Trilinolein inhibits proliferation of human non-small cell lung carcinoma A549 through the modulation of PI3K/Akt pathway

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Running title: anticancer effects of trilinolein on A549

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Abstract: Trilinolein has been identified as one of the active constituents isolated from *Panax notoginseng* used widely in traditional Chinese medicine. Protective actions of *Panax notoginseng* against cerebral ischaemia, beneficial effects on the cardiovascular system, and haemostatic, antioxidant, hypolipidaemic, hepatoprotective, renoprotective and estrogen-like activities have been illustrated. In the present study, the effects of trilinolein on the growth of non-small cell lung carcinoma A549 were investigated. It was found that the exposure of A549 cells to trilinolein resulted in growth inhibition and the induction of apoptosis in a dose- and time- dependent manner. Trilinolein treatment induced the upregulation of pro-apoptotic Bax, downregulation of anti-apoptotic Bcl-2 expression, which was associated with the proteolytic activation of caspases and the concomitant degradation of poly(ADP-ribose) polymerase (PARP) protein. Intracellular reactive oxygen species seem to play a role in the trilinolein-induced apoptosis, since ROS were produced early in the trilinolein treatment. Moreover, the activity of PI3K/Akt was downregulated in trilinolein-treated cells. Our results demonstrated that the most important regulators of trilinolein-induced apoptosis are Bcl-2 family and caspase-3,which are associated with cytochrome *c* release and dephosphorylation on the Akt signaling pathway.

Keywords: Chinese herbs; *Panax notoginseng*; apoptosis; trilinolein; PI3K/Akt

Introduction

Lung cancer is among the leading causes of death and its incidence is continuously increasing. Surgery, radiotherapy, and chemotherapy are currently the major treatments used to reduce lung cancer mortality (Saba and Khuri, 2005), however, these therapies have detrimental side effects on the normal healthy cells in the body. Therefore, it is important to discover new agents to treat lung cancer safely without affecting the body's healthy cells. Deregulation of signaling pathways like PI3K/Akt are often implicated in the pathogenesis of NSCLC (Li *et al.,* 2010). Therefore the need for accelerated development of effective NSCLC therapies is critical. At present, major work is being stressed on designing new therapeutic strategies targeting multiple signaling pathways for more effective disease management in NSCLC. We aimed to investigate how trilinolein affects several pathways including mitochondria-dependent, ROS, PI3k/Akt, and p53/p21 signals.

Certain Chinese herbs have been used as alternative therapeutic approaches, for treating lung cancer patients in Chinese population (Lu *et al.,* 2009; Sun *et al.,* 2010). *Panax notoginseng* Burk. F.H. Chen (Araliaceae) (*P. notoginseng*) is a highly valuable and important herb in oriental medicine for its therapeutic abilities. *P. notoginseng* has been widely used for hemostasis and protection of the cardiovascular system (Chen *et al.,* 2008; Yang *et al.,* 2005). *P. notoginseng* showed significant lowering effects on

serum total cholesterol and triglyceride levels (Joo *et al.,* 2010). The extract of the roots of *P. notoginseng* exhibited a significant anti-tumor-promoting activity on two-stage carcinogenesis of mouse skin tumors (Konoshima *et al.,* 1999). *P. notoginseng* extract was reported effective on precancerous patients (Yu, 1993). *P. notoginseng* extract and ginsenoside Rb1 increased the sensitivity of KHT sarcoma to ionizing radiation (Chen *et al.,* 2001). *P. notoginseng* was cytotoxic for the treatment of PC3 human prostate cancer cells (Chung *et al.,* 2004). There is a report that the serum of a dog fed with *P. notoginseng* extract inhibited proliferation of human gastric mucosa epithelium GES-1 cells (Wang *et al.,* 2004). A dammarane glycoside derived from ginsenoside Rb3 showed toxicity against breast cancer cells (He *et al.,* 2005). Also, *P. notoginseng* powder protects a precancerous stomach lesion (Shi *et al.,* 2003). The effects of crude *P. notoginseng* extract on tumor cells suggested that further purified or synthetic versions of *P. notoginseng* extract may be useful not only in vascular-related diseases, but also cancer therapy (Chen *et al.,* 2001).

The antitumor activity from *P. notoginseng* are mainly focused on its extract (Konoshima, *et al.,* 1999) and its constituent ginsenoside Rb1 (Chen *et al.,* 2001). Trilinolein has been focused on its antioxidant activity (Ng *et al.,* 2004), however, its anticancer activity has never been explored. Trilinolein is a candidate active ingredient isolated from *P. notoginseng* extract (Hong *et al.,* 1993). Trilinolein is a

triacylglycerol, which carries two unsaturated bonds (C 18:2, MW = 890; Fig. 1A), at all three esterified positions of glycerol (Hong *et al.,* 1993). Trilinolein has been reported to provide a number of beneficial effects including reducing thrombogenicity (Chan *et al.,* 2002), increasing erythrocyte deformability (Hong *et al.,* 1993), anti-ischemic (Chen *et al.,* 2008), anti-arrhythmic (Chan *et al.,* 1995), and displaying antioxidant effects in various experimental models (Chan *et al.,* 1997; Chan *et al.,* 2002). Additionally, trilinolein has been reported to reduce free radical damage associated with atherogenesis, and myocardial damage caused by ischaemia and reperfusion (Kritchevsky *et al.,* 2000). The sum of these pharmacologic effects may explain the benefits derived from treating circulatory disorders with the herb over the centuries. Therefore, we aimed to investigate the effects of trilinolein on human non-small cell lung cancer cells A549 and to explore the molecular mechanism through which trilinolein induces cell death

Materials and methods

Materials

Trilinolein was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). 3-(4,5-Dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), RNase A, propidium iodide (PI), trypsin, BSA, Tween-20,-80 and DMSO were purchased from Amresco Inc. (Solon, OH, USA). Dulbecco's Modified Eagle Medium (DMEM),

F-12 and fetal bovine serum (FBS) were purchased from GIBCO BRL (Rockville, MD, USA). Antibodies for pAkt, Akt, PIP3K, poly(ADPribose) polymerase (PARP), caspases-3 and -9 were purchased from Cell Singnaling (Boston, MA, USA). Antibody for cytochrome *c* was purchased from BioLegend (San Diego, CA, USA). Antibodies against p53, p21, Bax and Bcl-2 were purchased from Santa Cruz (Santa Cruz, CA, USA). Secondary antibodies were acquired from Santa Cruz (Santa Cruz, CA, USA).

Cell Lines and Cultures

Human renal cell carcinoma cell line A498, gastric adenocarcinoma MKN-45, and human NSCLC A549 cells were obtained from Food Industry Research and Development Institute (Hsinchu, Taiwan). A498 and MKN-45 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco BRL, Rockville, MD, USA), and A549 cell was grown in F-12 medium (Gibco BRL, Rockville, MD, USA) containing 10% FBS (Gibco BRL, Rockville, MD, USA), 100 U/mL of penicillin, and 100 mg/mL streptomycin mixed antibiotics (Gibco BRL, Rockville, MD, USA) at 37^{o}C in a humidified atmosphere comprised of 95% air and 5% CO_2. In all of the experiments, the medium was supplemented with 1% (v/v) fetal bovine serum (FBS).

Cell proliferation assay

MTT assay was performed in the A498, MKN-45 and A549 cell lines to measure the cytotoxicity of trilinolein. All cell lines were seeded in 96-well plates with 2×10^4 cells/well in culture medium. Trilinolein was dissolved in 0.8% (v/v) Tween 80 in PBS and sterilized by filtration. Cells were treated with various concentrations of trilinolein as indicated in each figure. After 24 h., the number of viable cells was determined. Briefly, 5 mg/mL MTT was added to each well, and the plate was incubated at 37^{o}C for 4 h. The medium was removed, and a 50 μL aliquot of DMSO was added, and the absorbance at 590 nm was measured for each well on ELISA reader. Data are presented as the mean ± SE of three independent experiments.

Flow cytometry analysis

2×10^5 A549 cells were seaded into each well of a 12-well plate (TPP; Techno Plastic Products AG, Trasadingen, Switz) 24 h before treatment with various concentrations of trilinolein for different time periods (0, 2, 12, 24, and 48 h). Cells were harvested with trysin-EDTA, washed twice with 10 ml ice-cold PBS, fixed in 70% (v/v) ethanol, and kept at 4 ^{o}C prior to propidium iodide (PI) staining [100 μg/mL PI, 0.2% (v/v) Nondiet P-40, and 1 mg/mL RNase A (DNase-free) in PBS lacking Ca^{2+} and Mg^{2+}; at a 1:1:1 ratio by volume] and analyzing DNA contents with flow cytometry (Becton Dickinson, San Jose, CA, USA). The intensity of PI

fluorescence was linearly amplified and both the area and width of the fluorescence pulse were measured. Ten thousand events were acquired, and the percentage of hypodiploid (apoptosis, sub-G1) events and percentages of cells in G0/G1, S and G2/M phases were determined using the DNA analysis software ModFitL T, version 2.0 (Verity Software, Topsham, ME, USA).

Measurement of intracellular ROS generation

A549 cells were incubated for 4 h in the presence of trilinolein (50, 75 and 100 μg/ml). In time course study, 75 μg/ml of trilinolein were incubated for 1, 2 and 4 h. Intracellular ROS production was measured by using a fluorescent dye, 2′,7′-dichlorodihydrofluorescein diacetate (H_2-DCF-DA) (Molecular Probes, Eugene, OR, USA), which can be converted to 2′,7′-dichlorofluorescein (DCF) by esterases when taken up. DCF reacts with ROS to generate a new highly fluorescent compound, dichlorofluorescein, which can be analyzed with FACS. The treated cells were incubated by H_2-DCF-DA (10 μM) at 37 °C for 30 min, washed twice with PBS, and then measured with FACS.

Western blotting analysis

A549 were plated in 10-cm dishes at a density of 3×10^6 cells and incubated with 75 μg/mL of trilinolein in F-12 containing 1% (v/v) FBS for 0, 2, 6, 12, 24, and 48 h. The cells were collected, then lysed in a lysis solution followed by incubation at 95^{o}C

for 5 min. Total proteins were separated using SDS-PAGE before being transferred to PVDF membranes, blocked with 5% (v/v) nonfat dry milk in PBS-Tween 20 and probed with the desired antibody (pAkt, Akt, PI3K, p53, p21, Bax, Bcl-2, caspase-3, caspase-9, cleaved PARP and cytochrome *c*) (dilution ratio = 1:1000) overnight at 4^{o}C. The blots were then incubated with horseradish peroxidase-linked secondary antibody for 1 h followed by development with the electrochemoluminsence (ECL) reagent and exposure to Hyperfilm (Amersham, Arlington Height, IL, USA). The data were analyzed by Gel-Logic 200 Imaging Systems, Molecular Imaging Software.

Statistical analysis

Values are presented as mean±SE relative to those of the control. Statistically significant differences from the control group were identified by one-way ANOVA for the data. $p<0.05$ was considered significant for all tests.

Results

Cytotoxic effect of trilinolein on A549 cells

In order to determine if trilinolein decreases cancer cell viability, the A549, MKN-45 and A498 cells were stimulated with various concentrations of trilinolein for 24 h and the cell viability was measured using the MTT assay. Trilinolein treatment significantly inhibited the cell viability of three cell lines in a concentration-dependent manner. And our results indicated that trilinolein exhibited stronger cytotoxic

properties against A549 cells (after 24 h treatment at 75 μg/mL, trilinolein decreased the A549 cell viability by ~ 52.8%, compared with control). (Fig. 1B).

Trilinolein induces apoptosis in A549 cells

Further experiments using flow cytometry analysis were carried out to determine if the anti-proliferative effects of trilinolein is the result of apoptotic cell death. 75 μg/mL trilinolein-treated cell demonstrate higher percentage of hypldiploid cells than in control cells (Fig. 2A). Also, 75 μg/mL trilinolein-treated cell showed time-dependent manner (Fig. 2B). This results indicate the the cytotoxic effects observed in response to trilinolein are correlated with the induction of apoptosis.

Modulation of PI3K/ Akt and activation of p53/p21 protein expression by trilinolein in A549 cells

The PI3k and phosphorylation status of Akt in A549 cells after trilinolein treatment was explored to determine if trilinolein-induced apoptosis is correlated with the Akt signal, which is a downstream effector of PI3K for survival signaling. The levels of PI3K and phosphorylation Akt were significantly decreased in a time-dependent manner, and demonstrated significant decrease at 12 and 24 h, respectively (Fig. 3A). Our results showed that the expression of p53 was markedly increased at earlier time period with trilinolein treatment and also in time-dependent manner as compared to the control (Fig. 3A). In Fig. 3A, our result also showed that

trilinolein-treated A549 cells exhibited an increase in p21 expression after 24 h of treatment.

Modulation of the expression of Bcl-2 family proteins by trilinolein in A549 cells

The expression of the pro-apoptotic factor Bax was significantly increased in A549 cells after 6 h incubation with 75 μg/mL trilinolein (Fig. 3A). Bcl-2 significantly decreased after 12 h incubation with 75 μg/mL trilinolein treatment in the A549 cancer cell lines. The Bax/Bcl-2 ratio was significantly elevated after 6 h treatment (Fig. 3C).

Activates caspases and degradation of PARP by trilinolein in A549 cells

In order to determine if trilinolein-induced apoptosis is associated with the activation of caspases, the protein expressions of caspase-3 and -9 in A549 cells were measured. Our results demonstrated that the expression of caspase-3 significantly increased after trilinolein treatment at 24 h. (Fig. 3B). Moreover, Western blotting studies suggest that apoptosis induction occurs via the intrinsic pathway because trilinolein induced the release of cytochrome *c* from mitochondria and stimulated the cleavage of inactive pro-caspase-9, resulting in 35-37 kDa active fragments (Fig. 3B). We also analyzed the effect of trilinolein on hydrolysis of the zymogen by Western blotting assay. Cleavage of pro-caspase-3 into 17–19 kDa fragments

significantly increased in trilinolein-treated cells (Fig. 3B) and PARP, a known substitute for caspase-3, was effectively hydrolyzed to the 85 kDa fragment.

Trilinolein increased intracellular ROS levels in A549 cells

Production of intracellular ROS in trilinolein-treated A549 cell was monitored by the oxidation-sensitive fluorescent dye DCFH-DA. An increase in DCFH fluorescence was detected in trilinolein-treated cells (Fig. 4A, 4B). A rapid production of ROS was detected at 1 h after treatment although the highest levels were not reached until 4 h (Fig. 4B). These findings suggest that ROS generation maybe crucial for trilinolein-induced cell death.

Discussion

Search for new chemopreventive and antitumor agents that are more effective but less toxic has great interest in phytochemicals. This is the first study to evaluate the cytotoxic properties of trilinolein in human non-small cell lung carcinoma A549 cells. A549 was more sensitive to trilinolein cytotoxicity (Fig. 1B). There are no normal cells or cell lines as controls in the present study. However, previous related studies indicated that 0.1~10 uM trilinolein have been shown protective effects in astrocytes and cardiomyocytes (Chiu *et al.*, 1999; Yang *et al.*, 2005).

Our results indicated that trilinolein can cause the accumulation of cells in the G1 phase of the cell cycle and apoptosis (Fig. 2B). In the present study we have

demonstrated that trilinolein was effective in inhibiting the growth of A549 cells in a dose- and time-dependent manner. Therefore, we investigated the biochemical mechanism underlying the pro-apoptotic activity of trilinolein in A549 cells. Trilinolein treatment was shown to induce release of mitochondria *c* and apoptosis in A549 cells through modulation of Bax and Bcl-2 proteins and activation of caspase-3. Moreover, p53-dependent downregulation of Akt may promote an apoptotic cell death.

Cell cycle analysis revealed that trilinolein caused a significant cell cycle arrest at the G_0/G_1 phase (Fig. 2B), accompanied by an increase in sub-G_1 (Fig. 2B), indicating cell death. Downstream target of p53 (e.g. p21) is known to play a role in cell cycle control by inducing G_1 or G_2 arrest in response to DNA damage (Yu *et al.,* 1999) or apoptosis associated with up-regulation of endogenous $p21^{WAF}$ (Kannan *et al.,* 2001). Our results showed that the level of p21 increased significantly in A549 cells when treated with trilinolein for 24 h following the increase of p53 (Fig. 3A). This suggests that p21 is involved in a p53-dependent pathway and plays a specific role in trilinolein-induced G_0/G_1 cell cycle arrest in A549 cells.

Activation of PI3K/Akt plays an important role in carcinogenesis by maintaining cancer cell proliferation, preventing apoptosis, and supporting the process of metastasis. As aberrant activation of the PI3K/Akt occurs frequently in NSCLC (Su *et*

al., 2010), recent efforts have focused on developing novel antitumor agents targeting this pathway. It is targeted by genomic aberrations including mutation, amplification and rearrangement more frequently than any other pathway in human cancer. Therefore, we studied the effects of trilinolein treatment on the PI3K/Akt signaling pathway. Trilinolein has been shown to inhibit PI3K and pAkt and upregulate p53 expressions. Our finding suggests that PI3K pathway may have been demonstrated as the critical mediator in p53 activation in response to trilinolein (Fig. 3A). Our results demonstrated that if efficient p53-dependent Akt cleavage is triggered, the Akt-mediated survival signals will be aborted and will not able to block p53-dependent apoptosis (Gottlieb *et al.*, 2002).

It has been suggested that apoptosis requires the activation of caspases in many cases (Ashkenazi and Dixit, 1998), we investigated the involvement of caspase activation in trilinolein-induced apoptosis in A549 cells. Treatment with trilinolein stimulated a time-dependent cleavage activation of procaspase 3 and PARP. To elucidate the mechanism of activation of caspase 3 by trilinolein, we examined the activation of its upstream activator, caspase 9. The activation of caspase 9 was evidenced by the degradation of its proenzyme. Considering the crucial role of the mitochondrial pathway in apoptosis, we examined changes in the levels of cytochrome *c* released into the cytosol in trilinolein-treated A549 cells. Since the

increased of cytosolic cytochrome *c* appeared earlier than activation of caspases (Fig. 3B), we think trilinolein may target to disrupt Bax/Bcl-2 ratio rather than directly damaging mitochondria integrity (Fig. 3C). When A549 cells were treated with trilinolein, a decrease in the level of pAkt was observed before caspase-3 activation (Fig. 3), indicating that Akt inhibition is an upstream event of caspase-3 activation in trilinolein-induced apoptosis.

ROS are persistently produced during the metabolic process. Under physiological conditions, the maintenance of an appropriate level of intracellular ROS is important in keeping redox balance and cell proliferation (Martin and Barrett, 2002). Excessive ROS accumulation, however, can lead to cellular injury (Mallis *et al.*, 2001). Recent evidence indicates that accumulated ROS causes sustained JNK activation and leads to apoptosis (van den Berg *et al.,* 2001). Cancer cells normally produce more ROS than do normal cells and addition of an agent that increases ROS may push a tumor cell beyond the breaking point (Schumacker, 2006). So, cancer cells might be vulnerable to damage by additional ROS stress, either through inhibiting ROS elimination or by adding exogenous ROS (Huang *et al.,* 2000). The cell-damaging property of ROS and the increased ROS generation in cancer cells may provide an opportunity to develop the cell killing potential of ROS by using exogenous ROS-stressing agents to increase the intracellular ROS to a toxic level, or

the threshold that triggers cell death. We attempted to measure changes in ROS levels in trilinolein-treated cells. The antiproliferative effect of trilinolein included in this study is associated with an increase in the intracellular level of ROS which was detectable at 1 h of treatment and remained elevated for at least 4 h (Fig. 4B). It was shown that ROS is decreased after trilinolein treatment in cardiomyocytes (Yang *et al.*, 2005;Chen *et al.*, 2005), however the mechanism responsible for the increase in ROS generation in trilinolein-treated A549 lung cancer cells is largely unclear. Oncogenic signals, mitochondrial dysfunction, and active metabolism are likely factors contributing to the increased production of ROS in cancer cells (Trachootham *et al.*, 2006). It is plausible that trilinolein could be involved in the regulation with the abovementioned signals. Other study indicated that elevated accumulation of resveratrol leads to increased intracellular ROS levels, which then subsequently induces glioma cell apoptosis (Shao *et al.*, 2009). The pro-oxidant property possibly results from the generation of phenoxyl radicals of resveratrol by the peroxidase-H_2O_2 system, which co-oxidizes cellular glutathione or NADH, accompanied by O_2 uptake to form ROS (Galati *et al.*, 2002). Thus, resveratrol probably acts as a pro-oxidant, disrupting intracellular redox balance and leading to apoptosis, which is the common postulated mechanism to explain resveratrol's anti-cancer effect. Further study should be conducted to explore the pro-oxidative effects of trilinolein. Also, it was suggested

that several mitogen-activated protein kinase (MAPKs) including c-Jun N-terminal kinase/stress activated protein kinase (JNK/SPK1/2) and $p38^{MAPK}$ play important roles in triggering apoptosis in response to oxidative stress (Tobiume *et al.*, 2001). We should further investigate whether MAPKs play a role in trilinolein-induced apoptosis on A549 cells.

In conclusion, our results suggested that trilinolein induced apoptosis in human lung carcinoma cells. The pro-apoptotic response was correlated with the increase of Bax, decrease of Bcl-2, cytochrome *c* release, caspase-3 activation and PARP degradation. Furthermore, the inactivation of Akt may play an important role in trilinolein-induced apoptosis. ROS could be another factor involved in the cell apoptosis. These results provide the possible mechanisms for the apoptotic activity of trilinolein.

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Figure legends:

Fig 1. (A) Chemical structure of trilinolein (B) Cytotoxic effects of trilinolein on A498, MKN-45 and A549 cells. These cancer cells were treated with various concentrations (0, 3.12, 6.25, 12.5, 25, 50, 75, and 100 μg/mL) of trilinolein for 24 h. Data are the mean ± SD of three independent experiments.

Fig 2. Determination of the proportion of sub-G1 and G0/G1 cells following trilinolein treatment of A549 cells, as determined by flow cytometry. (A) Distribution of cell cycle phase in A459 cells after treatment with various concentrations of trilinolein (0, 25, 50, 75, and 100 μg/mL) for 24 h (B) Distribution of cell cycle phase in A459 cells after treatment with 75 μg/mL trilinolein for 0, 2, 12, 24 and 48h

Fig 3. (A) Effects of trilinolein on the expression of PI3K, pAkt. Akt, p53 and p21 proteins in A549 Cells as determined using western blotting. Cells were treated with 75 μg/mL trilinolein for the times indicated. (B) Effect of trilinolein on the activity of apoptosis-associated proteins. A549 cells treated with 75 μg/ml trilinolein and proteins expression of Bax, Bcl-2, cytochrome *c*, poly(ADP-ribose) polymerase (PARP), caspase 9 and caspase 3 were analyzed after 0, 6, 12, 24 and 48 h exposure.

Trilinolein induced cleavage of procaspases 9 and 3 in the cytosol. β-Actin was used as an internal control. (C) The ratio of Bax/Bcl-2 prptein expression at 0, 6, 12, 24 and 48 h. Data are the mean ± SD of three independent experiments. *P < 0.05 compared with control.

Fig 4. Trilinolein-induced ROS generation. (A) A549 cells were incubated for 4 h in the presence of trilinolein (50, 75 and 100 μg/ml) or (B) with 75 μg/ml of trilinolein for 1, 2 and 4 h. The fluorescence of oxidized DCF was determined by flow cytometry.