Induction of cell cycle arrest and apoptosis in human non-small cell lung cancer A549 cells by casuarinin from the bark of *Terminalia arjuna* Linn.

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Casuarinin, a hydrolyzable tannin isolated from the bark of *Terminalia arjuna* Linn. (Combretaceae), inhibits human non-small cell lung cancer A549 cells by blocking cell cycle progression in the G_0/G_1 phase and inducing apoptosis. Enzyme-linked immunosorbent assay showed that the G_0/G_1 phase arrest is due to p53-dependent induction of p21/WAF1. An enhancement in Fas/APO-1 and the two forms of Fas ligand (FasL), membrane-bound FasL and soluble FasL, might be responsible for the apoptotic effect induced by casuarinin. Our study reports here for the first time that the induction of p53 and the activity of the Fas/FasL apoptotic system may participate in the antiproliferative activity of casuarinin in A549 cells.
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Introduction

Terminalia arjuna Linn. (Combretaceae), a large woody plant, has been reported to exhibit hypocholesterolemic, hypolipidemic, antioxidative, antimutagenic, antibacterial and antiviral activities [1–6]. Previous studies have shown that casuarinin can induce apoptosis in HL-60 cells [7]. It exhibited inhibitory effects on nitric oxide production by the murine macrophage-like cell line RAW 264.7 and cytotoxic effects on the proliferation of PRMI-7951 melanoma cells [8,9]. Furthermore, it was also shown to inhibit carbonic anhydrase activity [10]. To establish the anticancer mechanism of casuarinin from the bark of *T. arjuna*, 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 tumor cells to anticancer agents.

Materials and methods

Test compound

Casuarinin (Fig. 1) was isolated from the bark of *T. arjuna* as described previously [11]. Briefly, air-dried bark of *T. arjuna* was chipped into small pieces and extracted at room temperature with acetone:water (4:1, v/v). The extract was concentrated under reduced pressure (about 40°C) to give an aqueous solution which contained resinous precipitate. After removing the precipitate, the filtrate was concentrated and eluted with a solvent system of methanol:water:acetone to give four fractions. Fraction III was repeatedly chromatographed on a Sephadex LH-20, MCI-gel CHP 20P and Fuji-gel ODS G3 column to obtain casuarinin. The structure of casuarinin was confirmed by comparing its spectroscopic and physical data with the authentic sample as described by Lin *et al.* [11]. The stock solution of casuarinin was prepared at a concentration of 2 mg/ml 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 RPMI 1640. Control cultures received the carrier solvent [0.1% dimethylsulfoxide (DMSO)].

Reagents and materials

Fetal bovine serum (FBS), penicillin G, streptomycin and amphotericin B were obtained from Gibco/BRL (Gaithersburg, MD). DMSO, ribonuclease (RNase), propidium iodide (PI) and RPMI 1640 were purchased from Sigma (St Louis, MO). The {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 *N*-benzyloxycarbonyl-Ile-Glu-Thr-Asp-fluoromethylketone (z-IETD-fmk) were purchased from Calbiochem (Cambridge, MA). Anti-Fas antibody (ZB4) was obtained from Upstate Biotechnology (Lake Placid, NY).

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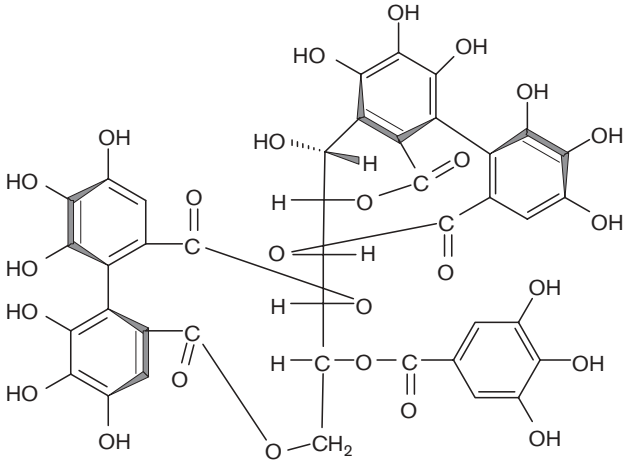
Fig. 1



Chemical structure of casuarinin isolated from the bark of *T. arjuna*.

Cell line and culture

Human non-small lung carcinoma cell line A549 (CCL185; ATCC, Manassas, VA) was maintained in monolayer culture at 37°C and 5% CO_2 in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B.

Cell proliferation assay

Inhibition of cell proliferation by casuarinin was measured by the 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 casuarinin (0, 1, 5, 10 and 20 μM) for 12, 24, 48 and 72 h. Then, 50 μl 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, Milford, MA) 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 casuarinin (0, 10 and 20 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 RNase and 50 μg/ml propidium iodide, incubated in the dark for 30 min at room temperature, and analyzed by an Epics flow cytometer. The data were analyzed using the Multicycle software (Phoenix Flow Systems, San Diego, CA).

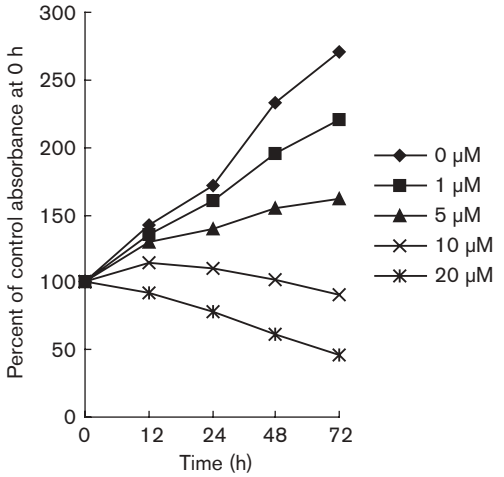
Measurement of apoptosis by ELISA

The induction of apoptosis by casuarinin 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, 10 and 20 μM casuarinin, for 6, 12, 24 and 48 h. The samples of cell lysate were placed in 96-well (1×10^6/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.

Assaying the levels of p53, p21, Fas/APO-1 and FasL (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 (mFasL) FasL. Briefly, A549 cells were treated with 0, 10 and 20 μM casuarinin for 6, 12, 24 and 48 h. The samples of cell lysate were placed in 96-well (1×10^6/well) microtiter plates coated with monoclonal detective antibodies and incubated for 1 (Fas/APO-1), 2 (p53 or p21/WAF1) or 3 h (FasL) at room temperature. It was necessary to determine the sFasL in cell culture supernatant by using the 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

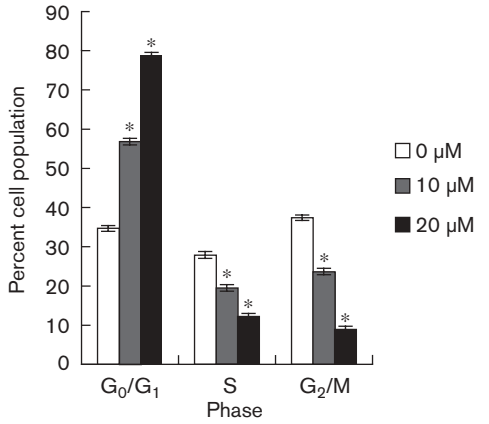
Fig. 2



The antiproliferative effect of casuarinin in A549 cells. Adherent cells plated in 96-well plates (10^4 cells/well) were incubated with different concentrations of casuarinin at various time intervals. Cell proliferation was determined by the XTT assay. Results are expressed as the percent of the cell proliferation of control at 0 h. The data shown are the mean obtained from three independent experiments. SDs were less than 10%.

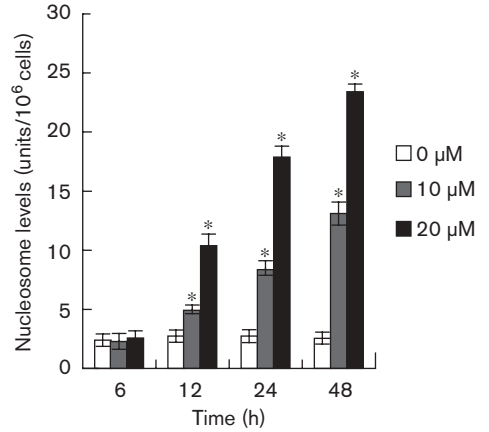
with color intensity proportional to the amount of protein present in the sample. The absorbance of each well was measured at 450 nm, and concentrations of p53, p21/WAF1, Fas/APO-1 and FasL were determined by interpolating from standard curves obtained with known concentrations of standard proteins.

Fig. 3



Effects of casuarinin on cell cycle distribution in A549 cells. Following treatment with 0, 10 and 20 μM casuarinin for 24 h, A549 cells were fixed and stained with propidium iodide, 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 casuarinin-treated cells as analyzed by Dunnett's test ($p < 0.05$).

Fig. 4



Induction of apoptosis in A549 cells by casuarinin. A549 cells were cultured with 0, 10 and 20 μM of casuarinin 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 asterisk indicates a significant difference between control and casuarinin-treated cells, as analyzed by Dunnett's test ($p < 0.05$).

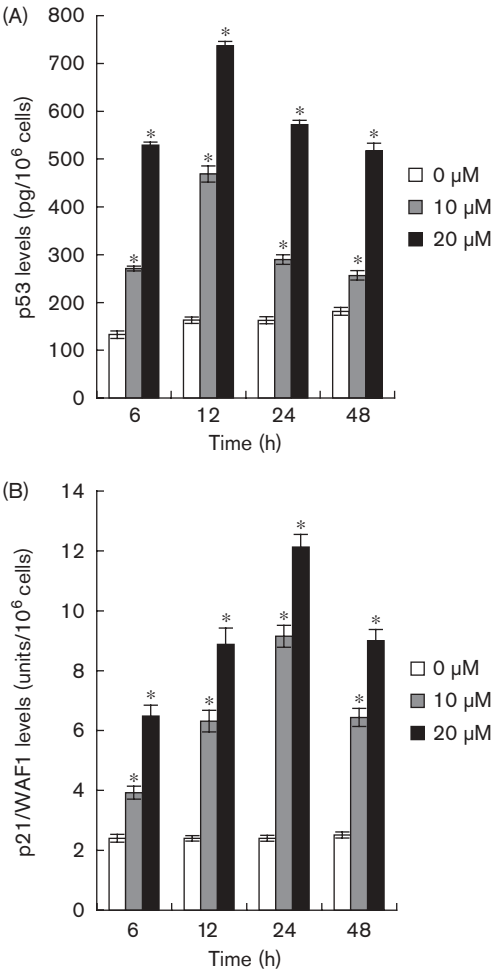
Assay for caspase-8 activity

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 and 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.

Statistical analysis

Data were expressed as means ± SD. Statistical comparisons of the results were made using analysis of variance

Fig. 5



Effects of casuarinin 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, 10 and 20 μM of casuarinin for 6, 12, 24 and 48 h. p53 and p21/WAF1 levels were determined by p53 pan-ELISA and WAF1 ELISA kits, respectively. The detailed protocol is described in Materials and methods. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between control and casuarinin-treated cells, as analyzed by Dunnett's test ($p < 0.05$).

(ANOVA). Significant differences ($p < 0.05$) between the means of control and casuarinin-treated cells were analyzed by Dunnett's test.

Results

Effect of casuarinin on A549 cell proliferation

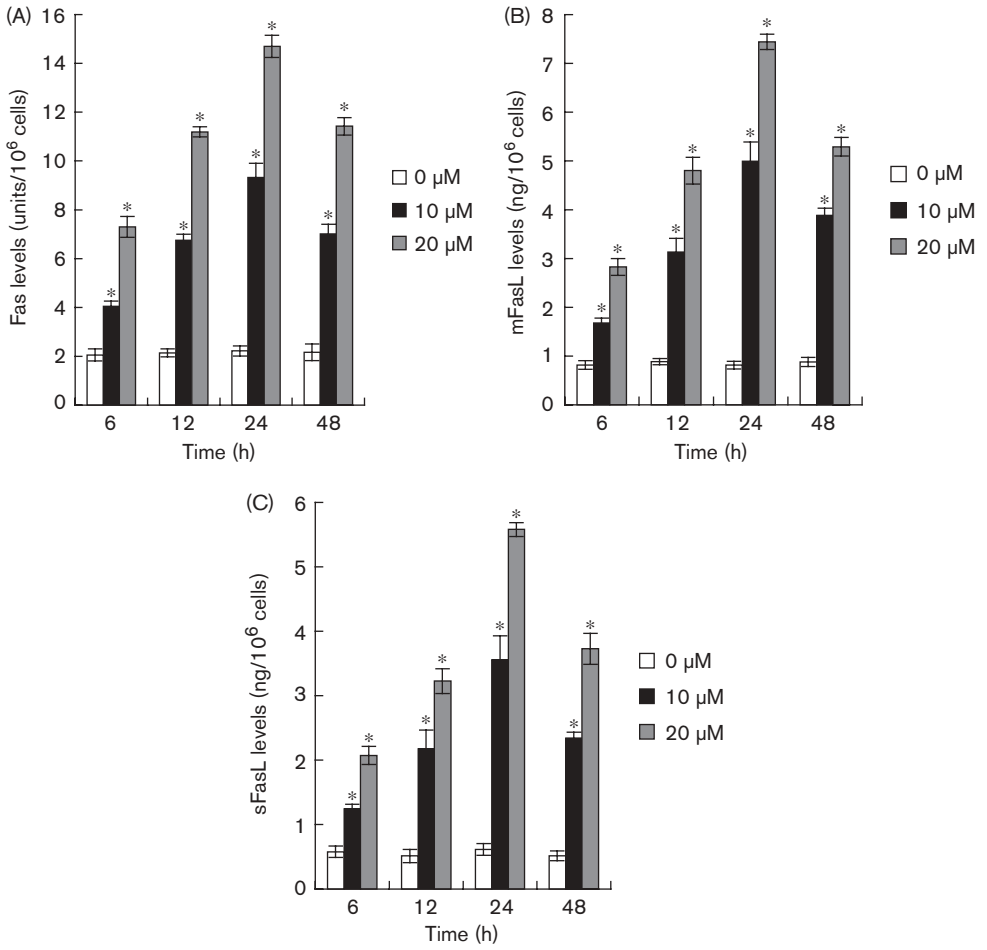
We first tested the antiproliferative effect of casuarinin in the lung cancer cell line, A549. As shown in Figure 2, the growth-inhibitory effect of casuarinin was observed in a dose- and time-dependent manner. At 48 h, the maximal effect on proliferation inhibition was observed with 20 μM casuarinin, which inhibited proliferation in 73.9% of A549 cells. The IC_{50} value was 10.7 μM. The maximal proliferation effect of 82.8% was reached by 20 μM casuarinin at 72 h.

Casuarinin-induced cell cycle arrest and apoptosis in A549 cells

The results on the effect of casuarinin on cell cycle progression of A549 are shown in Fig. 3. As compared to the control, 10 μM of casuarinin increased the population of G_0/G_1 phase from 34.7 to 56.8%. This effect was enhanced when A549 cells were treated by 20 μM of casuarinin (78.9% cell population in G_0/G_1 phase).

Figure 4 shows the time course of DNA fragmentation in continuous treatment with 10 and 20 μM of casuarinin. DNA fragmentation of A549 was found at 12 h and maximized at 48 h after addition of casuarinin. In contrast to the control, when cells were treated with casuarinin, the number of cells undergoing apoptosis increased from about 5.1- to 9.2-fold at 10 and 20 μM of casuarinin, respectively, at 48 h.

Fig. 6



The Fas/FasL apoptotic system was involved in casuarinin-mediated apoptosis. A549 cells were incubated with 0, 10 and 20 μM of casuarinin 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 ± SD of three determinations. The asterisk indicates a significant difference between control and casuarinin-treated cells, as analyzed by Dunnett's test ($p < 0.05$).

Casuarinin increases the expression of p53 and p21/WAF1 proteins in A549 cells

To determine whether tumor suppression factor p53 are involved in the casuarinin-mediated antiproliferative effect of A549 cells, the levels of p53 protein were assayed by ELISA. In the study of p53 protein expression, the p53-postive A549 [12] was treated with 10 and 20 μM casuarinin for 6, 12, 24 and 48 h. Marked induction of p53 protein was observed in a dose-dependent manner (Fig. 5A). The upregulation of p53 by casuarinin started to increase 6 h after treatment with casuarinin and reached maximum expression at 12 h.

The p21/WAF1 protein is first characterized as a downstream target of p53 and is thought to be responsible for G_0/G_1 cell cycle arrest [13]. Thus, we also assessed the p21/WAF1 expression of p53-expressing A549 cells using a WAF1 ELISA assay. Figure 5(B) shows that an increase in p21/WAF1 protein was apparent at 6 h and reached maximum induction at 24 h in casuarinin-treated A549 cells. Moreover, the induction of p21/WAF1 was in a dose-dependent manner. Based on these data, we suggest that casuarinin-mediated cell cycle arrest operates through the induction of p21/WAF1 protein on a p53-dependent event in A549 cells.

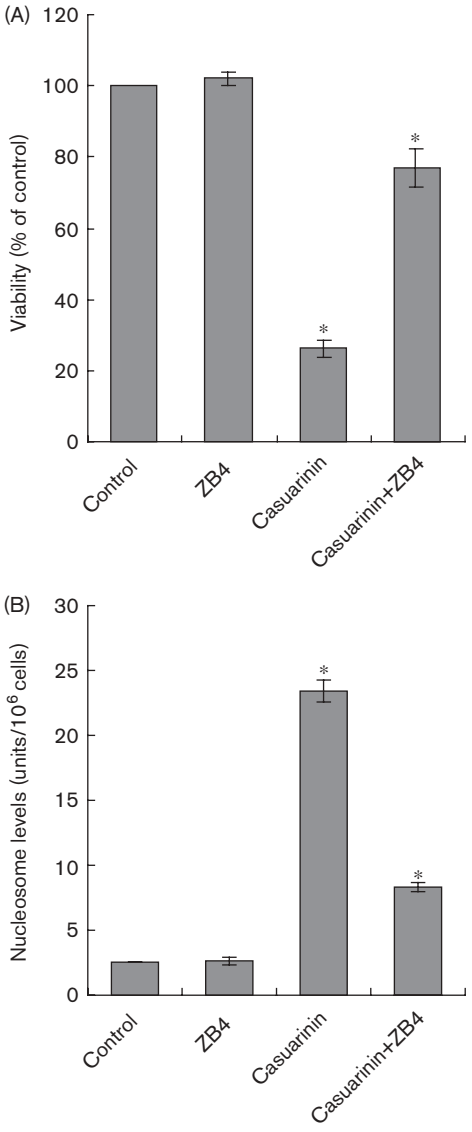
Fas/FasL apoptotic system might be a possible pathway of casuarinin-mediated apoptosis

It has been reported that over-expression of Fas-associating death domain protein could induce dose-dependent cell death in A549 cells [14]. By using Fas/APO-1 ELISA and FasL ELISA kits, we found that casuarinin increased expression of Fas/APO-1 receptor and soluble/membrane-bound FasL 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 casuarinin-induced apoptosis of A549 cells.

When A549 cells were pre-treated with an antagonistic anti-Fas antibody, ZB4, the antiproliferative and pro-apoptotic effects of casuarinin were effectively inhibited. At 20 μM of casuarinin, cell proliferation inhibition decreased from 73.9 to 23.2% (Fig. 7A). Compared to the control, the oligonucleosome DNA fragmentation of apoptosis induced by 20 μM of casuarinin decreased from about 9.2- to 3.3-fold at 48 h in ZB4 pretreated A549 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 24 h in 20 μM casuarinin-treated A549 cells (Fig. 8A). Furthermore, our results showed that the antiproliferative activity and induction of apoptosis by casuarinin were significantly decreased in the presence of inhibitor of caspase-8 (z-IETD-fmk) (Fig. 8B and C).

Fig. 7

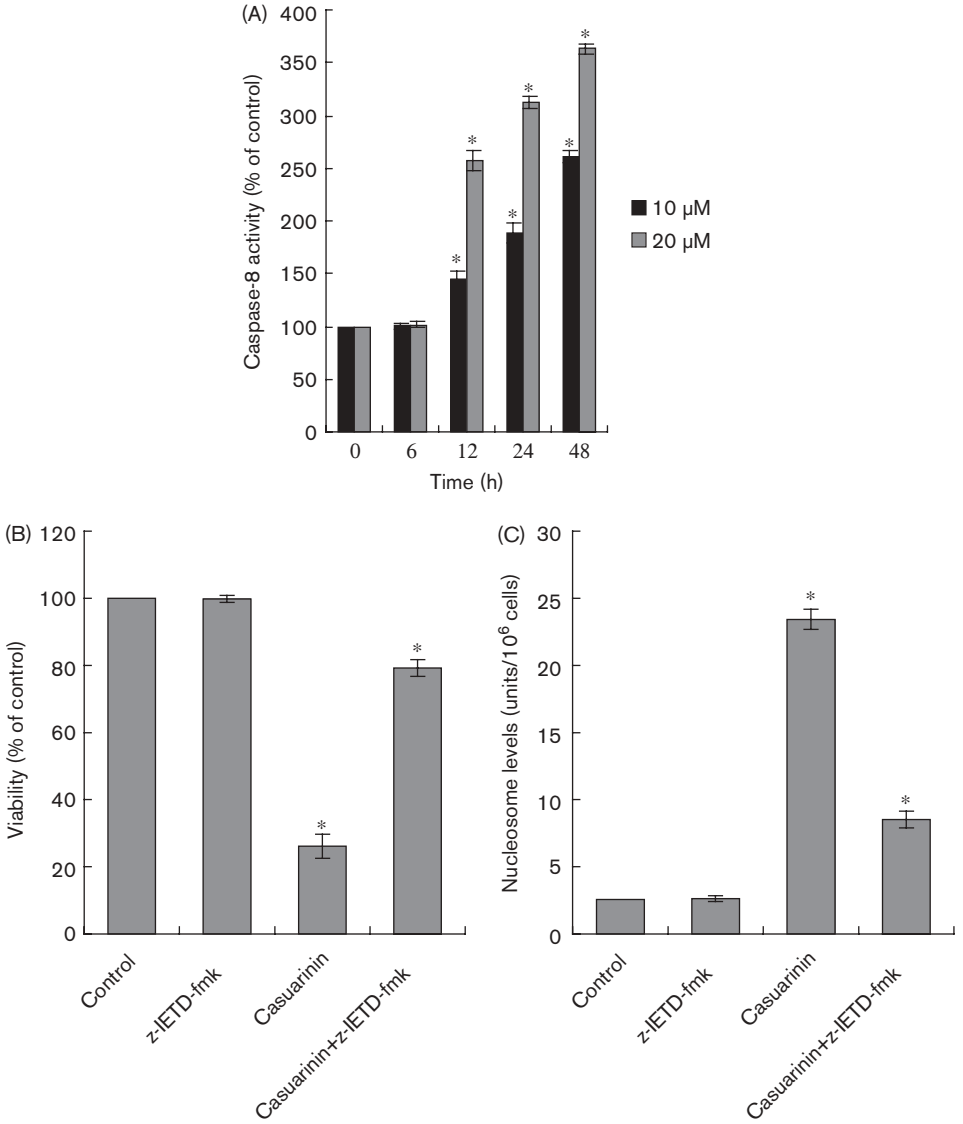


Effect of antagonistic anti-Fas antibody (ZB4) on casuarinin in A549 cells. (A) The antiproliferative and (B) pro-apoptotic effects of casuarinin were decreased by the Fas antagonist ZB4. For blocking experiments, cells were pre-incubated with 250 ng/ml ZB4 for 1 h and then treated with 20 μM of casuarinin for 48 h. Cell viability and apoptosis induction were examined by the XTT assay and nucleosome ELISA kit. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between control and casuarinin-treated cells, as analyzed by Dunnett's test ($p < 0.05$).

Discussion

Normal p53 function plays a crucial role in inducing apoptosis and cell cycle checkpoints in human and murine cells following DNA damage [13]. This has been

Fig. 8



(A) The activation of caspase-8 in A549 cells by casuarinin; (B) effect of caspase-8 inhibitor on casuarinin-mediated antiproliferation; (C) effect of caspase-8 inhibitor on casuarinin-induced apoptosis. A549 cells were incubated with various concentrations of casuarinin for the indicated times. For blocking experiments, cells were pre-incubated with z-IETD-fmk (10 μM) for 1 h before the addition of 20 μM casuarinin. After 48 h of treatment, cell viability and induction of apoptosis were measured by the XTT assay and the nucleosome ELISA kit. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between control and casuarinin-treated cells, as analyzed by Dunnett's test ($p < 0.05$).

further supported by the finding that p53 is the most commonly mutated tumor suppressor gene. Moreover, the chemosensitivity of cancer cells to chemotherapy agents is greatly influenced when the function of p53 is abrogated [15]. p21/WAF1 protein inhibits the activities of various cyclin-dependent kinase [16–18] and inhibits the phosphorylation of retinoblastoma (Rb) protein, thereby inhibiting the G_1–S phase transition [16,19]. Previous studies have shown that p21/WAF1 is transcriptionally regulated by p53-dependent and -independent pathways [20–22]. Our results demonstrate that p53 plays an important role in casuarinin-induced antiproliferative activity in A549 cells. Induction of p53 by casuarinin cannot only cause cell cycle arrest, but can also trigger apoptosis in A549 cells. This finding is supported by the following results. First, flow cytometry assays indicated that casuarinin arrested the cell cycle in the G_0/G_1 phase, which was attributed to the enhancement of p21/WAF1 protein that might be induced by p53. Second, the pro-apoptotic downstream target of p53, Fas/APO-1 protein [13], was increased by casuarinin. Moreover, the expression levels of these proteins were greatly increased (at 24 h) after maximal accumulation of p53 protein (at 12 h) in A549 cells.

The Fas/FasL system is a key signaling transduction pathway of apoptosis in cells and tissues [23]. Ligation of Fas by an 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 [23,24]. FasL is a tumor necrosis factor-related type II membrane protein [25]. Cleavage of mFasL by a metalloprotease-like enzyme results in the formation of sFasL [26]. Fas/APO-1 is expressed in human airway epithelial cells and plays a critical role in the pathophysiology of various pulmonary disorders [27]. Up-regulation of Fas/APO-1 expression has been demonstrated to induce apoptosis in hydrogen peroxide-treated A549 cells [27]. Serrao *et al.* have reported that neutrophils induce apoptosis of lung epithelial cells via release of soluble FasL [28]. Our study indicated that FasLs, mFasL and sFasL, increased in casuarinin-treated A549 cells. Moreover, the levels of Fas/APO-1 and the activity of caspase-8 were simultaneously enhanced in FasL-upregulating A549 cells. Furthermore, when the Fas/FasL system was blocked by ZB4, a decrease in cell growth inhibition and the pro-apoptotic effect of casuarinin was noted. Similarly, cell growth inhibition and apoptotic induction of casuarinin 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 casuarinin-mediated A549 cellular apoptosis.

In summary, our study suggests that the induction of p53 and activity of the Fas/FasL apoptotic system may participate in the antiproliferative activity of casuarinin in A549 cells. Our study has clearly demonstrated that casuarinin may be a promising chemopreventive agent for treating lung cancer.

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