DOI 10.1002/mnfr.200700252 Mol. Nutr. Food Res. 2008, *52*, 408–418

Research Article

The grape and wine constituent piceatannol inhibits proliferation of human bladder cancer cells via blocking cell cycle progression and inducing Fas/membrane bound Fas ligand-mediated apoptotic pathway

Po-Lin Kuo and Ya-Ling Hsu

Cell Biology Laboratory, Department of Biotechnology, Chia-Nan University of Pharmacy and Science, Tainan, Taiwan

Piceatannol (3,3′,4,5′-Tetrahydroxy-*trans*-stilbene) is a polyphenol present in grapes and wine. Piceatannol is a protein kinase inhibitor that modifies multiple cellular targets exerting immunosuppressive, antileukemic, and antitumorigenic activities in several cell lines and animal models. In this study, the antiproliferative activity of piceatannol was investigated. The results showed that piceatannol inhibited the proliferation of T24 and HT1376 human bladder cancer cells by blocking cell cycle progression in the G0/G1 phase and inducing apoptosis. ELISA showed that the G0/G1 phase arrest is due to an increased in the expression of p21/WAF1. An enhancement in Fas/APO-1 and membrane-bound Fas ligand (mFasL) might be responsible for the apoptotic effect induced by piceatannol. Our study reports the novel finding, that the induction of p21/WAF1 and activity of the Fas/mFasL apoptotic system may participate in the antiproliferative activity of piceatannol in T24 and HT1376 cells.

Keywords: Apoptosis / Bladder cancer / Cell cycle / Fas / Piceatannol

Received: July 4, 2007; revised: August 23, 2007; accepted: September 8, 2007

1 Introduction

Bladder cancer is the second most-common cancer of the genitourinary tract. Currently, there are a wide range of therapeutic modalities available based on the extent of the disease including intravesical chemotherapy, surgery, radiation therapy, and systemic chemotherapy. Unfortunately, patients with advanced bladder cancer face a 5 year survival rate of approximately 20–40% despite the wide range of treatment modalities [1–3]. This is because these classical treatments are hampered by unwanted side effects and, most importantly, by the development of tumor resistance [4]. There is an obvious urgent need for novel and effective therapies against bladder cancer.

Apoptosis plays an important role in homeostasis and development of tissue in the organism [4]. 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 [4, 5]. Several previous publications have reported that anti-cancer agents may induce apoptosis via the Fas/FasL system [6–8]. Fas is a cell surface receptor comprising a type I transmembrane receptor that expresses a cytoplasmic death domain and belongs to the tumor necrosis factor receptor superfamily [9]. Activation of Fas by its ligand (FasL) results in the oligomerization of its intracellular death domain and the recruitment of the intracellular adaptator Fas-associated death domain. Once bound, Fas-associated death domain is able to activate procaspases-8 and -10 in a death inducing signaling complex. In turn, caspases-8 and -10 activate downstream caspases, resulting in apoptotic cell death [10].

Correspondence: Dr. Ya-Ling Hsu, Cell Biology Laboratory, Department of Biotechnology, Chia-Nan University of Pharmacy and Science, No. 60, Erh-Jen Road, Sec.1, Jen-Te, Tainan 717, Taiwan
E-mail: hsuyl326@gmail.com
Fax: +886-6-266-2135

Abbreviations: FasL, ligand bound Fas; **FBS**, fetal bovine serum; **mFasL,** membrane-bound Fas ligand; **sFasL,** soluble Fas ligand; **PI**, propidium iodide; **XTT,** sodium 3-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene-sulfonic acid hydrate; **ZB4,** anti-Fas anitbody; **Z-IETD-FMK,** benzyloxy-carbonyl-Val-Ala-Asp-fluoromethylketone

Piceatannol (3,3′,4,5′-Tetrahydroxy-*trans*-stilbene) is a naturally occurring stilbene present in the skins of grapes, rhubarb, and sugar cane [11 – 14]. Piceatannol, a promising chemopreventive agent with anti-leukemic activity, is being extensively studied for treating various cancers and diseases, including colorectal and lung cancer as well as cardiovascular disease [15, 16]. Piceatannol acetate significantly inhibited the tumor growth Lewis lung carcinoma-bearing and carcinectomized mice [17]. Ferreira *et al.* revealed that piceatannol and its methylation derivatives increased the proapoptotic effect of doxorubicin in MDR1 gene-transfected mouse lymphoma cells [18]. Piceatannol is structurally similar to the anti-cancer stilbene resveratrol, which is also present in red wine, and preliminary data suggests that resveratrol is metabolized to piceatannol via cytochrome P4501B1 [19]. Piceatannol has been reported to be a protein-tyrosine kinase inhibitor with immunosuppressive activity, which could play an important role in preventing graft rejection [20 – 23]. Furthermore, piceatannol also has been indicated to possess anti-inflammatory properties, suppressing the activation of the nuclear transcription factor nuclear factor kappa B through inhibition of IκBα kinase and p65 phosphorylation [24]. However, the underlying mechanism of action of piceatannol in bladder cancer cells has remained largely unknown. In this study, we determined the antiproliferative activity of piceatannol, and examined its effect on cell cycle distribution and apoptosis in two human bladder cancer cell lines, T24 and HT1376. Furthermore, to establish the anticancer mechanism of piceatannol, we assayed the levels of p53, p21/WAF1, Fas/APO-1 receptor, and FasL, which are strongly associated with the signal transduction pathway of apoptosis and affect the chemosensitivity of tumor cells to anticancer agents.

2 Materials and methods

2.1 Chemicals and reagents

Fetal bovine serum (FBS), penicillin G, streptomycin, amphotericin B and DMEM were obtained from GIBCO BRL (Gaithersburg, MD, USA). Piceatannol, DMSO, ribonuclease, and propidium iodide (PI) were purchased from Sigma Chemical (St. Louis, MO, USA). Sodium 3-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene-sulfonic acid hydrate (XTT) and p53 pan ELISA kits were obtained from Roche Diagnostics (Mannheim, Germany). Nucleosome ELISA, WAF1 ELISA, FasL, Fas/APO-1 ELISA, and caspase-8 assay kits, and caspase-8 inhibitor, benzyloxy-carbonyl-Val-Ala-Asp-fluoromethylketone (Z-IETD-FMK) were purchased from Calbiochem (Cambridge, MA, USA). Anti-Fas antibody (ZB4) was obtained from Upstate Biotechnology (Lake Placid, NY, USA).

2.2 Preparation of piceatannol

Piceatannol was dissolved in DMSO and stored at −20°C. 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).

2.3 Cell culture

Bladder cancer cell line T24 (ATCC HTB-4) and HT1376 (ATCC CRL-1472) were obtained from the American Type Cell Culture Collection (Rockville, MD, USA). It was maintained in monolayer culture at 37°C and 5% CO_2 in DMEM supplemented with 10% FBS, 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 piceatannol 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 vehicle alone (0.1% DMSO) and piceatannol (0.5, 2.5, 5, and 10 μM) for 48 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; Thermo Electron Corporation, Milford, USA) 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 vehicle alone (0.1% DMSO) and piceatannol (4 and 8 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 ribonuclease and 50 μg/mL PI, 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, USA).

2.6 The analysis of apoptosis

Quantitative assessment of apoptosis was analyzed by an Annexin V assay kit (BD Biosciences PharMingen, San Jose, CA, USA). Briefly, cells grown in 10 cm Petri dishes were harvested with trypsin and washed in PBS. Cells were then resuspended in binding buffer (10 mmol/L HEPES/NaOH (pH 7.4), 140 mmol/L NaCl, 2.5 mmol/L $CaCl_2$) and stained

with Annexin V-FITC and PI at room temperature for 15 min in the dark. Cells were then analyzed in an EPICS flow cytometer (Coulter Electronics) within 1 h after staining. Data from 10 000 cells were collected for each data file. Apoptotic cells were defined as Annexin V-FITC-positive and PI-negative cells. Our definition of cellular status is as follows: unstained cells were classified as "live", cells stained for Annexin V only were "early apoptotic", cells stained for both Annexin V and PI were "late apoptotic", and cells stained for PI only were "dead". Apoptotic cells were the sum of early and late apoptotic cells [25 – 27].

Quantitative assessment of oligonucleosomal DNA fragmentation was also 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. For determination of apoptosis by ELISA, cells were treated with vehicle alone (0.1% DMSO) and piceatannol (4 and 8 μM) for the indicated time. The induction of apoptosis was evaluated by assessing the enrichment of nucleosome in cytoplasm, and determined exactly as described in the manufacturer's protocol [28].

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 FasL ELISA kits were used to detect p53, p21, Fas/APO-1 receptor and soluble (sFasL)/membrane-bound Fas ligand (mFasL). Briefly, T24 and HT1376 cells were treated with vehicle alone (0.1% DMSO) and piceatannol (4 and 8 μM) for 6, 12, 24, and 48 h. The samples of cell lysate were placed in 96 well (1×10^6 per well) microtiter plates that 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 sFasL in cell culture supernatant by using FasL 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 [29].

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 DTT, 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 to the untreated control [28].

2.9 Western blot assay

Cells (8×10^6/dish) were seeded in a 10 cm dish. After 24 h of incubation, cells were treated with 8 μM of piceatannol for the indicated times. Total cell extracts were prepared in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na_3VO_4, 0.5% NP-40, 1% Triton X-100, 1 mM PMSF, 5 μg/mL Aprotinin, and 5 μg/mL Leupetin). Equivalent amounts of protein were resolved by SDS-PAGE (10 – 12%) and transferred to PVDF membranes. After the membrane was blocked in Tris-buffer saline containing 0.05% Tween 20 and 5% non-fat powdered milk, the membranes were incubated with primary antibodies (the antibodies specific against p53 and p21) at 4°C for 16 h. After washing three times with Tris-buffer saline for 10 min each, the membrane was incubated with horseradish peroxidase-labeled secondary antibody for 1 h. The membranes were washed again, and detection performed using the enhanced chemiluminescence blotting detection system (Amersham, USA) [30].

2.10 Statistical analysis

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

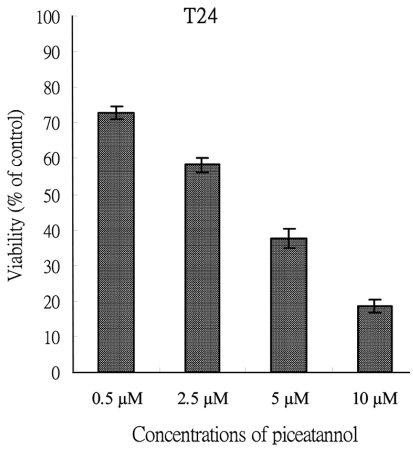
3 Results

3.1 Effect of piceatannol on T24 and HT1376 cell proliferation

We first tested the antiproliferative effect of piceatannol in two human bladder cancer cell lines, T24 and HT1376. As shown in Fig. 1, the proliferative inhibitory effect of piceatannol was observed in a dose-dependent manner. At 48 h, the maximal effect on proliferation inhibition was observed with 10 μM piceatannol, which inhibited proliferation in 81.5 and 77.5% of T24 and HT1376 cells. The IC_{50} values were 3.9 and 4.6 μM, respectively.

3.2 Piceatannol-induced cell cycle arrest and apoptosis in T24 and HT1376 cells

The results on the effects of piceatannol on cell cycle progression of T24 and HT1376 are shown in Fig. 2. As com-



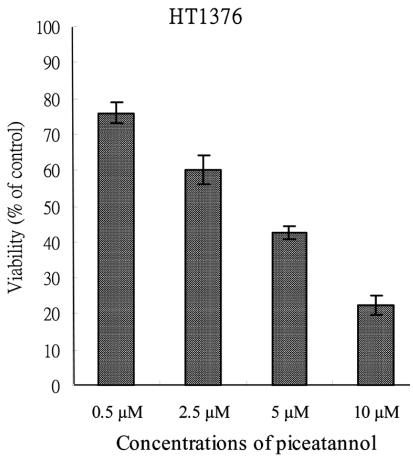
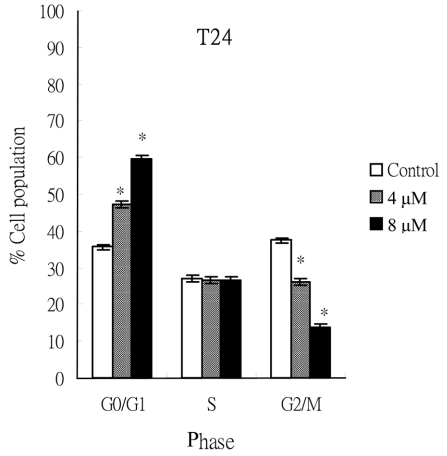


Figure 1. The anti-proliferative effect of piceatannol in T24 and HT1376 cells. Adherent cells plated in 96-well plates (10^4 cells/well) were incubated with different concentrations of piceatannol at 48 h. Cell proliferation was determined by XTT assay. Results are expressed as percent cell proliferation relative to the proliferation of control. Each value is the mean ± SD of three determinations.



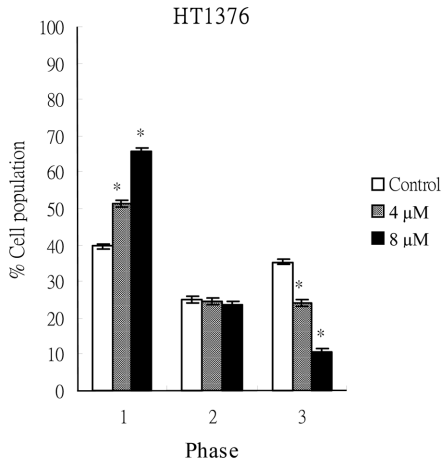


Figure 2. Effects of piceatannol on cell cycle distribution in T24 and HT1376 cells. Cells following treatment with vehicle alone (0.1% DMSO) and piceatannol (4 and 8 μM) for 24 h were fixed and stained with PI, and cell cycle distribution was then analyzed by flow cytometry. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between control and piceatannol-treated cells as analyzed by Dunnett's test ($p < 0.05$).

pared to the control, 4 μM of piceatannol increased the population of G0/G1 phase from 35.7 to 47.2% and 39.7 to 51.2% in T24 and HT1376 cells. This effect was enhanced when T24 and HT1376 cells were treated by 8 μM of piceatannol (59.6 and 65.6% cell population in G0/G1 phase of T24 and HT1376, respectively).

A quantitative evaluation of apoptosis was sought using an Annexin V-FITC dye to detect the translocation of phosphatidylserine from the inner (cytoplasmic) leaflet of the plasma membrane to the outer (cell surface). Compared with vehicle-treated cells, 8 μM piceatannol induced 42.7 and 34.7% of apoptotic cells in T24 and HT1376 at 48 h, respectively (Fig. 3(A)). Additionally, a quantitative evaluation was sought using ELISA to detect histone-associated oligonucleosome DNA fragments. Compared with vehicle-treated cells, 8 μM piceatannol induced 9.2- and 6.5-fold of cytoplasmic oligonucleosome in T24 and HT1376 at 48 h, respectively (Fig. 3(B)).

3.3 Piceatannol-mediated cell cycle arrest operates through the induction of p21/WAF1 protein in T24 and HT1376 cells

To determine whether tumor suppression factor p53 and its downstream molecule p21/WAF1 are involved in the piceatannol-mediated antiproliferative effects of T24 and HT1376 cells, the levels of these proteins were assayed by ELISA and western blot. T24 and HT1376 cells were treated with 4 and 8 μM piceatannol for 6, 12, 24, and 48 h. Treatment of piceatannol for up to 8 μM at 48 h did

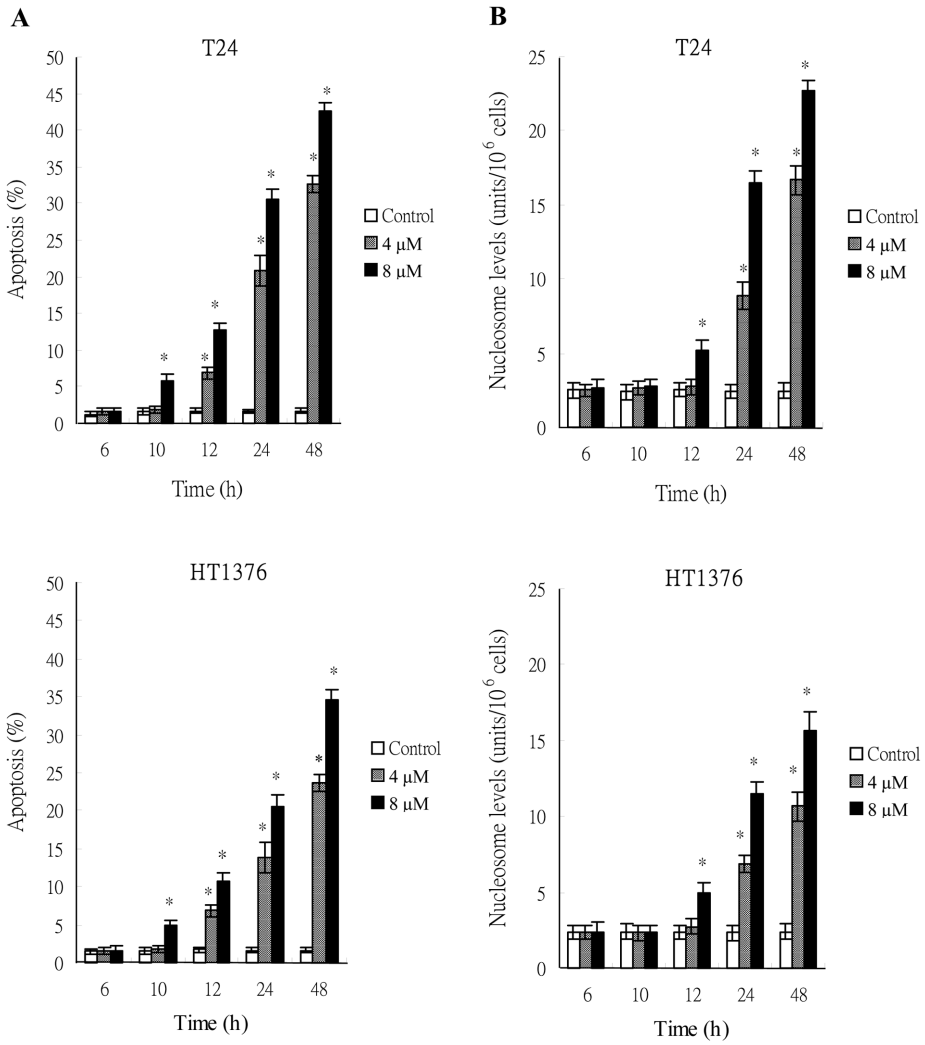


Figure 3. Induction of apoptosis in T24 and HT1376 cells by piceatannol. Piceatannol induced apoptosis in T24 and HT1376 cells determined by Annexin V-FITC/PI dye (A) and Nucleosome ELISA kit (B). For (A) and (B), cells were treated with vehicle and piceatannol for indicated times, and then the induction of apoptosis was assessed by Annexin V-FITC/PI dye and Nucleosome ELISA kit. The asterisk indicates a significant difference between control and piceatannol-treated cells as analyzed by Dunnett's test ($p < 0.05$).

not affect the protein expression of p53 (Figs. 4(A) and (C)). 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 [31]. Surprisingly, Figs. 4(B) and (C) show that an increase in p21/WAF1 protein was apparent at 6 h and was observed in a time-dependent manner. Moreover, the induction of p21/WAF1 was observed to be in a dose-dependent manner. Our results indicated that treatment of T24 and HT1376 cells with piceatannol did not induce any changes on the expression of p53. Furthermore, piceatannol-mediated cell cycle arrest operates through the induction of p21/WAF1 protein in T24 and HT1376 cells.

3.4 Fas/mFasL apoptotic system might be a possible pathway of piceatannol-mediated apoptosis

One study demonstrated immunohistochemistry Fas and Fas-ligand expression in tumor tissue of patient with bladder cancer [32]. By using Fas/APO-1 ELISA and FasL ELISA kits, we found that piceatannol increased expression of Fas/APO-1 receptor and mFasL in T24 and HT1376 cells as early as 6 h after treatment in a dose-dependent and time-dependent manner (Figs. 5(A) and (B)). We detected very low concentrations of the sFasL in culture supernatants of T24 and HT1376 cells (Fig. 5(C)). This result was similar to a previous report [33]. The time relationship between the

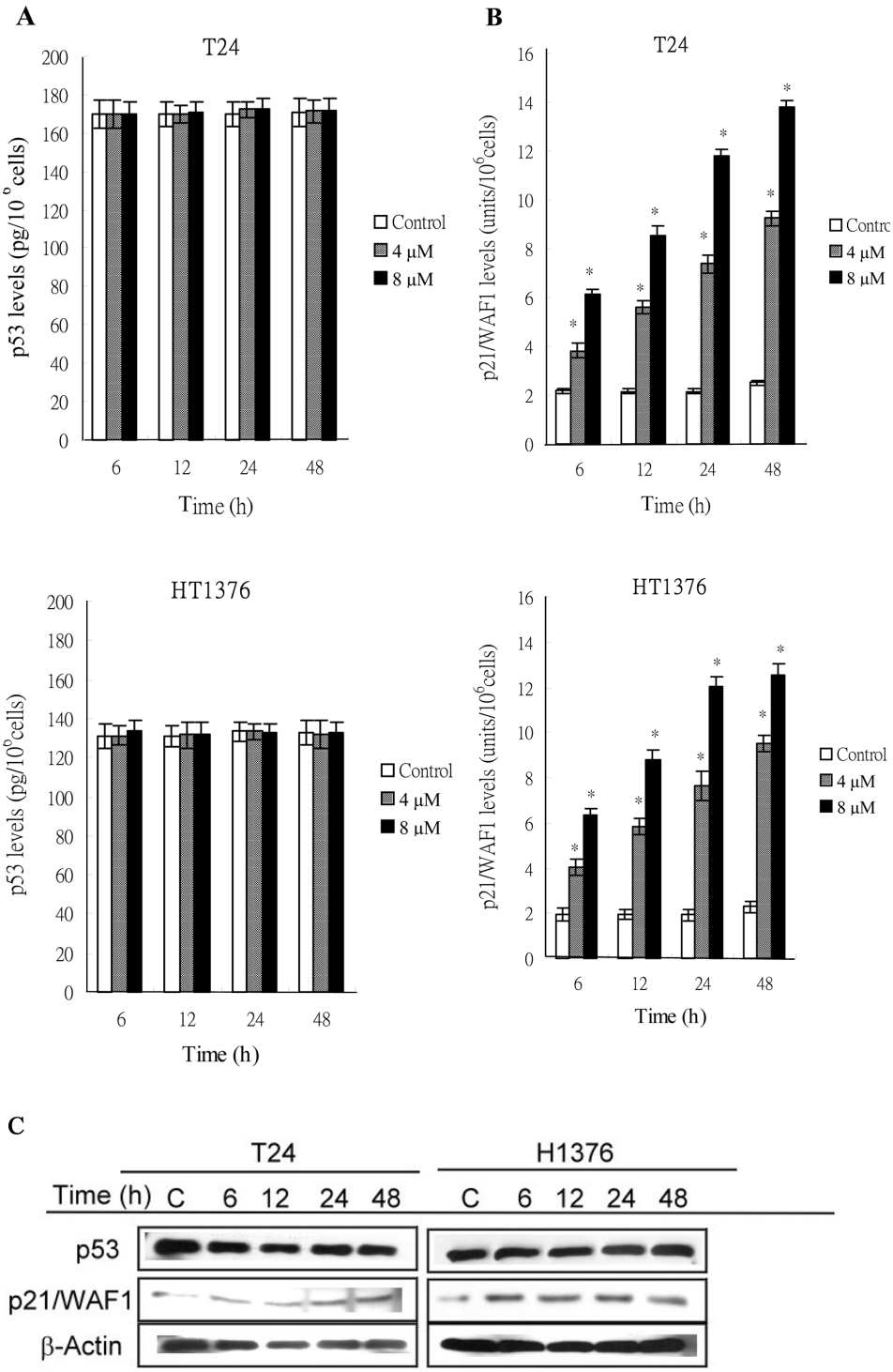


Figure 4. Effects of piceatannol on protein expression of p53 and p21/WAF1. (A) The level of p53 protein in T24 and HT1376 cells; (B) the level of p21/WAF1 in T24 and HT1376 cells. (C) The levels of p53 and p21/WAF1 in T24 and HT1376 examined by western blot. Cells were treated with vehicle alone (0.1% DMSO) and piceatannol (4 and 8 μM) for 6, 12, 24, and 48 h. p53 and p21/WAF1 levels were determined by ELISA and western blot assay. The detailed protocol is described in Section 2. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between control and piceatannol-treated cells, as analyzed by Dunnett's test ($p < 0.05$).

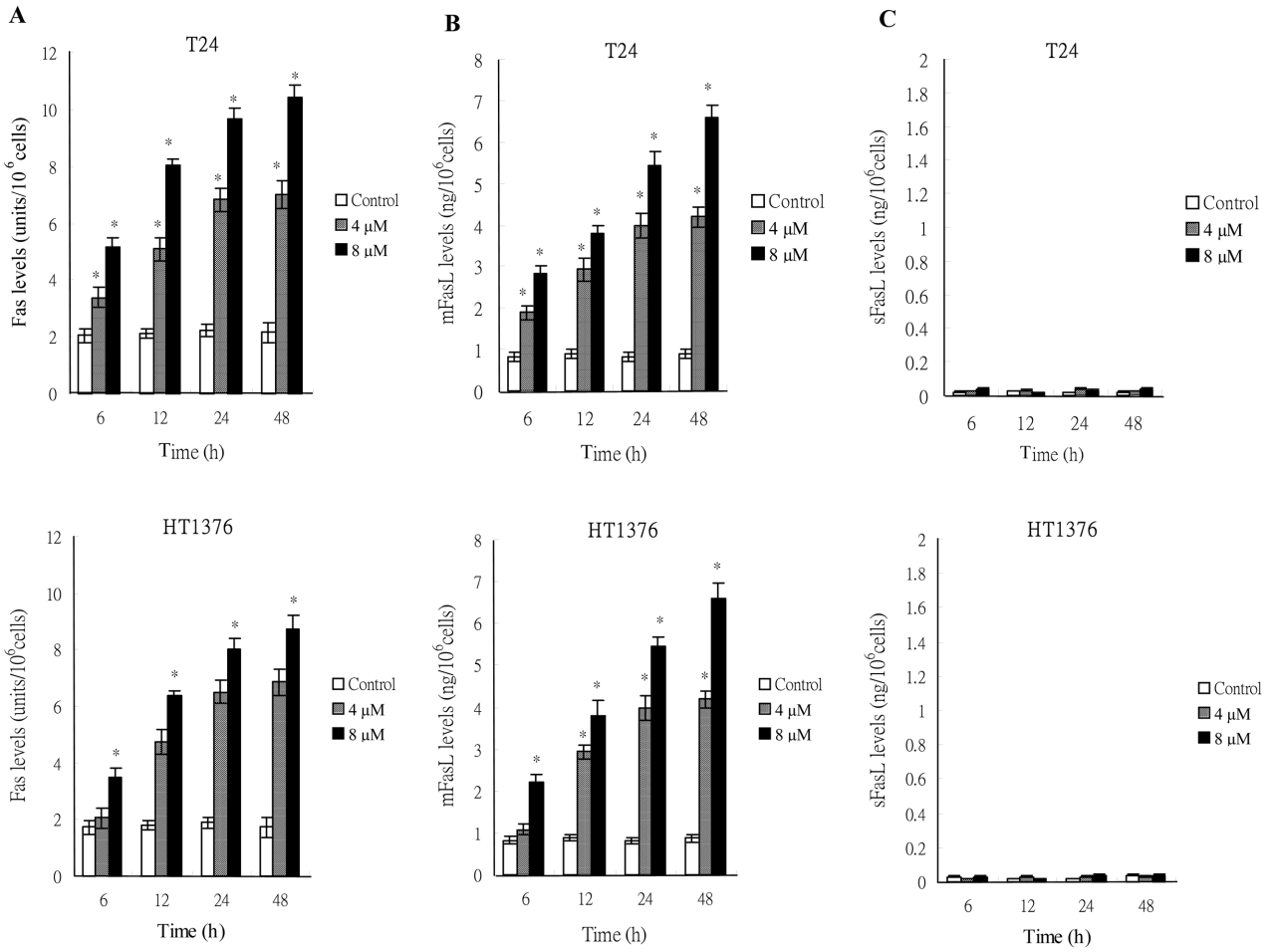


Figure 5. Fas/FasL apoptotic system was involved in piceatannol-mediated apoptosis. Cells were incubated with vehicle alone (0.1% DMSO) and piceatannol (4 and 8 μM) for 6, 12, 24, and 48 h. (A) The level of Fas/APO-1 receptor in T24 and HT1376 cells; (B) The amount of mFasL in T24 and HT1376 cells; (C) The amount of sFasL in T24 and HT1376 cells. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between control and piceatannol-treated cells, as analyzed by Dunnett's test ($p < 0.05$).

expression of Fas/mFasL at 6 h of treatment and the occurrence of oligonucleosome DNA fragmentation at 12 h of treatment could support the idea that the Fas/mFasL system might mediate piceatannol-induced apoptosis of T24 and HT1376 cells.

When T24 and HT1376 cells were pre-treated with an antagonistic ZB4 the antiproliferative and proapoptotic effects of piceatannol were effectively inhibited. At 8 μM of piceatannol, cell proliferation inhibition decreased from 81.5 to 13.2% and 77.5 to 19.2% in T24 and HT1376, respectively (Fig. 6(A)). Compared to the control, the oligonucleosome DNA fragmentation of apoptosis induced by 8 μM of piceatannol decreased from about 9.2- to 2-fold and 6.5- to 2.8-fold at 48 h in ZB4 pretreated T24 and HT1376 cells, respectively (Fig. 6(B)).

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 48 h in 8 μM piceatannol treated T24 and HT1376 cells (Fig. 7(A)). 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 piceatannol-induced apoptosis. To further provide this hypothesis, we assessed that the effect caspase-8 inhibitor (Z-IETD-FMK) on the piceatannol-mediated anti-proliferation and apoptosis. Our results showed that inhibition of caspase-8 not only decreased the piceatannol's antiproliferative activity, but also completely abolished induction of apoptosis in T24 and HT1376 cells (Figs. 7(B) 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 [31]. p21/WAF1

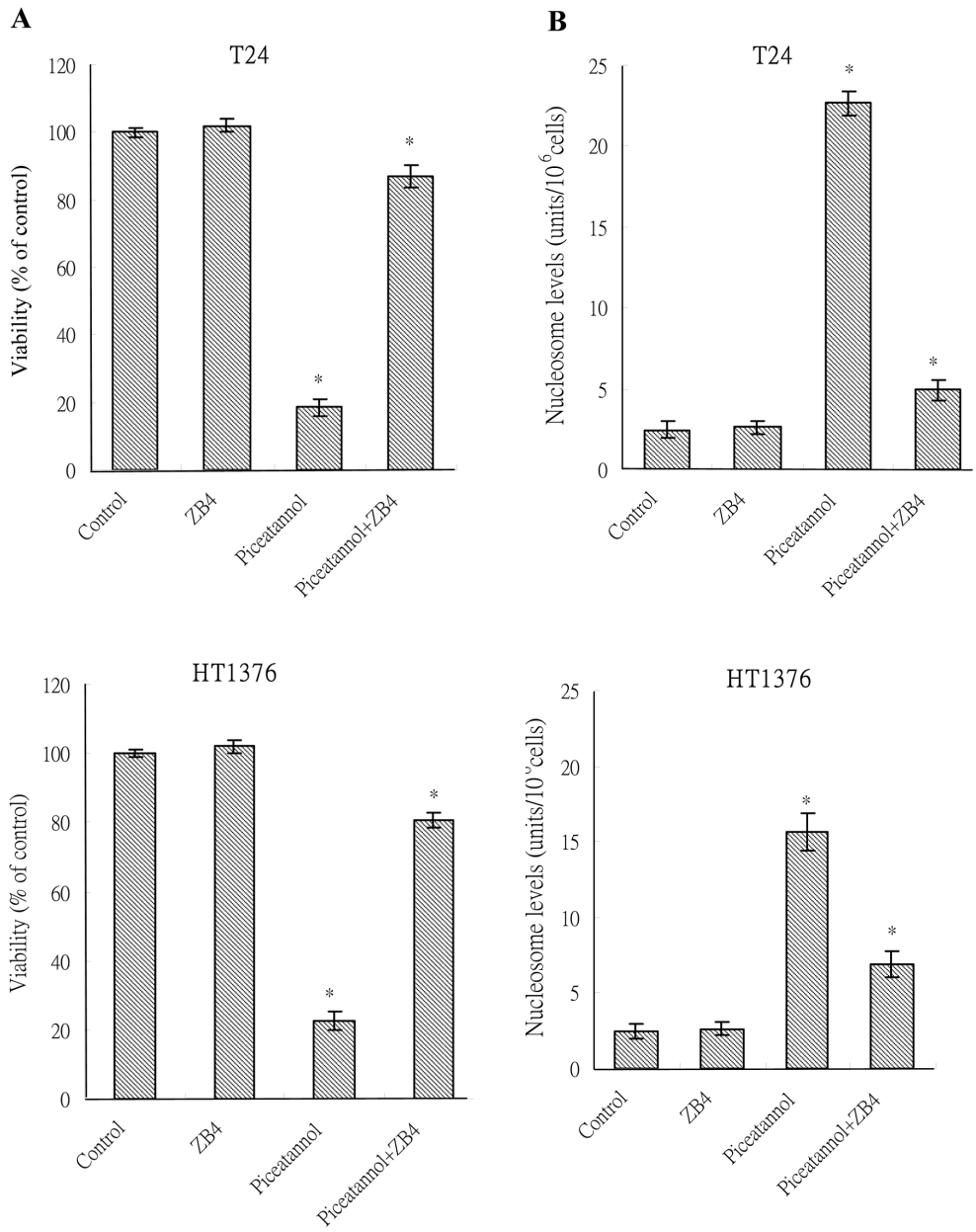


Figure 6. Effect of antagonistic ZB4 on piceatannol in T24 and HT1376 cells. (A) The antiproliferative and (B) proapoptotic effect of piceatannol was decreased by Fas antagonist ZB4. For blocking experiments, cells were preincubated with 250 ng/mL ZB4 for 1 h and then treated with 8 μM of piceatannol for 48 h. Cell viability and apoptosis induction were examined by XTT and Nucleosome ELISA kit. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between control and piceatannol-treated cells, as analyzed by Dunnett's test ($p < 0.05$).

protein inhibits the activities of various cyclin-dependent kinase [34–36], and inhibits the phosphorylation of retinoblastoma protein, thereby inhibiting the G1-S phase transition [35, 37]. Previous studies have shown that p21/WAF1 is transcriptionally regulated by p53-dependent and -independent pathways [38–40]. Our results show that treatment of T24 and HT1376 cells with piceatannol did not result in any changes on the expression of p53. In contrast, the amount of p21/WAF1 was increased by piceatannol treatment. Furthermore, flow cytometric analysis indicated that piceatannol could arrest T24 and HT1376 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 [41]. Thus, the actual role of p53 in piceatannol-mediated

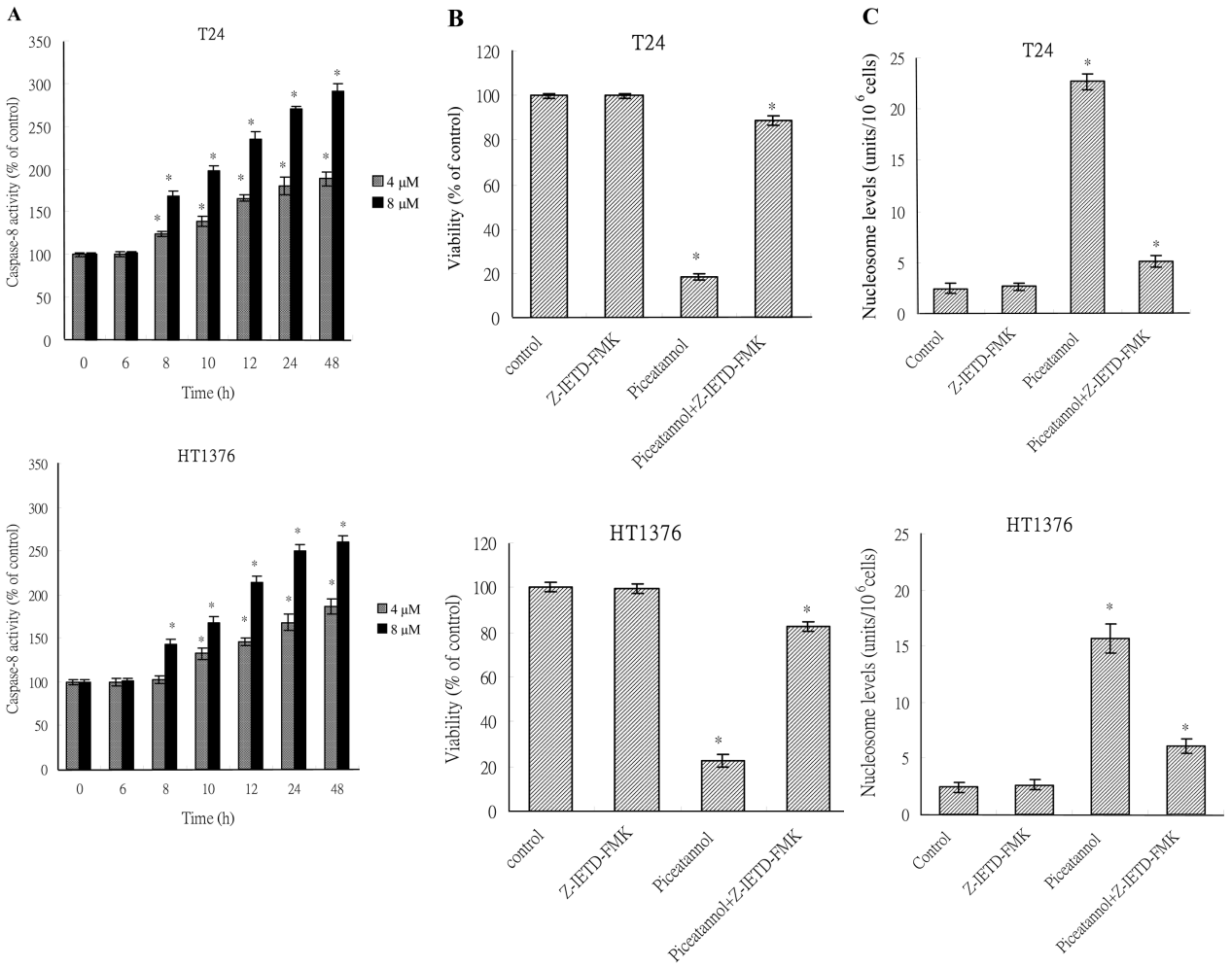


Figure 7. (A) The activation of caspase-8 in T24 and HT1376 cells by piceatannol; (B) Effect of caspase-8 inhibitor on piceatannol-mediated anti-proliferation; (C) Effect of caspase-8 inhibitor on piceatannol-induced apoptosis. Cells were incubated with various concentrations of piceatannol for the indicated times. For blocking experiments, cells were preincubated with Z-IETD-FMK (10 μM) for 1 h before the addition of 8 μM piceatannol. After 48 h of treatment, cell viability and induction of apoptosis were measured by XTT and Nucleosome ELISA kit. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between control and piceatannol-treated cells, as analyzed by Dunnett's test ($p < 0.05$).

apoptosis or cell cycle arrest needs to be assessed in additional studies.

Fas/FasL system is a key signaling transduction pathway of apoptosis in cells and tissues [9]. Ligation of Fas by agonistic antibody or by its natural 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 [9, 42]. FasL is a Tumor Necrosis Factor related type II membrane protein [43]. Cleavage of mFasL by a metalloprotease-like enzyme results in the formation of sFasL [44]. Investigation by Mizutani *et al*. revealed that the established bladder cancer cell line T24 was resistant to apoptosis following treatment with the agonistic Fas antibody CH-11. However, bladder cancer cells were sensitized to Fas-mediated cytotoxicity by CH-11 following cotreatment with either doxorubicin or adriamycin [45, 46]. Our study indicated that mFasL increased in piceatannol-treated T24 and HT1376 cells. Moreover, the levels of Fas/APO-1 and the activity of caspase-8 were simultaneously enhanced in mFasL-upregulating T24 and HT1376 cells following by apoptotic cell death (DNA fragmentation). Furthermore, when the Fas/mFas ligand system was blocked by ZB4, a decrease in cell growth inhibition and the proapoptotic effect of piceatannol was noted. Similarly, cell growth inhibition and apoptotic induction of piceatannol decreased in T24 and HT1376 cells treated with caspase-8 inhibitor. Thus, these findings are the first to show that the Fas/mFasL system plays an important role in piceatannol-mediated T24 and HT1376 cellular apoptosis.

In summary, our study has clearly demonstrated that piceatannol exerts a cytotoxic activity on the T24 and HT1376 cell lines. Therefore, the potential of this substance for the treatment of bladder cancer should be further investigated.

The authors have declared no conflict of interest.

5 References

[1] Cookson, M. S., Herr, H. W., Zhang, Z. F., Soloway, S., *et al.,* The treated natural history of high risk superficial bladder cancer: 15-year outcome, *J. Urol.* 1997, *58,* 62 – 67.

[2] Herr, H. W., Natural history of superficial bladder tumors: 10- to 20-year follow-up of treated patients, *World J. Urol.* 1997, *15,* 84 – 88.

[3] Sudarshan, S., Holman, D. H., Hyer, M. L., Voelkel-Johnson, C., *et al., In vitro* efficacy of Fas ligand gene therapy for the treatment of bladder cancer, *Cancer Gene Ther.* 2005, *12,* 12 – 18.

[4] Igney, F. H., Krammer, P. H., Death and anti-death: tumour resistance to apoptosis, *Nat. Rev. Cancer* 2002, *2,* 277 – 288.

[5] Kasibhatla, S., Tseng, B., Why target apoptosis in cancer treatment? *Mol. Cancer Ther.* 2003, *2,* 573 – 580.

[6] Lin, J. G., Chen, G. W., Li, T. M., Chouh, S. T., *et al.,* Aloe-emodin induces apoptosis in T24 human bladder cancer cells through the p53 dependent apoptotic pathway, *J. Urol.* 2006, *175,* 343 – 347.

[7] Perabo, F. G., Wirger, A., Kamp, S., Lindner, H., *et al.,* Carboxyamido-triazole (CAI), a signal transduction inhibitor induces growth inhibition and apoptosis in bladder cancer cells by modulation of Bcl-2, *Anticancer Res.* 2004, *24,* 2869 – 2877.

[8] Mizutani, Y., Yoshida, O., Bonavida, B., Sensitization of human bladder cancer cells to Fas-mediated cytotoxicity by cis-diamminedichloroplatinum (II), *J. Urol.* 1998, *160,* 561 – 570.

[9] Nagata, S., Golstein, P., The Fas death factor, *Science* 1995, *267,* 1449 – 1456.

[10] Juo, P., Kuo, C. J., Yuan, L., Blenis, J., Essential requirement for caspase-8/FLICE in the initiation of the Fas-induced apoptotic cascade, *Curr. Biol.* 1998, *8,* 1001 – 1008.

[11] Cantos, E., Espin, J. C., Tomas-Barberan, F. A., Postharvest stilbene-enrichment of red and white table grape varieties using UV-C irradiation pulses, *J. Agric. Food Chem.* 2002, *50,* 6322 – 6329.

[12] Matsuda, H., Tomohiro, N., Hiraba, K., Harima, S., *et al.,* Study on anti-Oketsu activity of rhubarb II. Anti-allergic effects of stilbene components from Rhei undulati Rhizoma (dried rhizome of Rheum undulatum cultivated in Korea), *Biol. Pharm. Bull.* 2001, *24,* 264 – 267.

[13] Roupe, K., Teng, X. W., Fu, X., Meadows, G. G., Davies, N. M., Determination of piceatannol in rat serum and liver microsomes: Pharmacokinetics and phase I and II biotransformation, *Biomed. Chromatogr.* 2004, *18,* 486 – 491.

[14] Waffo-Teguo, P., Hawthorne, M. E., Cuendet, M., Merillon, J. M., *et al.,* Potential cancer-chemopreventive activities of wine stilbenoids and flavans extracted from grape (Vitis vinifera) cell cultures, *Nutr. Cancer* 2001, *40,* 173 – 179.

[15] Kimura, Y., Baba, K., Okuda, H., Inhibitory effects of active substances isolated from Cassia garrettiana heartwood on tumor growth and lung metastasis in Lewis lung carcinoma-bearing mice (Part 1), *Anticancer Res.* 2000, *20,* 2899 – 2906.

[16] Wolter, F., Clausnitzer, A., Akoglu, B., Stein, J., Piceatannol, a natural analog of resveratrol, inhibits progression through the S phase of the cell cycle in colorectal cancer cell lines, *J. Nutr.* 2002, *132,* 298 – 302.

[17] Kimura, Y., Baba, K., Okuda, H., Inhibitory effects of active substances isolated from Cassia garrettiana heartwood on tumor growth and lung metastasis in Lewis lung carcinoma-bearing mice (Part 2), *Anticancer Res.* 2000, *20,* 2923 – 2930.

[18] Ferreira, M. J., Duarte, N., Gyemant, N., Radics, R., *et al.,* Interaction between doxorubicin and the resistance modifier stilbene on multidrug resistant mouse lymphoma and human breast cancer cells. *Anticancer Res.* 2006, *26,* 3541 – 3546.

[19] Potter, G. A., Patterson, L. H., Wanogho, E., Perry, P. J., *et al.,* The cancer preventative agent resveratrol is converted to the anticancer agent piceatannol by the cytochrome P450 enzyme CYP1B1, *Br. J. Cancer* 2002, *86,* 774 – 778.

[20] Fernandez, L. A., Torrealba, J., Yagci, G., Ishido, N., *et al.,* Piceatannol in combination with low doses of cyclosporine A prolongs kidney allograft survival in a stringent rat transplantation model, *Transplantation* 2002, *74,* 1609 – 1617.

[21] Geahlen, R. L., McLaughlin, J. L., Piceatannol (3,4,3′,5′-tetrahydroxy-trans-stilbene) is a naturally occurring protein-tyrosine kinase inhibitor, *Biochem. Biophys. Res. Commun.* 1989, *165,* 241 – 245.

[22] Larrosa, M., Tomas-Barberan, F. A., Espin, J. C., The grape and wine polyphenol piceatannol is a potent inducer of apoptosis in human SK-Mel-28 melanoma cells. *Eur. J. Nutr.* 2004, *43,* 275 – 284.

[23] Seow, C. J., Chue, S. C., Wong, W. S., Piceatannol, a Syk-selective tyrosine kinase inhibitor, attenuated antigen challenge of guinea pig airways *in vitro*, *Eur. J. Pharmacol.* 2002, *443,* 189 – 196.

[24] Ashikawa, K., Majumdar, S., Banerjee, S., Bharti, A. C., *et al.,* Piceatannol inhibits TNF-induced NF-kappaB activation and NF-kappaB-mediated gene expression through suppression of IkappaBalpha kinase and p65 phosphorylation, *J. Immunol.* 2002, *169,* 6490 – 6497.

[25] Xu, Z., Choudhary, S., Voznesensky, O., Mehrotra, M., *et al.,* Overexpression of COX-2 in human osteosarcoma cells decreases proliferation and increases apoptosis, *Cancer Res.* 2006, *66,* 6657 – 6664.

[26] Barton, B. E., Karras, J. G., Murphy, T. F., Barton, A., Huang, H. F., Signal transducer and activator of transcription 3 (STAT3) activation in prostate cancer: Direct STAT3 inhibition induces apoptosis in prostate cancer lines, *Mol. Cancer Ther.* 2004, *3,* 11 – 20.

[27] Kuo, Y. C., Kuo, P. L., Hsu, Y. L., Cho, C. Y., Lin, C. C., Ellipticine induces apoptosis through p53-dependent pathway in human hepatocellular carcinoma HepG2 cells, *Life Sci.* 2006, *78,* 2550 – 2557.

[28] Kuo, P. L., Hsu, Y. L., Lin, T. L., Lin, C. C., The antiproliferative activity of prodelphinidin B-2 3′-O-gallate from green tea leaf is through cell cycle arrest and Fas-mediated apoptotic pathway in A549 cells, *Food Chem. Toxicol.* 2005, *43,* 315 – 323.

[29] Hsu, Y. L., Kuo, P. L., Lin, L. T., Lin, C. C., Asiatic acid, a triterpene, induces apoptosis and cell cycle arrest through activation of extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways in human breast cancer cells, *J. Pharmacol. Exp. Ther.* 2005, *313,* 333 – 344.

[30] Kuo, P. L., Chen, C. Y., Hsu, Y. L., Isoobtusilactone A induces cell cycle arrest and apoptosis through reactive oxygen species/apoptosis signal-regulating kinase 1 signaling pathway in human breast cancer cells. *Cancer Res.* 2007, *67*, 7406 – 7420.

[31] May, P., May, E., Twenty years of p53 research: structural and functional aspects of the p53 protein, *Oncogene* 1999, *18,* 7621 – 7636.

[32] Lee, S. H., Lee, J. Y., Park, W. S., Kim, S. Y., *et al.,* Transitional cell carcinoma expresses high levels of Fas ligand *in vivo*, *BJU Int.* 1999, *83,* 698 – 702.

[33] Perabo, F. G., Mattes, R. H., Wirger, A., Kamp, S., *et al.,* Soluble Fas and Fas-ligand in bladder cancer *in vitro* and *in vivo*, *Urol. Oncol.* 2001, *6,* 163 – 169.

[34] Gu, Y., Turck, C. W., Morgan, D. O., Inhibition of CDK2 activity *in vivo* by an associated 20K regulatory subunit, *Nature* 1993, *366,* 707 – 710.

[35] Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K., Elledge, S. J., The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases, *Cell* 1993, *75,* 805 – 816.

[36] Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., *et al.,* p21 is a universal inhibitor of cyclin kinases, *Nature* 1993, *366,* 701 – 704.

[37] Dulic, V., Kaufmann, W. K., Wilson, S. J., Tlsty, T. D., *et al.,* p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest, *Cell* 1994, *76,* 1013 – 1023.

[38] el-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., *et al.,* WAF1, a potential mediator of p53 tumor suppression, *Cell* 1993, *75,* 817 – 825.

[39] Macleod, K. F., Sherry, N., Hannon, G., Beach, D., *et al.,* p53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage, *Genes Dev.* 1995, *9,* 935 – 944.

[40] Michieli, P., Chedid, M., Lin, D., Pierce, J. H., *et al.,* Induction of WAF1/CIP1 by a p53-independent pathway, *Cancer Res.* 1994, *54,* 3391 – 3395.

[41] Brooks, C. L., Gu, W., Ubiquitination, phosphorylation and acetylation: The molecular basis for p53 regulation, *Curr. Opin. Cell Biol.* 2003, *15,* 164 – 171.

[42] Hengartner, M. O., The biochemistry of apoptosis, *Nature* 2000, *407,* 770 – 776.

[43] Suda, T., Takahashi, T., Golstein, P., Nagata, S., Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family, *Cell* 1993, *75,* 1169 – 1178.

[44] Kayagaki, N., Kawasaki, A., Ebata, T., Ohmoto, H., *et al.,* Metalloproteinase-mediated release of human Fas ligand, *J. Exp. Med.* 1995, *182,* 1777 – 1783.

[45] Mizutani, Y., Yoshida, O., Effect of anticancer agents on Fas-mediated cytotoxicity against bladder cancer cells, *J. Infect. Chemother.* 1999, *5,* 139 – 143.

[46] Mizutani, Y., Okada, Y., Yoshida, O., Fukumoto, M., Bonavida, B., Doxorubicin sensitizes human bladder carcinoma cells to Fas-mediated cytotoxicity, *Cancer* 1997, *79,* 1180 – 1199.

