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Prodelphinidin B-2 3,3′-di-*O*-gallate from *Myrica rubra* inhibits proliferation of A549 carcinoma cells via blocking cell cycle progression and inducing apoptosis

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Abstract

In this study, the antiproliferative activity of prodelphinidin B-2 3, 3′-di-*O*-gallate (PB233′OG) isolated from the bark of *Myrica rubra* (Myricaceae) was investigated. The results showed that PB233′OG inhibited the proliferation of A549 by blocking cell cycle progression in the G0/G1 phase and inducing apoptosis. Enzyme-linked immunosorbent assay (ELISA) showed that the G0/G1 phase arrest is due to increase the expression of p21/WAF1. An enhancement in Fas/APO-1 and its two form ligands, membrane-bound Fas ligand (mFasL) and soluble Fas ligand (sFasL), might be responsible for the apoptotic effect induced by PB233′OG. Our study reports here for the first time that the induction of p21/WAF1 and activity of the Fas/Fas ligand apoptotic system may participate in the anti-proliferative activity of PB233′OG in A549 cells.

Keywords: Prodelphinidin B-2 3; 3′-di-*O*-gallate; A549 cell; p53; Fas/APO-1; Fas ligand; Apoptosis

1. Introduction

Lung cancer is the leading cause of cancer death in the world, and non-small cell lung carcinoma (NSCLC) accounts for approximately 75–85% of these cancers. Non-small cell lung cancers commonly develop resistance to radiation and chemotherapy, and they often present at stages beyond surgical remedy. Since current treatment modalities are inadequate, novel therapies are necessary to reduce the effects of the increasing incidence of pulmonary neoplasm (Cheng et al., 2003a,b; Kim et al., 2003). Efforts to improve the efficacy of chemotherapy might change the poor prognosis for lung cancer.

Apoptosis has been characterized as a fundamental cellular activity to maintain the physiological balance of the organism. It is also involved in immune defense machinery (Hengartner, 2000) and plays a necessary role as a protective mechanism against carcinogenesis by eliminating damaged cells or abnormal excess cells proliferated owing to various chemical agents' induction (Hengartner, 2000; Brown and Wouters, 1999). Emerging evidence has demonstrated that the anticancer activities of certain chemotherapeutic agents are involved in the induction of apoptosis, which is regarded as the preferred way to manage cancer (Hengartner, 2000; Brown and Wouters, 1999).

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-diarrhea, and also as a dyeing and tanning agent in Japan and China (Nonaka et al., 1983). Previous 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) (Fig. 1) is a proanthocyanidin gallate that has been reported to exhibit

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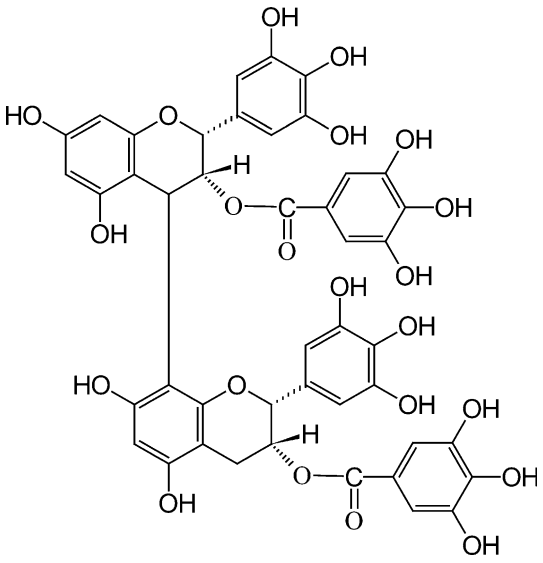

Fig. 1. Chemical structure of prodelphinidin B-2 3, 3′-di-*O*-gallate.

antioxidant and antiviral activities (Cheng et al., 2003a,b; Ohta et al., 1992; Takechi et al., 1985). To establish the anticancer mechanism of prodelphinidin B-2 3, 3′-di-*O*-gallate from *M. rubra*, 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 chemo-sensitivity of tumor cells to anticancer agents.

2. Materials and methods

2.1. Test compound

PB233′OG (purity>95%) was isolated from the bark of *M. 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 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 Roswell Park Memorial Institute-1640 (RPMI-1640). Control cultures received the carrier solvent (0.1% DMSO).

2.2. Reagents and materials

Fetal bovine serum, penicillin G, streptomycin, and amphotericin B were obtained from GIBCO BRL (Gaithersburg, MD). DMSO, ribonuclease (RNase), propidium iodide, and RPMI-1640 were purchased from Sigma (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 (Germany). Nucleosome ELISA, WAF1 ELISA, Fas Ligand, Fas/APO-1 ELISA, and caspase-8 assay kits, and caspase-8 inhibitor *N*-benzyloxycarbonyl-Ile-Glu-Thr-Asp-fluoromethylketone (Z-IETD-FMK) were purchased from Calbiochem (Cambridge, MA). Anti-Fas Ab (ZB4) was obtained from Upstate Biotechnology, (Lake Placid, NY).

2.3. Cell line and culture

Human non-small lung carcinoma cell line, A549 (American Type Culture Collection [ATCC] CCL185) was maintained in monolayer culture at 37 °C and 5% CO_2 in RPMI-1640 supplemented with 10% Fetal bovine serum, 100 units/ml of penicillin G, 100 μg/ml of streptomycin, and 0.25 μg/ml of amphotericin B.

2.4. 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 PB233′OG (0, 0.5, 2.5, 5 and 10 μM) for 12, 24, 48, and 72 h. 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 incubation, the absorbance was measured on an ELISA reader (Multiskan EX, Labsystems) at a test wavelength of 492 nm and a reference wavelength of 690 nm.

2.5. 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,

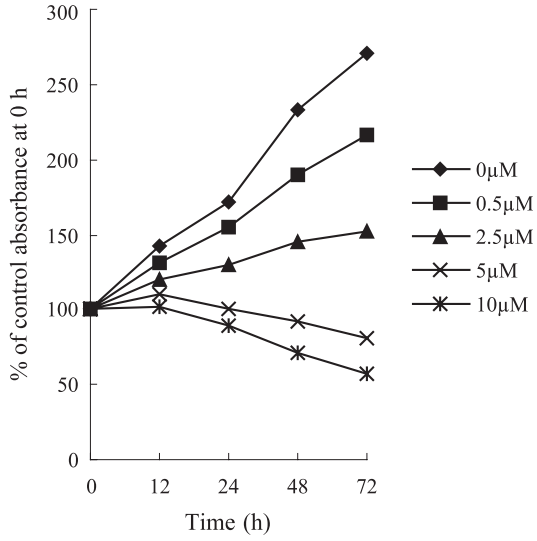

Fig. 2. The anti-proliferative effect of PB233′OG in A549 cells. Adherent cells plated in 96-well plates (10^4 cells/well) were incubated with different concentrations of PB233′OG at various time intervals. Cell proliferation was determined by XTT assay. Results are expressed as the percent of the cell proliferation of control at 0 h. Data shown are the mean obtained from three independent experiments. Standard deviations were less than 10%.

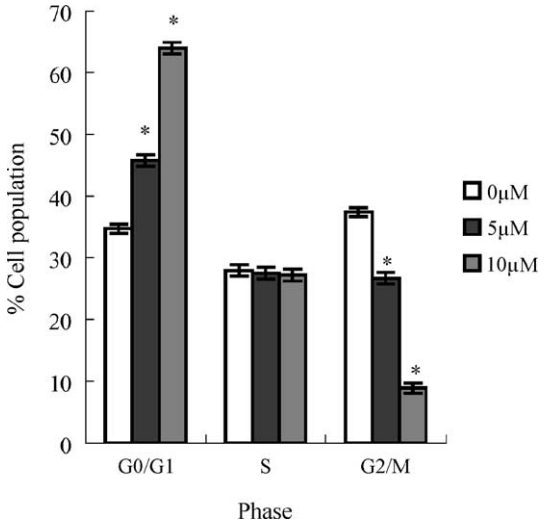

Fig. 3. Effects of PB233′OG on cell cycle distribution in A549 cells. A549 cells following treatment with 0, 5, and 10 μM PB233′OG for 24 h were fixed and stained with propidium iodide, and cell cycle distribution was then analyzed by flow cytometry. Each value is the mean±S.D. of three determinations. The asterisk indicates a significant difference between control and PB233′OG-treated cells as analyzed by Dunnett's test ($P<0.05$).

and 10 μM) for 24 h. After treatment, the cells were collected by trypsinization, fixed in 70% ethanol, washed in phosphate-buffered saline (PBS), re-suspended in 1 ml of PBS containing 1 mg/ml RNase and 50 μg/ml propidium iodide, incubated in the dark for 30 min at room temperature, and analyzed by EPICS flow cytometer. The data were analyzed using the Multicycle software (Phoenix Flow Systems, San Diego, CA).

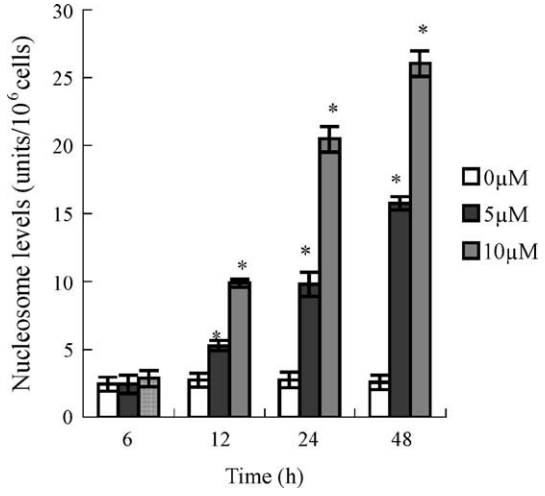

Fig. 4. Induction of apoptosis in A549 cells by PB233′OG. A549 cells were cultured with 0, 5, and 10 μM of PB233′OG for 6, 12, 24, and 48 h. Cells were harvested and lysed with lysis buffer. Cell lysates containing cytoplasmic oligonucleosomes of apoptotic cells were analyzed by means of Nucleosome ELISA. Each value is the mean±S.D. of three determinations. The asterisk indicates a significant difference between control and PB233′OG-treated cells, as analyzed by Dunnett's test ($P<0.05$).

2.6. 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. A549 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) 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.

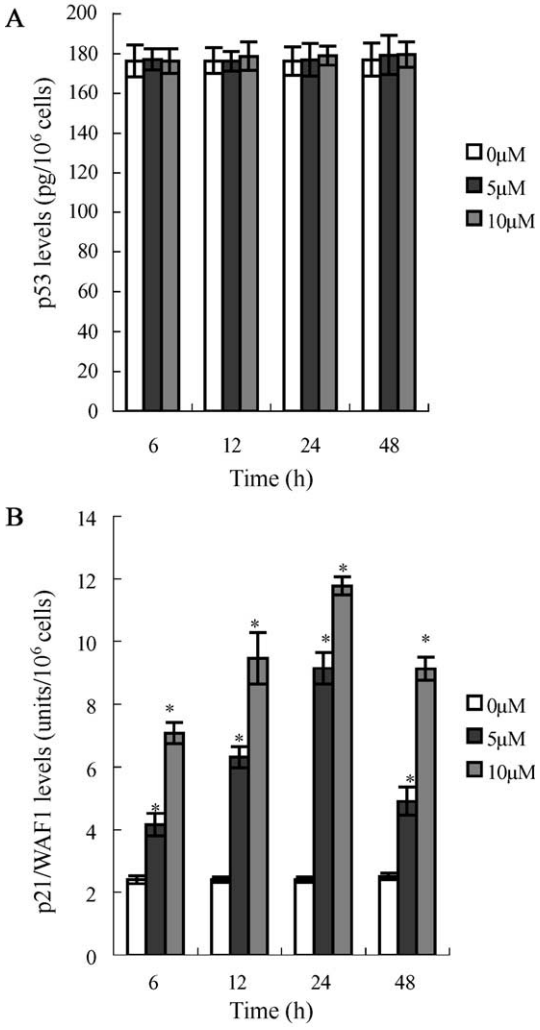

Fig. 5. Effects of PB233′OG on protein expression of p53 and p21/WAF1. (A) The level of p53 protein in A549 cells; (B) the level of p21/WAF1 in A549 cells. Human non-small cell lung cancer A549 cells were treated with 0, 5, and 10 μM of PB233′OG for 6, 12, 24, and 48 h. p53 and p21/WAF1 levels were determined 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. The asterisk indicates a significant difference between control and PB233′OG-treated cells, as analyzed by Dunnett's test ($P<0.05$).

2.7. 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). Briefly, A549 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) microtiter 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 catalyzed the conversion of a chromogenic substrate (tetramethylbenzidine) to a colored solution with 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.

2.8. 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, 1mM EDTA, 10% glycerol, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (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 to the untreated control.

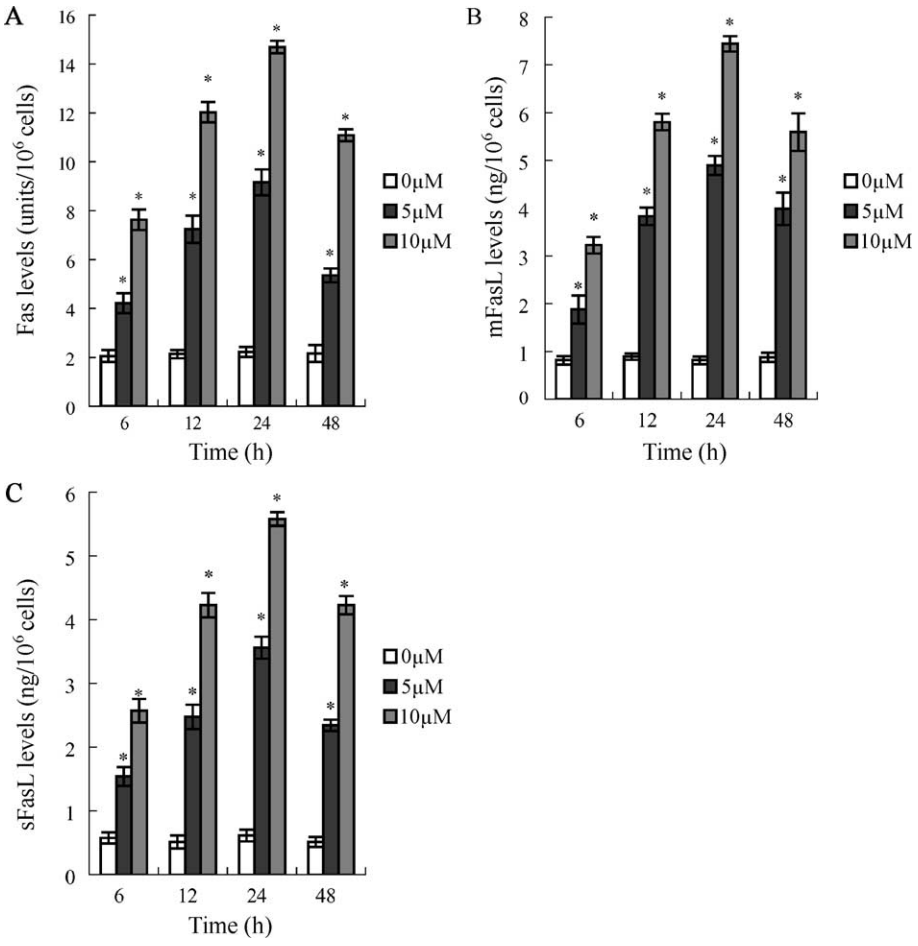

Fig. 6. Fas/FasL apoptotic system was involved in PB233′OG-mediated apoptosis. A549 cells were incubated with 0, 5, and 10 μM of PB233′OG for 6, 12, 24, and 48 h. (A) The level of Fas/APO-1 receptor in A549 cells; (B) The amount of mFasL in A549 cells; (C) The amount of sFasL in A549 cells. Each value is the mean±S.D. of three determinations. The asterisk indicates a significant difference between control and PB233′OG-treated cells, as analyzed by Dunnett's test ($P<0.05$).

2.9. Statistical analysis

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

3. Results

3.1. Effect of PB233′OG on A549 cell proliferation

We first tested the antiproliferative effect of PB233′OG in the lung cancer cell line, A549. As shown in Fig. 2, the growth inhibitory effect of PB233′OG was observed in a dose and time-dependent manner. At 48 h, the maximal effect on proliferation inhibition was observed with 10 μM PB233′OG, which inhibited proliferation in 69.6% of A549 cells. The IC_{50} value was 5.2 μM. The maximal proliferation effect of 79% was reached by 10 μM PB233′OG at 72 h.

3.2. PB233′OG-induced cell cycle arrest and apoptosis in A549 cells

The results on the effect of PB233′OG on cell cycle progression of A549 are shown in Fig. 3. As compared to the control, 5 μM of PB233′OG increased the population of G0/G1 phase from 34.7% to 45.8%. This effect was enhanced when A549 cells were treated by 10 μM of PB233′OG (63.9% cell population in G0/G1 phase).

Fig. 4 shows the time course of DNA fragmentation in continuous treatment with 5 and 10 μM of PB233′OG. DNA fragmentation of A549 was found at 12 h and maximized at 48 h after addition of PB233′OG. In contrast to the control, when cells were treated with PB233′OG, the number of cells undergoing apoptosis increased from about 6.1 fold to 10.2 fold at 5 and 10 μM of PB233′OG, respectively, at 48 h.

3.3. PB233′OG-mediated cell cycle arrest operates through the induction of p21/WAF1 protein in A549 cells

To determine whether tumor suppression factor p53 and its downstream molecule p21/WAF1 are involved in the PB233′OG-mediated antiproliferative effect of A549 cells, the levels of these proteins were assayed by ELISA. In the study of p53 protein expression, the p53-postive A549 (Lehman et al., 1991) was treated with 5 and 10 μM PB233′OG for 6, 12, 24, and 48 h. Treatment of PB233′OG for up to 10 μM at 48 h did not affect the protein expression of p53 (Fig. 5A). The p21/WAF1 protein is first characterized as a downstream target of p53, and is thought to be responsible for G0/G1 cell cycle arrest (May and May, 1999). Surprisingly, Fig. 5B shows that an increase in p21/WAF1 protein was apparent at 6 h and reached maximum induction at 24 h in PB233′OG treated A549 cells. Moreover, the induction of p21/WAF1 was observed to be in a dose-dependent manner. Our results indicated that treatment of A549 cells with PB233′OG was not observed any changes on the expression of p53. Furthermore, PB233′OG-mediated cell cycle arrest operates through the induction of p21/WAF1 protein in A549 cells.

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

It has been reported that over-expression of Fas-associating death domain protein could induce dose-

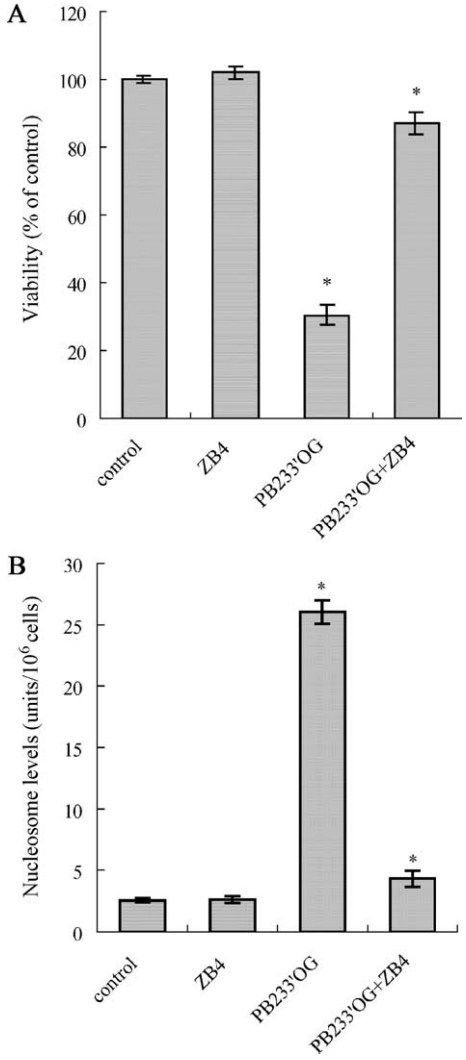

Fig. 7. Effect of antagonistic anti-Fas antibody (ZB4) on PB233′OG in A549 cells. (A) The antiproliferative and (B) proapoptotic effect of PB233′OG was decreased by Fas antagonist ZB4. For blocking experiments, cells were preincubated with 250 ng/ml ZB4 for 1 h and then treated with 10 μM of PB233′OG for 48 h. Cell viability and apoptosis induction were examined by XTT and Nucleosome ELISA kit. Each value is the mean±S.D. of three determinations. The asterisk indicates a significant difference between control and PB233′OG-treated cells, as analyzed by Dunnett's test ($P<0.05$).

dependent cell death in A549 cells (Kim et al., 2003). 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 A549 cells as early as 6 h after treatment in a dose-dependent manner (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 PB233′OG-induced apoptosis of A549 cells.

When A549 cells were pre-treated with an antagonistic anti-Fas antibody, ZB4, the antiproliferative and proapoptotic effects of PB233′OG were effectively inhibited. At 10 μM of PB233′OG, cell proliferation inhibition decreased from 69.6% to 13.2% (Fig. 7A). Compared to the control, the oligonucleosome DNA fragmentation of apoptosis induced by 10 μM of PB233′OG decreased from about 10.2-fold to 1.7-fold at 48 h in ZB4 pretreated A549 cells (Fig. 7B).

We next measured the downstream caspase of Fas/FasL system. The results showed that caspase-8 activity increased at 8 h, and reached maximum induction at 24 h in 10 μM PB233′OG treated A549 cells (Fig. 8A). The activation of caspase-8 (at 8 h) was before the production of oligonucleosome DNA fragmentation (at 12 h) showing caspase-8 activation was required in PB233′OG-induced apoptosis. To further provide this hypothesis, we assessed that the effect caspase-8 inhibitor (Z-IETD-FMK) on the PB233′OG-mediated anti-proliferation and apoptosis. Our results showed that inhibition of caspase-8 not only decreased the PB233′OG's antiproliferative activity, but

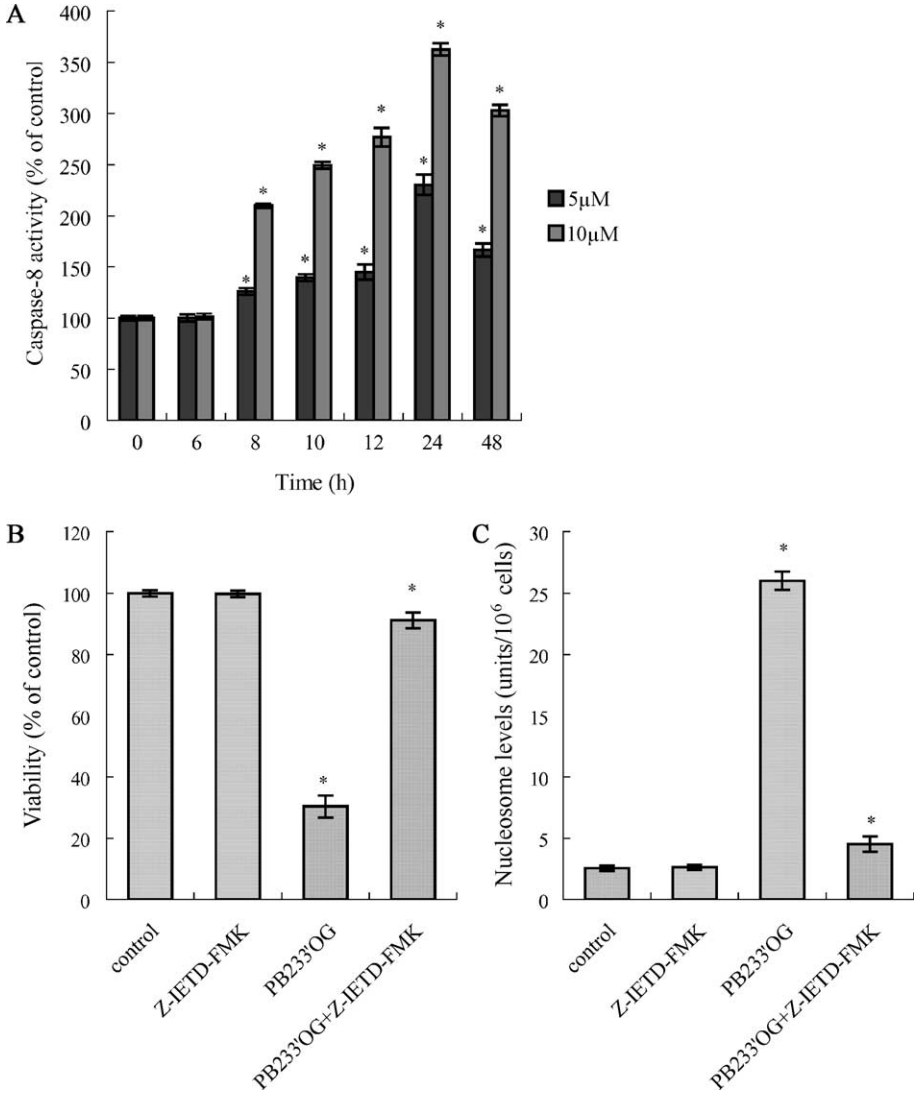

Fig. 8. (A) The activation of caspase-8 in A549 cells by PB233′OG; (B) Effect of caspase-8 inhibitor on PB233′OG-mediated anti-proliferation; (C) Effect of caspase-8 inhibitor on PB233′OG-induced apoptosis. A549 cells were incubated with various concentrations of PB233′OG for the indicated times. For blocking experiments, cells were preincubated 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. Each value is the mean±S.D. of three determinations. The asterisk indicates a significant difference between control and PB233′OG-treated cells, as analyzed by Dunnett's test ($P<0.05$).

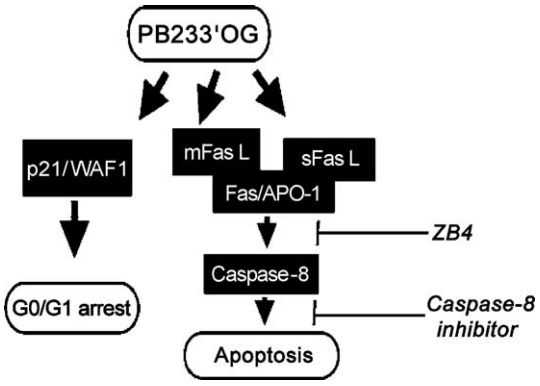

Fig. 9. Proposed model for PB233′OG-mediated cell cycle arrest and apoptosis.

also abolished induction of apoptosis in A549 cells (Figs. 8B and C).

4. Discussion

Normal p53 gene is well known to play a crucial role in inducing apoptosis and as cell cycle checkpoints in human and murine cells following DNA damage (May and May, 1999). p21/WAF1 protein inhibits the activities of various cyclin-dependent kinase (Gu et al., 1993; Harper et al., 1993; Xiong et al., 1993), and inhibits the phosphorylation of retinoblastoma protein, thereby inhibiting the G1-S phase transition (Dulic et al., 1994; Harper et al., 1993). Previous studies have shown that p21/WAF1 is transcriptionally regulated by p53-dependent and -independent pathways (El-Deiry et al., 1993; Macleod et al., 1995; Michieli et al., 1994). Our results indicated that treatment of A549 cells with PB233′OG was not observed any changes on the expression of p53. In contrast, the amount of p21/WAF1 was increased by PB233′OG treatment. Furthermore, flow cytometric analysis indicated that PB233′OG could arrest A549 cells in the G0/G1 phase. Therefore, we suggest that the blockade of cell cycle progression was attributed to the amount of enhancement of p21/WAF1 protein. However, it has been reported that not only the total amount of p53 protein but also its phosphorylation state, which in turn regulates it transcriptional activity, plays a key role in p53-mediated function (Brooks and Gu, 2003). Thus, the actual role of p53 in PB233′OG-mediated apoptosis or cell cycle arrest need to be assessed in additionally study.

Fas/FasL system is a key signaling transduction pathway of apoptosis in cells and tissues (Nagata and Golstein, 1995). Ligation of Fas by agonistic antibody or its mature ligand induces receptor oligomerization and formation of death-inducing signaling complex (DISC), followed by activation of caspase-8, then further activating a series caspase cascades resulting in cell apoptotic death (Hengartner, 2000; Nagata and Golstein, 1995). FasL is a Tumor Necrosis Factor (TNF) related type II membrane protein (Suda et al., 1993). Cleavage of membrane-bound Fas ligand (mFasL) by a metalloprotease-like enzyme results in the formation of soluble Fas ligand (sFasL) (Kayagaki et al., 1995). Fas/APO-1 is expressed in human airway epithelial cells, and plays a critical role in the pathophysiology of various pulmonary disorders (Fujita et al., 2002). Up-regulation of Fas/APO-1 expression has been demonstrated to induce apoptosis in hydrogen peroxide-treated A549 cells (Fujita et al., 2002). Serrao et al. (2001) have reported that neutrophils induce apoptosis of lung epithelial cells via release of soluble FasL. Our study indicated that Fas ligands, mFasL and sFasL, increased in PB233′OG-treated A549 cells. Moreover, the levels of Fas/APO-1 and the activity of caspase-8 were simultaneously enhanced in FasL-upregulating A549 cells following by apoptotic cell death (DNA fragmentation). Furthermore, when the Fas/Fas ligand system was blocked by ZB4, a decrease in cell growth inhibition and the proapoptotic effect of PB233′OG was noted. Similarly, cell growth inhibition and apoptotic induction of PB233′OG decreased in A549 cells treated with caspase-8 inhibitor. Thus, these findings are the first to show that the Fas/FasL system plays an important role in PB233′OG-mediated A549 cellular apoptosis.

In summary, our study suggests that PB233′OG inhibits the cell cycle progression at G0/G1 phase is through the induction of p21/WAF1. In addition, it further induces apoptotic cell death by initiation of the Fas/FasL death receptor system in A549 cells (Fig. 9). Our study has clearly demonstrated that PB233′OG may be a promising chemopreventive agent for treating lung cancer.

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