Toona Sinensis Extracts Induced Cell Cycle Arrest and Apoptosis in the Human Lung Large Cell Carcinoma

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Toona sinensis extracts have been shown to exhibit anti-cancer effects in human ovarian cancer cell lines, human promyelocytic leukemia cells and human lung adenocarcinoma. Its safety has also been confirmed in animal studies. However, its anti-cancer properties in human lung large cell carcinoma have not been studied. Here, we used a powder obtained by freeze-drying the supernatant of centrifuged crude extract from *Toona sinensis* leaves (TSL-1) to treat the human lung carcinoma cell line H661. Cell viability was evaluated by the 3-(4-,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. Flow cytometry analysis revealed that TSL-1 blocked H661 cell cycle progression. Western blot analysis showed decreased expression of cell cycle proteins that promote cell cycle progression, including cyclin-dependent kinase 4 and cyclin D1, and increased the expression of proteins that inhibit cell cycle progression, including p27. Furthermore, flow cytometry analysis showed that TSL-1 induced H661 cell apoptosis. Western blot analysis showed that TSL-1 reduced the expression of the anti-apoptotic protein B-cell lymphoma 2, and degraded the DNA repair protein, poly(ADP-ribose) polymerase. TSL-1 shows potential as a novel therapeutic agent or for use as an adjuvant for treating human lung large cell carcinoma.

Key Words: apoptosis, cell cycle, lung cell carcinoma, *Toona sinensis*
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Lung cancer is the leading cause of cancer-related deaths in men and women worldwide and has a very poor prognosis [1]. There are two major types of lung cancer: small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). Large cell carcinoma has the worst prognosis of all NSCLCs [2,3]. Chemotherapy has many intolerable side effects and reached a therapeutic plateau for treating lung cancer [1]. It is essential to find novel agents with potent anti-cancer activity and few side effects. According to epidemiological data, there is an inverse relationship between the frequency of vegetable consumption and lung cancer [4–7]. Many natural food compounds have been demonstrated to exert a protective effect against lung cancer, including lycopene [8] and selected vegetables [9,10] but, to date, no specific type of vegetable seems to be particularly efficacious.

Toona sinensis Roem is a widely distributed native plant of South-East Asia. The leaves and young shoots

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of *Toona sinensis Roem* have long been used as an edible vegetable in China and Malaysia [11]. In traditional medicine, it is used to treat many diseases such as enteritis, dysentery, itchiness, abdomen tumor, diabetes and hypertension [11–14]. However, few studies have been done to evaluate its anti-cancer properties. Crude water extracts from *Toona sinensis* leaves (TSL-CE) inhibit cell proliferation and induce apoptosis of human lung adenocarcinoma (A549) cells [12]. TSL-1, a powder obtained by freeze-drying the supernatant of centrifuged TSL-CE, has powerful antioxidant activity and can also inhibit proliferation and induce apoptosis of human promyelocytic leukemia cells [15,16]. TSL-2, a powder obtained by freeze-drying the supernatant of the centrifuged extract of TSL-1 in 99.5% ethanol, was shown to induce apoptosis of human ovarian cancer cells and inhibit tumor growth in a murine xenograft model [14]. Moreover, TSL-1 was shown to be safe in mice at a highest tested dose of 5 mg/g body weight [17].

Different cancer cell lines with different genetic characteristics may show different responses to the same compound, even when they originate from the same organ [14,18–20]. For example, tea polyphenols were shown to be more effective on the human lung carcinoma cell line H661 than on the human lung adenocarcinoma cell line H441 [20]. In the era of targeted therapy, targeting specific tumors with specific drugs is expected to produce the greatest effects with the least side effects. Of the three types of NSCLC cell lines tested, we found that TSL-1 was most effective on H661 cells. Further study revealed that TSL-1 can arrest cell cycle progression, inhibit proliferation and induce apoptosis of H661 cells.

Materials and Methods

Preparation of extracts of Toona sinensis Roem and other chemicals

Toona sinensis leaves were obtained from *Toona sinensis Roem* grown in Tuku (Yunlin County, Taiwan) and were picked and washed thoroughly with water. TSL-CE was prepared by immersing 1 kg of fresh Toona sinensis in 1 L of water, and boiling them until 100 mL of fluid remained, as previously described [12]. The TSL-CE was then centrifuged at 3,000 rpm for 12 minutes and the supernatant was lyophilized by freeze-drying to obtain a powder, which we called TSL-1.

Cell culture

Human lung adenocarcinoma cell line H441, human lung squamous cell carcinoma cell line H520, and human lung large cell carcinoma cell line H661 were purchased from the Institute of Food Science (Hsinchu, Taiwan). These cells were cultured in RPMI-1640 medium (Gibco, Invitrogen Corporation, Carlsbad, CA, USA), supplemented with 10% FBS, 1% penicillin-streptomycin, 2 mM L-glutamine, 10 mM HEPES buffer solution and 1 mM MEM sodium pyruvate solution, and incubated in a 5% CO_2 incubator at 37°C.

Colorimetric assay for cytotoxicity test

Colorimetric assay using 3-(4-,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma, St Louis, MO, USA) was used to determine cell proliferation and viability [21]. Cells were treated with different concentrations of TSL-1 (0, 0.125, 0.25, 0.50, or 1 mg/mL) for 24 or 48 hours, and untreated cells were prepared as a control group. After treatment, the media were replaced with MTT reagent (2 mg/mL and the cells were incubated in a 5% CO_2 incubator at 37°C for 1.5 hours. The media were then aspirated and dimethyl sulfoxide was added to each well. After shaking the plates for 5 minutes, light absorbance values were determined at the wavelength of 540 nm using a microplate reader to calculate the 50% inhibitory concentration (IC_{50}), i.e. the drug concentration at which the light absorbance value of the experimental group is 50% of that of the control group.

Western blot analysis

Cells were treated with 0.5 mg/mL or 1 mg/mL TSL-1 for 24 hours, washed twice with ice-cold phosphate-buffered saline (PBS) and then harvested in a lysis buffer (50 mM Tris-base, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.1 mM sodium orthovanadate, pH 7.4, and 1% Triton X-100 in 5 mL) containing protease inhibitors (1 M PMSF, 1 mg/mL leupeptin, 100 mM benzidine, 2 μL/mL aprotinin, and 2 mg/mL pepstatin A) for 10 minutes at 4°C. The cell lysates were centrifuged at 12,000 rpm for 10 minutes. Total protein content was determined using the protein assay dye reagent concentrate (Bio-Rad, Hercules, CA, USA), with bovine serum albumin as a standard. The protein extracts were subjected to a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to the PVDF membranes, and the blots were blocked in 5% non-fat milk at 37°C for 1 hour.

The blots were then probed with anti-actin antibody (Chemicon, Temecula, CA, USA); anti-cyclin D1, cyclin-dependent kinases (CDK)-4 and p27 antibodies (Santa Cruz, CA, USA); anti-Bcl2 antibody (Cell Signaling, Beverly, MA, USA) and anti-poly(ADP-ribose) polymerase (PARP) antibody(Zymed, San Francisco, CA, USA) for 1 hour at room temperature. After washing in 1×tris-buffered saline/Tween buffer, blots were incubated for 1 hour with the appropriate secondary antibody. The membranes were developed using an enhanced chemiluminescence system (Amersham International, UK) and exposed to high sensitivity film. The images were analyzed by computer-assisted densitometry and Bio-1D V.97 software (Vilber Lourmat, France).

Determination of cell cycle by flow cytometry

Cells were treated with TSL-1 for 24 hours. After washing with PBS, the cells were detached from the culture dish using trypsin/EDTA and fixed with 70% alcohol at 4°C. The samples were then incubated with 10 μg/mL RNase A and 50 μg/mL propidium iodide (PI) for 30 minutes at 37°C. DNA content and cell cycle progression was studied by flow cytometry and results were analyzed using Multi-Cycle DNA analysis software.

Measurement of cell apoptosis by flow cytometry

Annexin-V staining was performed to detect apoptotic cells. After treating with TSL-1, cells were washed with HEPES buffer (pH 7.4, 10 mM Hepes/NaOH, 140 mM NaCl, 5 mM $CaCl_2$) and centrifuged at 1,200 rpm for 5 minutes at 4°C. The cell pellets were resuspended in 100 μL of staining solution (2 μL of Annexin-V fluorescein labeling reagent, and 2 μL of 0.5 mg/mL PI in 100 μL of Hepes buffer) and incubated for 15 minutes at room temperature in the dark. Samples were centrifuged again at 1,200 rpm for 5 minutes at 4°C and the cells were filtered through a 30 μm mesh to individual cell. Necrotic cells were double-stained with Annexin-V and PI, while apoptotic cells were only stained with Annexin-V. The relative fluorescent intensities of Annexin-V and PI were analyzed by flow cytometry with WINDI 2.5 software.

Statistical analysis

All studies were done with six duplicates. Results are presented as mean ± standard deviation. One-tailed Student's *t* test was used to analyze the difference between two groups. Significance was defined as $p < 0.05$ for all tests.

Results

Effects of TSL-1 on cell survival of H661 cells

Cells were exposed to 0, 0.125, 0.25, 0.5, or 1 mg/mL TSL-1 for 24 or 48 hours. TSL-1 induced cell death in a dose- and time-dependent manner in all three human lung carcinoma cell lines (H441, H520 and H661), as determined by the MTT assay. The IC_{50} of TSL-1 at 24 hours was 1.2 mg/mL, 0.73 mg/mL and 0.29 mg/mL for H441, H520 and H661 cells, respectively. H661 was then selected for further studies because H661 is derived from a human lung large cell carcinoma, which has the worst prognosis of all NSCLC types. Moreover, TSL-1 showed the most potent inhibitory activity against H661 IC_{50} values of 0.29 mg/mL at 24 hours and 0.12 mg/mL at 48 hours (Figure 1).

Effects of TSL-1 on cell cycle progression and cell cycle protein expression

H661 cells were incubated with 0.5 mg/mL TSL-1 for 24 hours and flow cytometry was used to analyze cell cycle progression. We found that TSL-1 effectively arrested the cell cycle at the G1 phase (Figure 2). TSL-1 also significantly decreased the expression of cyclin D1 and CDK4 in a dose-dependent manner, when compared with the control group. Conversely, TSL-1 significantly increased the expression of p27 in a dose-dependent manner when compared with

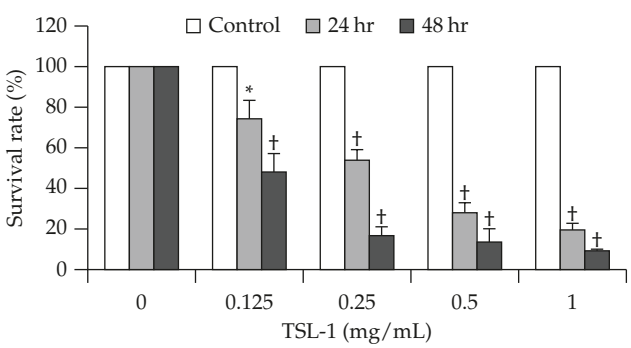

Figure 1. *Effect of TSL-1 on cell survival of H661 cells. TSL-1 induced cell death in a dose- and time-dependent manner. *$p < 0.05$ versus control; †$p < 0.01$ versus control. TSL-1 = the supernatant of centrifuged crude extract from Toona sinensis leaves.*

the control group ($p < 0.05$) (Figure 3). These data suggest that TSL-1 caused cell cycle arrest in H661 cells in association with decreased expression of proteins that promote cell cycle progression (cyclin D1 and CDK4) and increased expression of the cell cycle inhibitory protein p27.

Effects of TSL-1 on apoptosis of H661 cells

H661 cells were incubated in RPMI 1640 medium with or without 0.5 mg/mL TSL-1 for 24 hours after which apoptosis was determined based on Annexin-V and PI staining. The proportion of cells that had undergone apoptosis was significantly greater compared with cells in the control group ($p < 0.01$) (Figure 4).

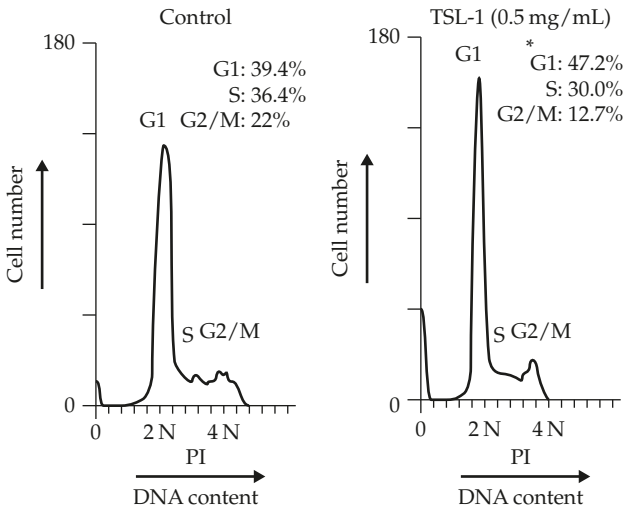

Figure 2. *Effects of TSL-1 on cell cycle progression of H661 cells. Compared with the control group, TSL-1 significantly arrested the cell cycle of H661 cells in the G1 phase. *$p < 0.05$ versus control cells. TSL-1 = the supernatant of centrifuged crude extract from Toona sinensis leaves.*

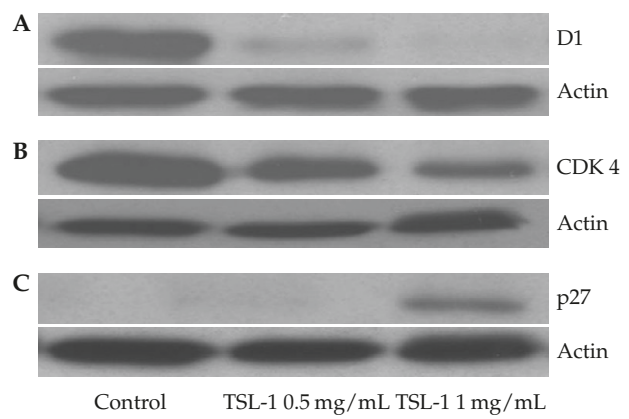

Figure 3. *Effect of TSL-1 on cyclin D1, cyclin-dependent kinase 4 and p27 protein expression. TSL-1 significantly reduced the protein expression of (A) cyclin D1 and (B) cyclin-dependent kinase 4, and (C) increased the expression of p27 in a concentration-dependent manner. Actin was used as an loading control. TSL-1 = the supernatant of centrifuged crude extract from Toona sinensis leaves.*

Effect of TSL-1 on apoptosis-related proteins

To clarify the mechanisms by which TSL-1 induced apoptosis of H661 cells, H661 cells were incubated in 0.5 mg/mL or 1 mg/mL TSL-1 for 24 hours, and the proteins were extracted for Western blotting. We found that TSL-1 reduced the expression of Bcl2 in a concentration-dependent manner (Figure 5). It is well known that Bcl2 is a potent anti-apoptotic protein that is expressed in lung cancer [22,23]. We also found that TSL-1 promotes the cleavage of PARP (Figure 5). It is also well known that PARP cleavage is vital for appropriate apoptosis activity [24]. Thus, TSL-1 may induce H661 cell apoptosis through pathways involving Bcl2 and PARP.

Discussion

Cell cycle progression in mammalian cells is controlled by positive regulators such as cyclins and CDK, and by negative regulators such as CDK inhibitors. Cyclin D1 is over-expressed in almost 60% of resectable NSCLCs [25]. The over-expression of cyclin D1 is associated with lymph node metastasis [26] and is a predictor of shorter survival time of patients with NSCLC [27,28]. By contrast, the absence of cyclin D1 expression may have a favorable prognostic value [29]. CDK inhibitors, which can block the cell cycle, have been proposed as tumor suppressor genes [30]. The p27 gene, one such CDK inhibitor, is also a prognostic factor that is positively associated with the overall survival time of patients with NSCLC [31,32]. In our study, TSL-1 reduced the expression of cyclin D1, increased the expression of p27 and arrested cell cycle progression. Thus, TSL-1 may be beneficial for treating human lung large cell carcinoma.

Apoptosis, programmed cell death, is a physiological process of cell suicide and plays an important role in the maintenance of tissue homeostasis. DNA damage and cellular injury caused by drugs or other mechanisms initiate signaling pathways leading to apoptosis [33]. Inhibition of apoptosis is one of the main characteristics of cancer. Defects in apoptosis are

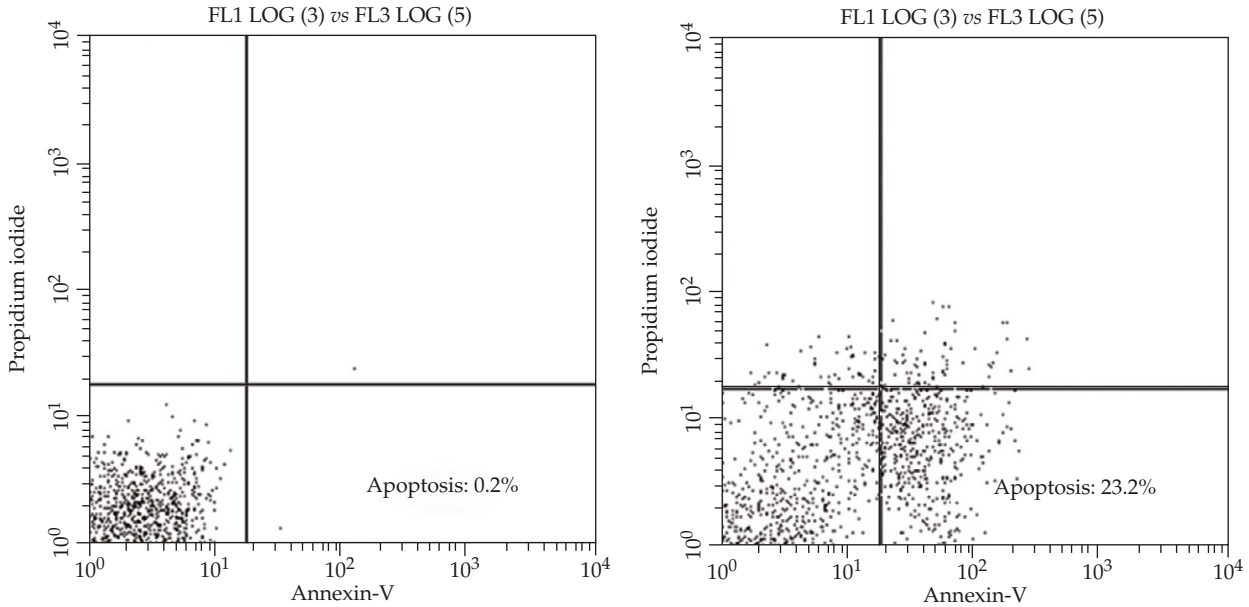

Figure 4. *Effect of the supernatant of centrifuged crude extract from Toona sinensis leaves on apoptosis of H661 cells. Quadrant 1 (lower left) represents necrotic cells, with double-staining for Annexin-V and PI. Quadrant 4 (upper right) represents apoptotic cells stained with Annexin-V. Results showed that TSL-1 significantly induced apoptosis compared with the control group (n = 6, *p < 0.01 versus control cells).*

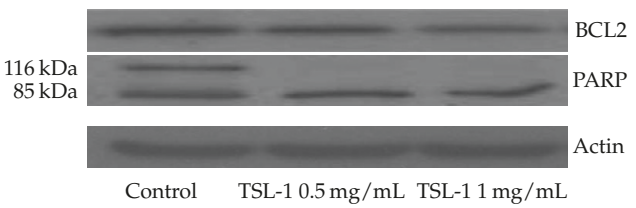

Figure 5. *Effects of TSL-1 on Bcl2 expression and PARP cleavage. Expression of Bcl2 protein was significantly reduced in a dose-dependent manner by TSL-1. PARP was degraded into two components (116 kDa and 85 kDa) after treatment with both doses of TSL-1 for 24 hours. Actin was used as an loading control.*

implicated in tumorigenesis and drug resistance, and these defects are also responsible for chemotherapy failure [34]. Bcl2 is a potent anti-apoptotic protein that is expressed in SCLC and NSCLC cells [35–38]. Bcl2 over-expression is related to resistance to anti-cancer agents [22]. PARP is an abundant nuclear enzyme that mediates the repair of DNA breaks and is activated in response to DNA damage induced by ionizing radiation, oxidative stress and DNA-binding anti-cancer drugs [39,40]. PARP activation can rescue tumor cells from therapeutic DNA damage induced by chemotherapy agents [24]. In our study, TSL-1 induced cancer cell apoptosis, which was associated with reduced expression of Bcl2 and degradation of PARP. Thus, TSL-1 could be used to support the effects of standard chemotherapy and overcome drug resistance of cancer cells.

H661 cells are derived from human lung large cell carcinoma, which has the worst prognosis of all NSCLCs. When compared with other NSCLC cell lines, H661 cells are more resistant to several standard chemotherapy drugs [41]. Interestingly, the H661 cells were found to be the most vulnerable cell line to TSL-1 in this study. In another study, tea polyphenols also showed better effectiveness in H661 cells than in H441 cells [20]. By contrast, solamargine showed better effectiveness in H441 cells than in H661 cells [18]. Therefore, the so-called "drug resistance" is not the real cause for treatment failure, it only means that we have not found the most appropriate drug for the cancer being treated. The reasons for the differences in response of these cells lines may involve the distinct genetic characteristics of the different cancer cell lines. For example, it is known that H441 has a mutation in exon 5, codon 158 of p53 [42], while H520 and H661 express non-functional p53 [43], H441 and H520 do not express p16, and H661 produces a smaller p16 protein [44,45]. However, the underlying mechanisms still remain unknown. In actual clinical practice, all three types of NSCLC are treated with similar combinations of standard chemotherapy drugs, so it is little wonder that the prognosis is poor. In the era of targeted

therapy and tailored therapy, it is now feasible to conduct further studies to evaluate the effect of TSL-1 specific to human lung large cell carcinoma.

In conclusion, we found that TSL-1 had effects on cell cycle regulation and apoptosis. In terms of cell cycle regulation, TSL-1 arrested the cell cycle in the G1 phase by inhibiting the expression of cyclin D1 and CDK4 and by increasing the expression of the CDK inhibitor p27. With respect to apoptosis, TSL-1 promoted apoptotic cell death by decreasing the expression of Bcl2 and enhancing the cleavage of PARP. Therefore, TSL-1 may hold some potential as a novel therapeutic agent or could be used as an adjuvant in combination with traditional chemotherapy to treat human lung carcinoma.

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香椿萃取物已被證實在人類卵巢癌細胞株、人類白血病細胞株及人類肺腺癌細胞株 A549 具有抗癌作用。其安全性也已在動物實驗證實。但香椿萃取物在人類大細胞肺癌的效果尚未被研究。我們將香椿葉水萃物離心後之上清液加以冷凍乾燥，製成香椿葉水萃物第一區分（TSL-1），試驗其使用在人類大細胞肺癌細胞株 H661 的效果。細胞存活以 MTT assay 加以分析。對細胞週期的影響以流式細胞儀加以分析，並以西方墨點法偵測細胞週期蛋白。我們發現 TSL-1 可以阻斷 H661 細胞週期的進行，下降促細胞週期進行的蛋白質，CDK4 及 cyclin D1 的表現，增加抑制細胞週期進行的蛋白質 p27 的表現。再者，TSL-1 可誘發 H661 細胞株的凋亡，下降抗凋亡蛋白 Bcl-2 的表現，並促進 DNA 修復蛋白 PARP 的分解。TSL-1 可以經由阻斷細胞週期的進行及促進細胞凋亡來抑制人類大細胞肺癌細胞株 H661，加以無明顯毒性，顯示其有潛力發展成治療人類大細胞肺癌的輔助藥物。

關鍵詞：凋亡，細胞週期，大細胞肺癌，香椿

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