the breast cancer cell line, MCF-7. The growth inhibitory effect of PB233′OG was observed in a dose- and time-dependent manner (Figure 2). At 48 h, the maximal effect on proliferation inhibition was observed with 10 μM PB233′OG, which inhibited proliferation in 72.2% of MCF-7 cells. The IC50 value was 5.2 μM. The maximal proliferation effect of 83.3% was reached by 10 μM PB233′OG at 72 h.

PB233′OG induced apoptosis in MCF-7 cells without affecting the cell cycle distribution

To clarify the mechanism of anti-proliferative effect, EPICS flow cytometer and apoptotic ELISA kits were used to analyse cell cycle distribution and apoptosis, respectively. In cell cycle distribution, our results did not show any significant change between the control group and the PB233′OG-treated group for up to 10 μM at 24 h (Figure 3). By using Nucleosome ELISA kit, we demonstrated that PB233′OG induced apoptosis of MCF-7 cells in a dose- and time-dependent manner (Figure 4). In contrast to the controls, when cells were treated with PB233′OG, the number of cells undergoing apoptosis increased from about 4 fold at 5 μM PB233′OG to 8 fold at 10 μM PB233′OG at 48 h.

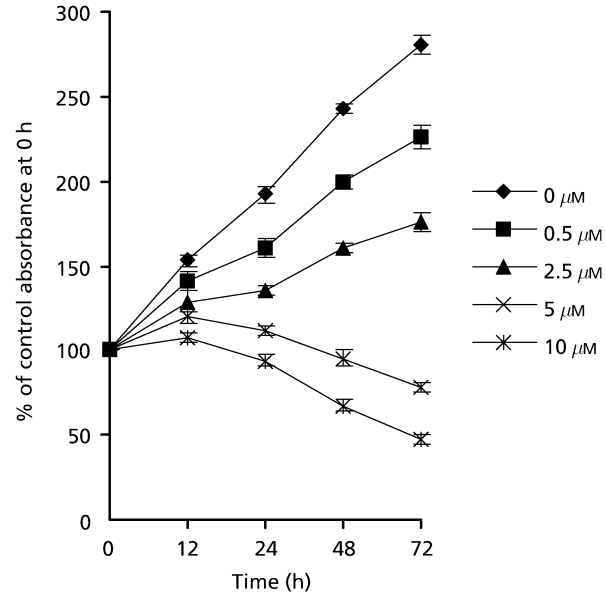

Figure 2 The anti-proliferative effect of PB233′OG in MCF-7 cells. Adherent cells plated in 96-well plates (10^4 cells/well) were incubated with different concentrations of PB233′OG at various time intervals. Cell proliferation was determined by XTT assay. Results are expressed as the percent of the cell proliferation of control at 0 h. Standard deviations were less than 10%.

p53 and p21/WAF1 were not involved in PB233′OG-mediated inhibition of cell proliferation

To understand the molecular mechanism of how PB233′OG works to induce apoptosis, the p53 pan ELISA and WAF1 ELISA kits were used to analyse p53 and its downstream molecule p21/WAF1. Treatment with PB233′OG for up to 10 μM at 48 h did not affect the protein expression of p53 or p21/WAF1 (Figure 5). Therefore, PB233′OG-induced apoptosis might not be regulated by p53 and p21/WAF1.

The Fas/FasL apoptotic system might be a possible pathway of PB233′OG-mediated apoptosis

By using Fas/APO-1 ELISA and Fas Ligand ELISA kits, we found that PB233′OG increased expression of Fas/APO-1 receptor and soluble/membrane-bound Fas ligand in MCF-7 cells as early as 6 h post treatment in a

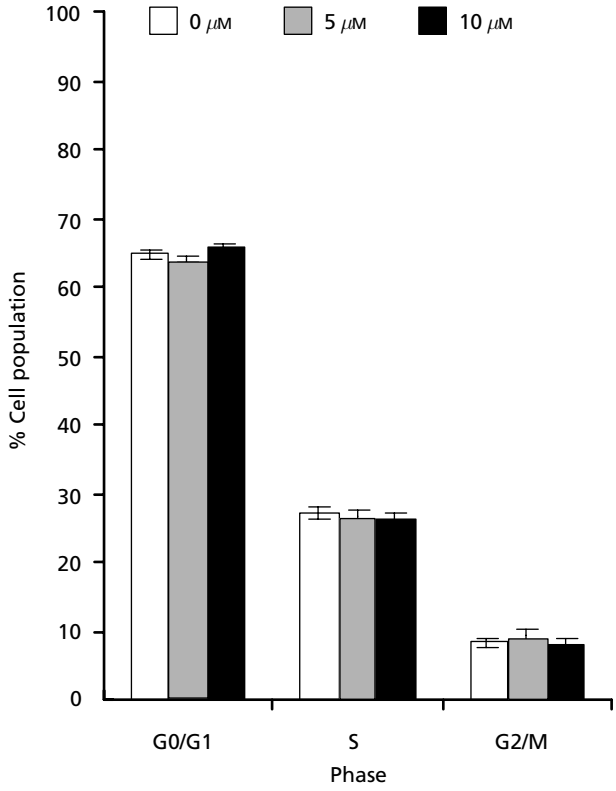

Figure 3 The effect of PB233′OG on cell cycle distribution in MCF-7 cells. MCF-7 cells following treatment with 0, 5 and 10 μM PB233′OG for 24 h were fixed and stained with propidium iodide, and cell cycle distribution was then analysed by flow cytometry. The data indicate the percentage of cells in G0/G1, S and G2/M phases of the cell cycle ($P > 0.05$). The results represent the mean values ± s.d. from three individual experiments.

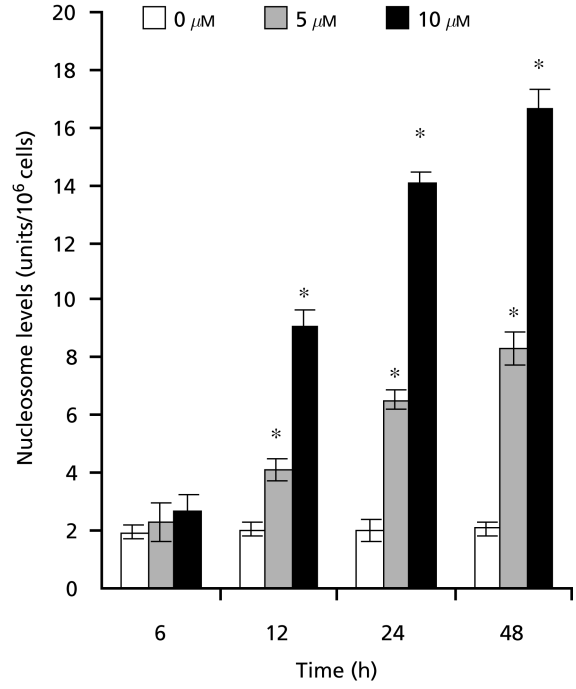

Figure 4 Induction of apoptosis in MCF-7 cells by PB233′OG. MCF-7 cells were cultured with 0, 5 and 10 μM of PB233′OG for 6, 12, 24 and 48 h. Cells were harvested and lysed with lysis buffer. Cell lysates containing cytoplasmic oligonucleosomes of apoptotic cells were analysed by means of Nucleosome ELISA. Each value is the mean ± s.d. of three determinations; $*P < 0.05$, vs control cells (Dunnett's test).

dose-dependent fashion (Figure 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 PB233′OG-induced apoptosis of MCF-7 cells.

When MCF-7 cells were pre-treated with an antagonistic anti-Fas antibody, ZB4, the anti-proliferative and pro-apoptotic effects of PB233′OG were effectively prevented. At 10 μM of PB233′OG, cell proliferation inhibition decreased from 72.2% to 14.2% (Figure 7A). Compared with the control, the oligonucleosome DNA fragmentation of apoptosis induced by 10 μM of PB233′OG decreased from about 8 fold to 2.3 fold at 48 h in ZB4-pretreated MCF-7 cells (Figure 7B).

We next measured the downstream caspase of Fas/FasL system. The results showed that caspase-8 activity increased at 12 h, and reached maximum induction at 48 h in 10 μM PB233′OG-treated MCF-7 cells (Figure 8A). Furthermore, our results showed that the anti-proliferative activity and induction of apoptosis by PB233′OG were significantly decreased in the presence of inhibitor of caspase-8 (Z-IETD-FMK) (Figures 8B and 8C).

Discussion

Normal p53 gene is well known to play a crucial role in inducing apoptosis and acting as a cell cycle checkpoint in human and murine cells following DNA damage (May & May 1999). p21/WAF1 protein blocks the activity of various cyclin-dependent kinases (Gu et al 1993; Harper et al 1993; Xiong et al 1993) and inhibits the phosphorylation of retinoblastoma (RB) protein, thereby preventing the G1-S phase transition (Harper et al 1993; Dulic et al 1994). Previous studies have shown that p21/WAF1 is transcriptionally regulated by p53-dependent and -independent pathways (El-Deiry et al 1993; Michieli et al 1994; Macleod et al 1995). Our results did not show any significant change between the control group and the PB233′OG-treated group for up to 10 μM at 48 h when assayed for protein expression of p53 and p21/WAF1 (Figure 5), so it is clear that p53 and p21/WAF1 may not participate in PB233′OG-affected cell cycle distribution and PB233′OG-induced apoptosis in MCF-7 cells.

The Fas/FasL system is a key signalling transduction pathway of apoptosis in cells and tissues (Nagata & Golstein 1995). Ligation of Fas by agonistic antibody or its mature ligand induces receptor oligomerization and formation of death-inducing signalling complex (DISC), followed by activation of caspase-8, then further activating

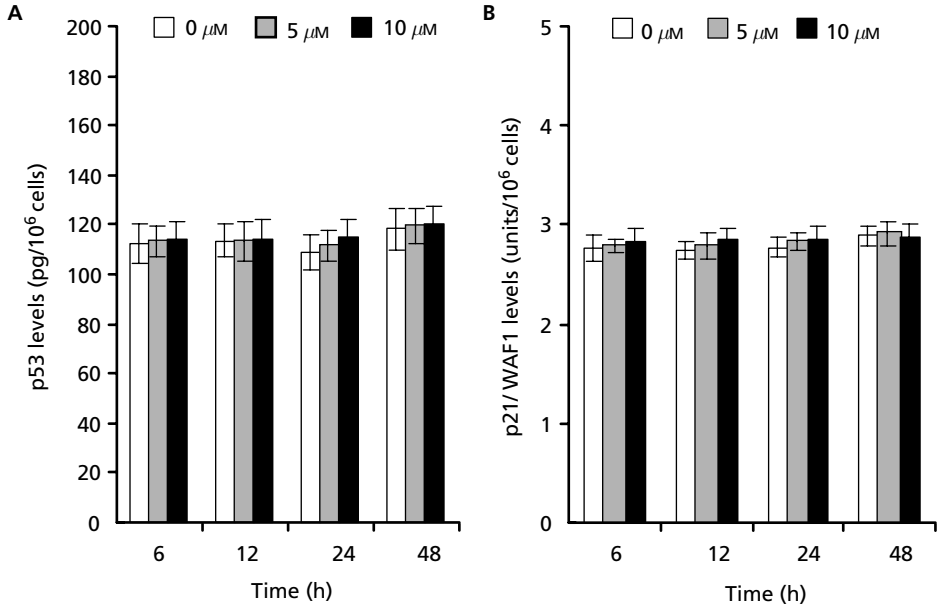

Figure 5 Effect of PB233′OG on protein expression of p53 and p21/WAF1. A. The level of p53 protein in MCF-7 cells. B. The level of p21/WAF1 in MCF-7 cells. Human breast adenocarcinoma MCF-7 cells were treated with 0, 5 and 10 μM of PB233′OG. p53 and p21/WAF1 levels were determined by p53 pan ELISA and WAF1 ELISA kit, respectively. The detailed protocol is described in Materials and Methods. Each value is the mean ± s.d. of three determinations.

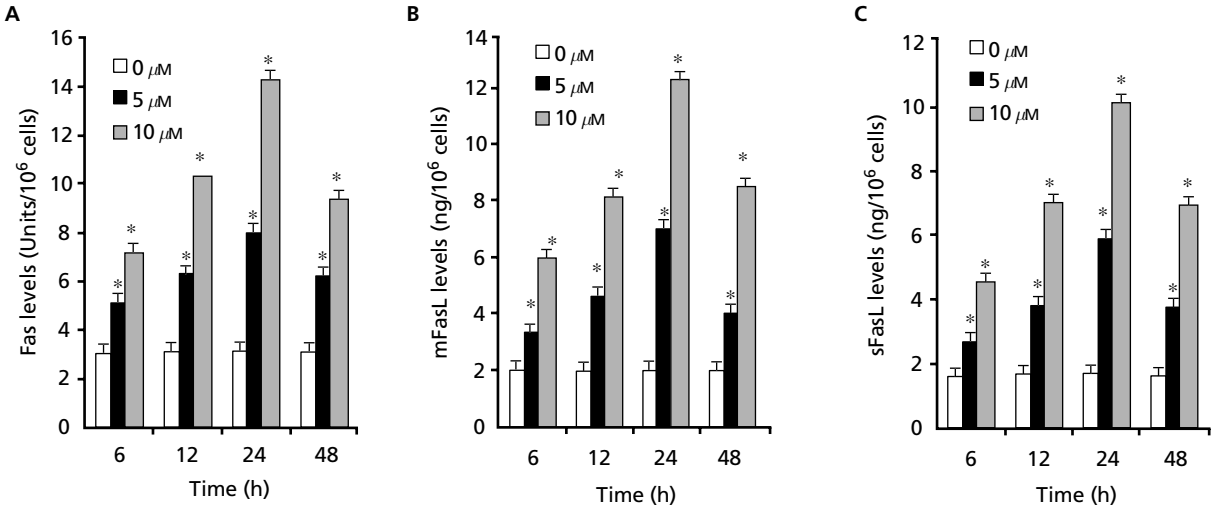

Figure 6 The Fas/FasL apoptotic system was involved in PB233′OG-mediated apoptosis. MCF-7 cells were incubated with 0, 5 and 10 μM of PB233′OG for 6, 12, 24 and 48 h. A. The level of Fas/APO-1 receptor in MCF-7 cells. B. The amount of mFasL in MCF-7 cells. C. The amount of sFasL in MCF-7 cells. Each value is the mean ± s.d. of three determinations; *$P < 0.05$, vs control cells (Dunnett's test).

a series of caspase cascades resulting in cell apoptotic death (Nagata & Golstein 1995; Hengartner 2000). FasL is a TNF related type II membrane protein (Suda et al 1993), and cleavage of the membrane-bound Fas ligand (mFasL) by a metalloprotease-like enzyme results in the formation of soluble Fas ligand (sFasL) (Kayagaki et al 1995). Defects in the Fas/FasL apoptotic signalling pathway provide a survival advantage to cancer cells and may be implicated in tumourigenesis. 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 (Landowski et al 1997; Toillon et al 2002). Our study indicated that Fas ligands mFasL and sFasL increased in PB233′OG-treated MCF-7 cells. Moreover, levels of Fas/APO-1 and the activity of caspase-8 were simultaneously enhanced in FasL-upregulating MCF-7

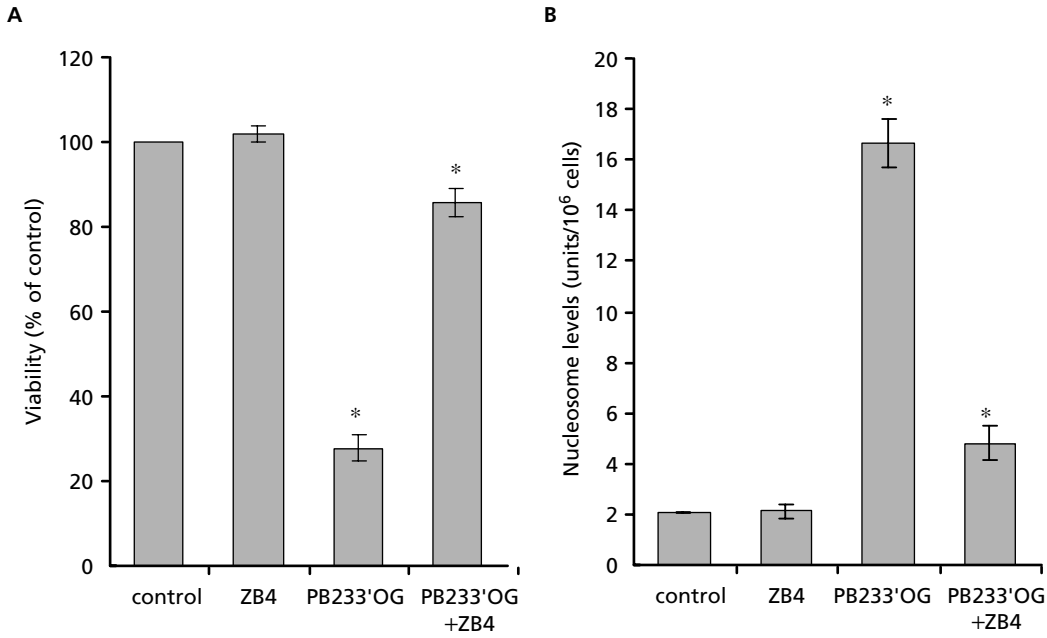

Figure 7 Effect of antagonistic anti-Fas antibody (ZB4) on PB233′OG in MCF-7 cells. The anti-proliferative (A) and pro-apoptotic effect (B) of PB233′OG was decreased by Fas antagonist ZB4. For blocking experiments, cells were pre-incubated with 250 ng mL^{-1} ZB4 for 1 h and then treated with 10 μM of PB233′OG for 48 h. Cell viability and apoptosis induction were examined by XTT and Nucleosome ELISA kit, respectively. The data shown are the means ± s.d. of three determinations; *$P < 0.05$, vs control cells (Dunnett's test).

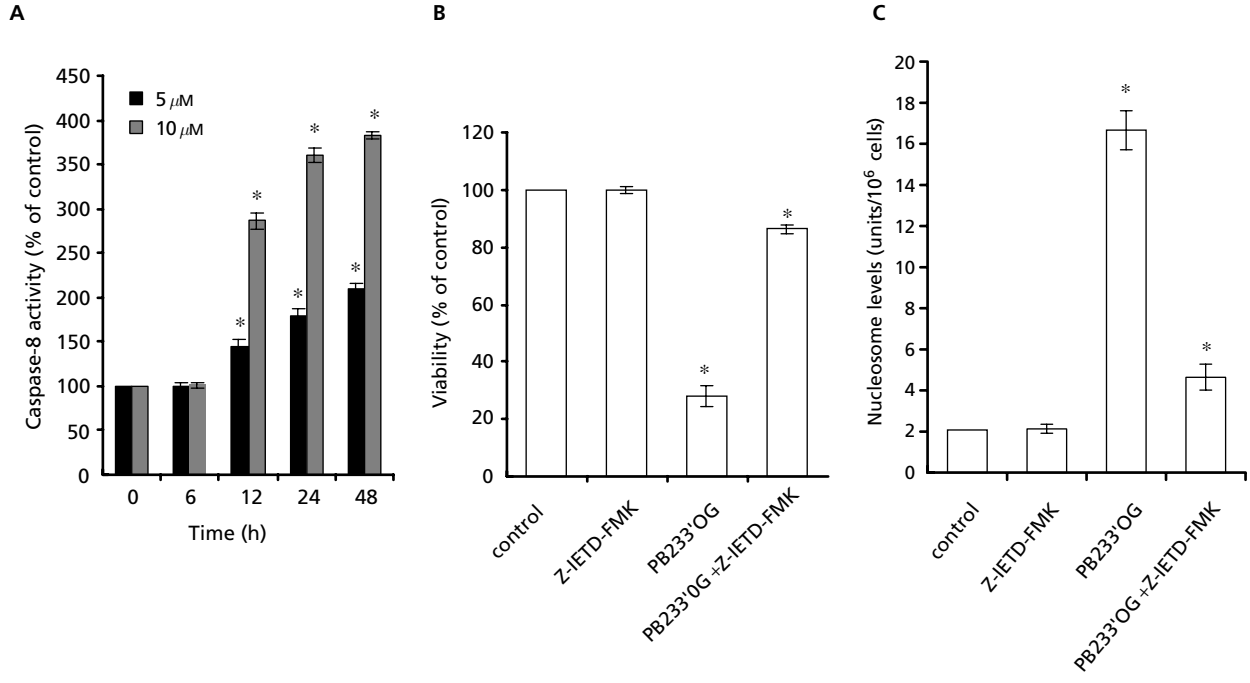

Figure 8 A. The activation of caspase-8 in MCF-7 cells by PB233′OG. B. Effect of caspase-8 inhibitor on PB233′OG-mediated anti-proliferation. C. Effect of caspase-8 inhibitor on PB233′OG-induced apoptosis. MCF-7 cells were incubated with various concentrations of PB233′OG for the indicated times. For blocking experiments, cells were pre-incubated with Z-IETD-FMK (10 μM) for 1 h before the addition of 10 μM PB233′OG. After 48 h of treatment, cell viability and induction of apoptosis were measured by XTT and Nucleosome ELISA kit, respectively. Each value is the mean ± s.d. of three determinations; *$P < 0.05$, vs control cells (Dunnett's test).

cells. Furthermore, when the Fas/Fas ligand system was blocked by ZB4, a decrease in both cell growth inhibition and the pro-apoptotic effect of PB233′OG was noted. Similarly, cell growth inhibition and apoptotic induction of PB233′OG decreased in MCF-7 cells treated with caspase-8 inhibitor. These findings are the first to show that the Fas/FasL system plays an important role in PB233′OG-mediated MCF-7 cellular apoptosis.

Overall, our results have demonstrated that PB233′OG inhibits cell growth 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 cancer (Reimer et al 2000; Chopin et al 2002), it remains to be determined whether PB233′OG treatment will prove useful in the fight against advanced breast cancer.

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