國 立 交 通 大 學
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碩士論文

PT-262 誘發細胞凋亡與抑制腫瘤形成的作用

Induction of apoptosis and tumorigenesis inhibition by PT-262

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中文摘要

本論文為探討一種由 6,7-dichloro-quinoline-5,8-dione 所衍生的新合成化合物，稱為 7-chloro-6-piperidin-1-yl-quinoline-5, 8-dione，簡稱 PT-262，研究其抗癌的能力。PT-262 具有誘發 A549 人類肺癌細胞產生細胞毒性及細胞凋亡的作用。處理 PT-262 後會促使細胞凋亡蛋白 caspase 3 被活化與其下游分子 PARP 蛋白的切割。同時，我們發現 PT-262 會降低抗細胞凋亡蛋白，包括 survivin 及 XIAP 的表達，相反地，處理 PT-262 之後，會誘發參與調控細胞凋亡的調控蛋白 p38 與 H2AX 的蛋白磷酸化活化量增加。有趣地，PT-262 會增加具有抑制腫瘤功能 E-cadherin 蛋白表達，並且表現於 A549 肺癌細胞的細胞膜，然而 PT-262 並不影響 E-cadherin 基因的表達。再者，PT-262 具有抑制先天免疫缺失的老鼠體內之異種移植人類肺癌腫瘤的形成能力。綜合以上結果，我們提供 PT-262 具有誘發人類肺癌細胞凋亡及抑制腫瘤形成的抗癌活性，並推測 PT-262 是一種有潛力的化合物，可提供作為開發未來肺癌的治療。

Abstract

A new synthetic compound 7-chloro-6-piperidin-1-yl-quinoline-5, 8-dione (designed as PT-262) derived from 6, 7-dichloro-quinoline-5, 8-dione on its anticancer ability was investigated in this study. PT-262 induced cytotoxicity and apoptosis in A549 lung carcinoma cells. The activated caspase 3 and the cleaved form of PARP proteins were induced by treatment with PT-262. Meanwhile, we found that PT-262 reduced anti-apoptosis proteins expression including survivin and XIAP. In contrast, the phosphorylation of p38 and H2AX, the apoptotic-regulated proteins, were activated by PT-262. Interestingly, PT-262 increased a tumor suppressor protein E-cadherin expression, which located in the membrane of A549 lung cancer cells; however, the gene expression of E-cadherin was not altered by PT-262. Furthermore, PT-262 inhibited tumorigenesis of xenograft lung cancer cells in severe combined immunodeficiency (SCID) mice. Together, our findings provide that PT-262 exerts the anticancer activities on apoptosis induction and tumorigenesis inhibition in human lung cancer cells. We suggest that PT-262 is a novel and potential chemical for developing cancer therapy in lung cancer.

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Abbreviations

IAP	inhibitor of apoptosis protein
MAPK	mitogen-activated protein kinase
NSCLC	non-small cell carcinoma
PARP	poly (ADPribose) polymerase
PI	propodium iodide
SCLC	small cell carcinoma
XIAP	X-linked inhibitor of apoptosis protein
SCID	severe combined immunodeficiency
PBS	phosphate buffered saline
FITC	fluorescein isothiocyanate
MTT	3-(4-, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide
FBS	fetal bovine serum
RT-PCR	reverse transcriptase PCR

1. Introduction

1.1 Lung cancer

Cancers are the major cause of mortality in the world. In Taiwan, lung cancer has been become the leading reason of death (Department of Health, Executive Yuan, 2009). Lung cancers are divided into two types: small cell carcinoma (SCLC) and non-small cell lung cancer (NSCLC) (Ramalingam and Belani, 2008; Sun et al., 2007). SCLC patients contained about 20 % of all lung cancer cases; whereas NSCLC accounts for approximately 80–85 % of all cases of lung cancer (Dempke et al., 2010; Ramalingam and Belani, 2008). With the greater tendency of rapidly spreading to other tissues by the time of diagnosis, SCLC usually takes chemotherapy combined with radiation; while surgery is the treatment of choice for early-stage localized NSCLC (Jeremic et al., 2004; Manser et al., 2005). However, treatment with chemotherapeutic drugs becomes the recommended remedy for advanced metastatic cases (Dempke et al., 2010). Thus, development of effective therapeutic drugs for cancer treatments is highly desired.

1.2 Derivatives of 5, 8-quinolinediones

The 5, 8-quinolinediones have been used as precursors to create many derivatives possessing biological activities such as anticancer and antimicrobial functions (Hsu et al., 2008; Humphries et al., 1974; Inouye et al., 1987; Lown and Sim, 1976; Porter et al., 1973; Ryu and Kim, 1994; Shaikh et al., 1986). Moreover, 6, 7-dihaloquinoline-5, 8-diones also display potential starting molecules for synthesis of bioactive compounds (Yoon et al., 2000). It has been shown that amine addition as the modifying strategy by the reason of electron deficiency on the 6-position of 5,8-quinolinedione for synthesizing various derivatives (Boger et al., 1987). 6-Anilino-5, 8-quinolinedione (LY83583), a benzoquinone derivative of 5, 8-quinolinedione, inhibits guanylyl cyclase activity to reduce the levels of cGMP (Kumagai et al., 1998). LY83583 has been used as an agent to reduce nitric oxide (NO)-dependent cGMP responses, which can be utilized as a pharmaceutical chemical for evaluating the roles of cGMP in biological events (Kumagai et al., 1998; Schmidt et al., 1985). Moreover, NSC 663284 is a potential CDC25 inhibitor that exhibits highly inhibiting activity against human breast cancer cell growth (Lazo et al., 2001; Pu et al., 2002). Additionally, two novel quinolinedione derivatives, 6-(4-fluorophenyl)-amino-5, 8-quinolinedione (OQ1) and 6-(2,3,4-

Trifluoro-phenyl)-amino-5,8-quinolinedione (OQ21) were reported to inhibit L-arginine-induced endotheliumin-dependent relaxation of aortic rings and the inhibition of LPS-induced production of NO in rats (Lee et al., 2000; Lim et al., 2009).

1.3 Anticancer activities of 5, 8-quinolinediones

The derivatives of 5, 8-quinolinedione have been exhibited antitumor activities in a variety of cancers. LY83583 has been shown to induce cell senescence by up-expression of p21 in a p53-independent manner in colorectal cancer cells (Lodygin et al., 2002). Furthermore, NSC 663284 can inhibit CDC25 activity by covalently binding to serine residue of CDC25 protein that mediates the proliferation inhibition of tumor cells (Braud et al., 2008). TAS-103 is a novel quinoline derivative possessing remarkable antitumor activity in mouse and human models and currently is in phase I clinical trials (Azuma and Urakawa, 1997; Utsugi et al., 1997; Yoshida et al., 2008). TAS-103 is a specific topoisomerase inhibitor that functions on topoisomerase II-mediated DNA cleavage (Azuma and Urakawa, 1997; Byl et al., 1999; Utsugi et al., 1997). 7-Chloro-6-piperidin-1-yl-quinoline-5, 8-dione designated as PT-262 is a new 5, 8-quinoline-dione derivative that can inhibit phosphorylation of

ERK and suppress cell proliferation in both p53-wild type and p53-null lung cancer cells (Hsu et al., 2008). Furthermore, previous study has also shown that PT-262 exhibits inhibition activity of CDC2 phosphorylation and induction of G2/M phase arrest in lung cancer cells (Hsu et al., 2008). In addition, natural analogues of 5,8-quinolinediones such as streptonigrin and lavendamycin isolated from microorganisms have been shown to possess anticancer activities (Boger et al., 1987).

1.4 Apoptosis

Apoptosis is a form of cellular suicide that is widely observed in nature. Originally, apoptosis refers to dying cells exhibiting a characteristic pattern of changes, including cytoplasmic shrinkage, active membrane blebbing, chromatin condensation and fragmentation into membrane-enclosed vesicles (Wyllie et al., 1980). In mammalian cells, apoptosis is mediated by a family of cysteine proteases known as caspases (Riedl and Shi, 2004; Shi, 2002). The inactive procaspases become cleaved active form when cells are stimulated by death signals (Earnshaw et al., 1999). Activation of caspases can cleave specific cellular substrates, including cytoplasmic structural proteins such as actin and cytokeratins and/or nuclear proteins such as poly (ADP-ribose) polymerase (PARP) and lamins for inducing cell death (Caulin et al.,

1997; Mashima et al., 1997; Tewari et al., 1995). This cascade leads to cell catastrophe with numerous of biochemical and morphological changes (Fangusaro et al., 2006).

Besides to the apoptosis-promoting proteins, there are some inhibitors of apoptosis protein (IAP) which exhibit opposite activities of anti-apoptosis (Liston et al., 2003). X-linked inhibitor of apoptosis (XIAP) is the best characterized and most potent member of the inhibitor of apoptosis (IAP) family (Deveraux and Reed, 1999; Deveraux et al., 1997). Its caspases-inhibitory activity by direct binding to the caspase substrates accounts for the protective effect against several apoptotic triggers including irradiation and various anticancer drugs (LaCasse et al., 1998). Among the IAP family members, survivin is abundantly expressed in the embryonic tissues and in most of the tumors, but in the normal differentiated cells it is almost absent (Ambrosini et al., 1997). Over- expression of survivin in cancer may overcome cell cycle checkpoints to facilitate aberrant progression of transformed cells through mitosis (Sah et al., 2006). Several studies have demonstrated resistance of survivin-expressing cells to anticancer drug-induced apoptosis (Rodel et al., 2003; Tran et al., 2002). It is an efficient strategy to block the survival pathways and to induce apoptotic pathways in cancer therapy (Kuo et al., 2004; Tamura et al., 2000;

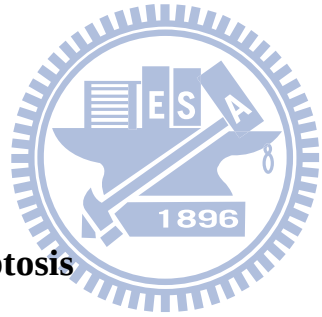
Wall et al., 2003).

1.5 Apoptosis-regulation proteins and apoptosis

Mitogen-activated protein (MAP) kinases, including ERK, c-Jun NH2-terminal kinases (JNK) and p38 MAPK are important intermediates of the signal-transduction pathway associated with proliferation or apoptosis (Lim et al., 2009; Tominaga et al., 2004). Abundant evidence for p38 involvement in apoptosis exists to date and is based on concomitant activation of p38 and apoptosis induced by a variety of agents (Juo et al., 1997; Zarubin and Han, 2005). Some studies have shown that p38 activation can be blocked by caspase inhibitors suggesting p38 functions downstream of caspase activation (Huang et al., 1997); whereas, overexpression of MKK6b, the upstream molecule of p38 can also induce caspase activity and cell death thus implying that p38 may function both upstream and downstream of caspases in apoptosis (Hanks and Hunter, 1995) Furthermore, it has been reported that p38 activation may be reduced in tumors and that loss of components of the p38 pathway such as MKK3 and MKK6 resulted in increased proliferation and likelihood of tumorigenic conversion (Brancho et al., 2003).

H2AX is a histone H2A variant that plays essential role in the recruitment and

accumulation of DNA repair proteins to sites of double-strand breaks (DSB) damage. When cells are exposed to γ-irradiation (IR), Ser-139 is rapidly γ-phosphorylated (Rogakou et al., 1998). Phosphorylated H2AX (γ-H2AX) forms nuclear foci at the sites of induced DSBs and is essential in the recruitment of repair factors to the damaged DNA sites (Paull et al., 2000; Rogakou et al., 1998) Moreover, γ-H2AX is formed during apoptosis initiated by DNA damage (Rogakou et al., 2000). Therefore, assessment of H2AX phosphorylation can be used in clinical practice as a marker of premalignant lesions and to predict cell sensitivity to radiotherapy and chemotherapy (Podhorecka, 2009).



1.6 E-cadherin and apoptosis

E-cadherin is a transmembrane glycoprotein that belongs to the classical cadherin family of Ca^{2+}-dependent adhesion proteins (Gooding et al., 2004). It plays essential roles in epithelial cell behavior, tissue formation, and suppression of cancer formation (van Roy and Berx, 2008). E-cadherin comprises the major part of adherins junction with cytoplasmic domain binding of proteins termed catenins that connect the cadherin complex to the actin cytoskeleton and several signaling pathways (Gu et al., 2009). The cytoplasmic domain of E-cadherin can regulate endocytosis,

intracellular signaling and gene transcription, and local control of the actin cytoskeleton (Halbleib and Nelson, 2006; Perez-Moreno and Fuchs, 2006). The role of E-cadherin on regulating apoptosis is still unclear. Some studies have shown that wild-type E-cadherin expressing cells are more sensitive to the effect of apoptotic agents; while E-cadherin-mutated cells acquired resistance to apoptosis (Ferreira et al., 2005; Witta et al., 2006). E-cadherin is involved in the pro-apoptotic process via the regulation of the caveolin-1-mediated down regulation of survivin, an apoptosis inhibitor, in HT 29 and B16-F10 melanomas (Torres et al., 2007) However, some studies showed that the disruption of adherins junction may affect susceptibility to cell death by secondary aopototic stimuli (Jones et al., 2004; Li et al., 2008; Yi et al., 2009). In particular, survival signals can be produced when cells adhere to the extracellular matrix or when intercellular adhesions are formed (Comoglio et al., 2003)

1.7 E-cadherin and tumorigenesis

E-cadherin is a well-known tumor suppressor protein (Pecina-Slaus, 2003). In human cancers, cell-cell adhesion is generally perturbed and it is considered as the indispensible step for cancer cells to escape from the normal growth control signals

(St Croix et al., 1998). The abnormal regulation of these intercellular interactions is also involved in invasion and metastasis, the latter cases of tumor progression but the most life-threatening steps (Hirohashi, 1998). It is believed that adhesion could contribute to tumor suppression either by physically joining cells or by facilitating other juxtacrine signaling via other receptor systems (Gottardi et al., 2001). E-cadherin is not only an invasion suppressor but also a proliferation suppressor (Vleminckx et al., 1991). It is also found that the effect of E-cadherin on proliferation suppression depends on its ability to prevent growth factor induced reduction in p27 protein level (St Croix et al., 1998; Vleminckx et al., 1991).

1.8 E-cadherin and β-catenin

An intact E-cadherin-catenin complex is required for maintenance of normal intercellular adhesion (Wijnhoven et al., 2000). Many studies have shown that loss of expression of the E-cadherin/β-catenin complex on the membrane is correlated with poor prognosis in human cancers such as ampullary cancer, meningioma and lung cancer (Hsu et al., 2010; Tseng et al., 2010; Zhou et al., 2010). Loss of E-cadherin leads to invasive behavior which allows β-catenin to enter nuleus where it regulates the transcription of EMT-related and proliferation-regulatory genes (Moreno-Bueno et

al., 2008). Alternatively, E-cadherin tumor suppressor activity could result from binding and antagonizing the nuclear signaling function of β-catenin (Gottardi et al., 2001).

1.9 Purpose of this study

In this study, the anticancer abilities of PT-262 on apoptosis and anti-tumorigenesis effects were investigated in human lung carcinoma cells. Understanding the anticancer mechanisms of PT-262 by which mediate the apoptosis and anti-tumorigenesis in human lung cancer cells may contribute to the therapy of lung cancers.

2. Materials and Methods

2.1 Chemicals and antibodies

PT-262 was synthesized and kindly provided by our collaborator Dr. Chinpiao Chen (National Dong Hwa University, Hualien, Taiwan). Hoechst 33258, propidium iodide and 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical (St. Louis, MO). BODIPY FL phallacidin (B-607) was purchased from Invitrogen (Carlsbad, CA). Anti-p38, anti-survivin, anti-E-cadherin and anti-ERK-2 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-XIAP and anti-phospho-p38 (Thr180/Tyr182) were purchased from Cell Signaling Technology, Inc. (Beverly MA, USA). Anti-caspase-3 was purchased from BioVision, Inc. (San Francisco, CA). Anti-PARP was purchased from Clontech Laboratories, Inc. (Mountain view, CA). Anti-GAPDH was purchased from Novus Biologicals Inc. (Littleton, CO). Anti-γ-H2AX was purchased from BD Bioscience (Franklin Lakes, NJ). Anti-β-catenin was purchased from Abcam Inc. (San Francisco, CA).

2.2 Cell culture

The A549 cell line (ATCC, #CCL-185) was derived from human lung carcinoma. A549 cells were cultured in complete RPMI-1640 medium (Gibco, Life Technologies, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, and L-glutamine (0.03 %, w/v). The cells were maintained at 37 °C and 5 % CO2 in a humidified incubator (310/Thermo, Forma Scientific, Inc., Marietta, OH).

2.3 Cytotoxicity MTT assay

The cells were plated in 96-well plates at a density of 1×10^4 cells/well for 16-20 h. Thereafter the cells were treated with various concentrations of PT-262 for 24 h in complete RPMI-1640 medium. After treatment, the cells were washed once with phosphate buffered saline (PBS) and were re-cultured in complete RPMI-1640 medium for two days. Subsequently, the cells were incubated with 0.5 mg/ml of MTT in fresh complete RPMI-1640 medium for 4 h. The surviving cells converted MTT to formazan by forming a blue-purple color when dissolved in dimethyl sulfoxide. The intensity of formazan was measured at 565 nm using a microplate reader

(VERSAmax, Molecular Devices Inc., CA). The relative percentage of surviving cells was calculated by dividing the absorbance of treated cells by that of the control in each experiment.

2.4 Annexin V-propidium iodide analysis

For detection of apoptotic events, floating and adherent cells were collected. A549 cells were treated with 0-30 μM PT-262 for 24 h at 37 ℃. After PT-262 treatment, the cells were centrifuged and then the cell pellets were suspended in 1 × Annexin V binding buffer. Samples were incubated with fluorescein isothiocyanate (FITC)-conjugated-Annexin V and propidium iodide (PI) according to the manufacturer's instruction (BioVision, Mountain View, CA) for 5 min at room temperature. Then cells were analyzed immediately using a flow cytometer (FACS Calibur, BD Biosciences, Heidelberg, Germany). For each measurement, 10,000 cells were analyzed. Dot plots and histograms were analyzed by CellQuest software (BD Biosciences). The cells showed Annexin V positive and PI negative indicate at early apoptosis; while the cells showed Annexin V positive and PI positive indicate at late apoptosis. Annexin V and PI negative cells were termed viable.

2.5 Western blotting

After the end of drug treatment, the cells were lysed in the ice-cold whole cell extract buffer containing the protease inhibitors. The lysate was vibrated for 30 min at 4 ℃ and centrifuged at 10,000 rpm for 30 min. Protein concentration was measured by BCA protein assay kit (Pierce, Rockford, IL). Equal amounts of proteins (40–60 μg/well) were subjected to electrophoresis using 10 to 12 % sodium dodecyl sulfate -polyacrylamide gels. To verify equal protein loading and transfer, ERK-2 or GAPDH were used as the protein loading control. Proteins were then transferred to polyvinylidene difluoride membranes and the membranes were blocked overnight at 4℃ using blocking buffer (5 % non-fat dried milk in solution containing 50 mM Tris/HCl (pH 8.0), 2 mM $CaCl_2$, 80 mM sodium chloride, 0.05 % Tween 20 and 0.02 % sodium azide). The membranes were then incubated for 2 h at 25 ℃ with specific primary antibody followed by anti-rabbit or anti-mouse immunoglobulin G-horseradish peroxidase conjugated secondary antibodies. The membranes were washed three times for 10 min with washing solution. Finally, the protein bands were visualized on the X-ray film using the enhanced chemiluminescence detection system (PerkinElmer Life and Analytical Sciences, Boston, MA). A gel-digitizing software, Un-Scan-It gel (ver. 5.1; Silk Scientific, Inc., Orem, UT), was used to quantify the

intensity of each band on the X-ray film.

2.6 RT-PCR

Cells were plated at a density of 2×10^6 cells per 60-mm petri dish in culture medium. Total cellular RNA was purified by Trizol reagent (Invitrogen) according to the manufacturer's protocol. RNA concentrations were determined by spectrophotometry. cDNAs were synthesized by SuperScript™ III reverse transcriptase with oligo $(dT)_{12-18}$ primer (Invitrogen) Each reverse transcript was amplified with GAPDH as an internal control. The following primer pairs were used for amplification, E-cadherin forward primer: 5'-CAGTCAAAAGGCCTCTACGG-3' and E-cadherin reverse primer: 5'-GTGTATGTGGCAATGCGTTC-3'; GAPDH forward primer: 5'-CGGAGTCAACGGATTTGGTC GTAT-3' and GAPDH reverse primer: 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'. RT-PCR was performed by a DNA thermal cycler (Mastercycler gradient, Hamburg, Germany). The initial denaturation was performed at 94 °C for 2 min, followed by 30 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 40 s; and 72 °C for 5 min. The PCR products were visualized on 1.2 % agarose gels with ethidium bromide staining under UV transillumination, and photograph was taken by a camera (DH27-S3, Medclub,

Taoyuan, Taiwan).

2.7 Immunofluorescence staining and confocal microscopy

To view the protein localization and expression of β-catenin after PT-262 treatment, the cells were subjected to immunofluorescence staining and confocal microscopy. After fixation with 4 % paraformaldehyde solution, the cells were washed three times with PBS, and non-specific binding sites were blocked in PBS containing 10 % FBS and 0.3 % Triton X-100 for 1 h. Thereafter, the cells were separately incubated with rabbit anti-β-catenin (1:200) antibody in PBS containing 10 % FBS overnight at 4°C, and washed three times with 0.3 % Triton X-100 in PBS. Then the cells were incubated with goat anti-rabbit Cy3 (1:200) in PBS containing 10 % FBS for 1 h at 37 °C. F-actin and nuclei were stained with BODIPY FL phallacidin and Hoechst 33258, respectively. The samples were examined under a confocal microscope Fluoview 300 (Olympus, Tokyo, Japan).

2.8 The cell surface expression of E-cadherin analyzed by flow cytometry

After treatment with PT-262 for 24 h, A549 cells were harvested and incubated

with 10 % bovine serum albumin in PBS for 30 min at 4 ℃. Subsequently, the samples were incubated with mouse anti-E-cadherin antibody (1:100), and followed incubated with a FITC-coupled goat anti-mouse antibody (1:200) for 30 min at 4 °C. At the end of incubation, the cells were resuspended in 1 × PBS and immediately analyzed by a flow cytometer (FACS Calibur, BD Biosciences). The fluorescence intensities of E-cadherin were quantified the green fluorescence using CellQuest software (BD Biosciences).

2.9 Anchorage-independent assay

To evaluate the effect of PT-262 on colony forming ability of lung cancer cells in soft agar, anchorage-independent assay was analyzed in this study. A549 cells were treated with or without 20-30 μM PT-262 for 24 h. After treatment, the cells were then seeded 2500 cells/well in 1 ml of 0.33 % agarose in 1 × RPMI medium with 1 ml of 0.5 % agarose in 1 × RPMI medium as the based layer . The cultures were maintained in a 37 °C, 5 % CO_2 incubator for 20 days. Cells were fed 1-2 times per week with fresh culture medium. Finally, the colonies and numbers were counted under a phase contrast microscope. Colony numbers (more than 10 cells existing in a colony) were calculated from 10 random fields of each treatment.

2.10 Animal studies

CB17/Icr-Prkdcscid/CrlBltw mice (4-week-old male) were purchased from BioLASCO (Taipei, Taiwan). After 1-2 weeks for environmental adaption, the mice were used for human lung cancer cell inoculation. Two treatment methods were executed as followed. For pre-treatment of PT-262, A549 cells were treated with or without 20-30 μM PT-262 prior to inoculation into flanks of the severe combined immunodeficiency (SCID) mice (1×10^6 cells / mouse). The tumor size and the body weight of the mice were recorded every four days since then until day 64. For post-treatment of PT-262, solid A549 flank tumors were established by subcutaneous injection of 2×10^6 cells. When tumors reached volumes of 100 mm^3, they received a 100 μL intra-tumoral injection of Tween 80/PBS (vehicle) or 5 mg/kg of PT-262. The injections administrated every four days starting at day 0 and ending on day 8. The tumor volume was continuously measured until the mice were sacrificed due to excessive tumor size or serious trauma was observed at the surface of the tumors. Tumor measurement was performed using calipers, and tumor size was calculated using the ellipsoid formula 1/2 (LW^2), where L represents length (the longest part of the tumor) and W represents width (the widest part).

2.11 Statistical analysis

Data were analyzed using Student's *t* test or analysis of variance (a comparison of multiple groups), and a *p* value of <0.05 was considered statistically significant in each experiment.

3. Results

3.1 PT-262 induces cytotoxicity and apoptosis in human lung cancer cells

Fig. 1 shows the chemical structure of PT-262. Treatment with 5–30 μM PT-262 for 24 h reduced the cell viability via a concentration-dependent manner in A549 cells (Fig. 2). The cell viability was 27.2 % in A549 cells after treatment with 30 μM PT-262 for 24 h. To further examine the apoptosis induction by PT-262, the PT-262-treated cells were analyzed by Annexin V-FITC/PI staining. The apoptotic cells (Annexin V^+ / PI^+) were increased following treatment with PT-262 at 20 μM and 30 μM 24 h (Fig. 3A). PT-262 (30 μM for 24 h) significantly induced the apoptosis level at average of 37.03 % in A549 cells (Fig. 3B). In addition, PT-262 also increased the cell necrosis at average of 39.32 % by 30 μM treatment (Fig. 3A)

3.2 PT-262 inceases the protein levels of active caspase-3 and cleavage of PARP in human lung cancer cells

To examine the induction of apoptosis pathway following PT-262 treatment, A549 lung cancer cells were analyzed the protein levels of the caspase-3 activation and PARP cleavage using Western blot. The active forms of caspase-3 (12 kD and 17

kD) were induced following treatment with PT-262 (Fig. 4). GAPDH protein is an internal control protein that was not altered by PT-262 treatment. Moreover, the cleaved form proteins of PARP (89 kD) were increased by exposure to PT-262 in A549 cells (Fig. 4).

3.3 PT-262 inhibits anti-apoptosis proteins expression

To analyze the effect of PT-262 on the expression of anti-apoptosis proteins, the PT-262-treated cells were subjected to Western blot. Survivin and XIAP proteins, which belong to the IAP family, were examined in this study. After treatment with 5-30 μM PT-262 for 24 h, survivin protein levels were markedly reduced in A549 cells via concentration-dependent manner (Fig. 5A). The XIAP proteins expression were slightly increased by PT-262 at low concentrations of 5-10μM; in contrast, PT-262 inhibited XIAP protein expression at higher concentrations of 20-30 μM (Fig. 5B).

3.4 PT-262 induces γ-H2AX and phosphorylated p38 protein expression in human lung cancer cells

To analyze the apoptosis-regulated protein expressions whether involve in

PT-262-induced apoptosis, the phosphorylated H2AX and p38 protein were analyzed by Western blot. As shown in Fig. 6A, treatment with 5–30 μM PT-262 for 24 h significantly induced γ-H2AX proteins in A549 cells. Furthermore, the phosphorylation of p38 proteins was increased following treatment with PT-262 (5-30 μM for 24 h) (Fig. 6B). The total protein levels of p38 and GAPDH were not altered by PT-262 (Fig. 6A and Fig. 6B).

3.5 PT-262 increases E-cadherin protein expression but not on the gene expression in human lung cancer cells

To examine the expression of E-cadherin in A549 cells, total cellular proteins were extracted and subjected to Western blot analysis. As shown in Fig. 7, the protein expression of E-cadherin was elevated after treatment with 5-30 μM PT-262 for 24 h. To further investigate the effect of PT-262 on transcriptional level of E-cadherin, the total RNA extracts were collected for RT-PCR analysis. The qualities of total RNA extracts were presented by the contents of 28S rRNA and 18S rRNA (Fig. 8A). The gene expression of E-cadherin was not significantly affected by PT-262 treatment (Fig. 8B and 8C). We have further investigated the cellular membrane location of E-cadherin by indirect E-cadherin immunofluorescence staining and flow cytometer.

The fluorescence intensity of membranous E-cadherin proteins was increased by PT-262 (Fig. 9A). The fluorescence intensity of membranous E-cadheirn proteins was elevated to 2-folds by 30 μM PT-262 treatment comparing to untreated A549 cells (Fig. 9B).

3.6 The protein expression of β-catenin is located in the membrane of human lung cancer cells by PT-262 treatment

To examine the influence of PT-262 on the membranous complex partner of E-cadherin β-catenin, the protein expression and location of β-catenin after treatment with PT-262 were analyzed by Western blot and confocal microscope. As shown in Fig. 10A, the protein expression of β-catenin was induced by PT-262. The PT-26-treated A549 cells showed significantly increased β-catenin protein expression than the control cells (Fig. 10B). To examine whether PT-262 affects the cell morphology and cellular location of β-catenin proteins, A549 cells were treated with or without PT-262 (10 μM for 24 h) and subjected to immunofluorescence staining and confocal microscopy. The red fluorescence intensity exhibited by β-catenin, the green fluorescence intensity exhibited by actin proteins of the cytoskeleton, and the blue colour indicated nuclei by staining with Hoechst 33258. Treatment with PT-262

markedly altered the cytoskeleton with elongated shape. The membranous accumulation of β-catenin was also observed in PT-262-treated cells comparing with the control cells.

3.7 PT-262 inhibits Anchorage-independent ability and tumorigenesis of human lung cancer cells

To evaluate the antitumor abilities of PT-262, the anchorage-independent soft agar assay and xenograft lung tumor in SCID mice were analyzed. The number of colonies was inhibited by PT-262 in a concentration-dependent manner (Fig. 12A). The volume of cell populations was also reduced by PT-262. After counting colony number, PT-262 significantly inhibited the colony formation ability in soft agar (Fig. 12B). In the animal model, pretreatment of PT-262 in A549 cells followed by cell inoculation into SCID mice reveals inhibitory effect on tumorigenesis comparing to the control group (Fig. 13A). post-treatment of PT-262 (5 mg/kg) also showed smaller tumor size in treatment group than control group (Fig. 13 B).

4. Discussion

The derivatives of 5, 8-quinolinedione have been shown to exert different bio-activities including anti-microorganism and anti-neoplasm (Hsu et al., 2008; Inouye et al., 1987; Ryu and Kim, 1994). Apoptosis is a cell suicide mechanism that requires specialized cellular machinery. A central component of this machinery is a proteolytic system involving caspases, a highly conserved family of cysteine proteinases with specific substrates (Schwenger et al., 1997). In this study, PT-262 exerts cyototoxicity to human lung cancer cells. PT-262 also induces significant level of apoptosis. Furthermore, PT-262 induces the activation of caspase-3 and cleavage of PARP. Accordingly, we suggest PT-262 induces apoptosis pathway that mediates caspase-dependent pathway for cancer cell death in human lung cancer cells. However, we also found that PT-262 induced partial necrosis in lung cancer cells. Thus, the precise mechanism of PT-262-induced cell death should be further investigated.

Anticancer agents can display their inhibitory activity via the induction of apoptosis or the suppression of survival-regulated protein expression in tumor cells (Jiang et al., 2010; Kuo et al., 2004; Lodygin et al., 2002; Tamura et al., 2000). Survivin, a specifically expressed protein in human cancer cells, overcomes cell cycle

checkpoints to facilitate aberrant proliferation of cancer cells (Ambrosini et al., 1997; Sah et al., 2006). Over-expression of survivin in transformed cells cause resistance to anticancer drug-induced apoptosis (Rodel et al., 2003; Tran et al., 2002). In human lung cancer cells, PT-262 exhibited the promising activity of survivin inhibition. Accordingly, we suggest that the inhibition of survivin expression by PT-262 may be involved in the regulation of PT-262-induced apoptosis.

Activation of p38 MAPK has been associated with the induction of apoptosis in response to various cellular stresses (Chao and Yang, 2001; Schwenger et al., 1997; Xia et al., 1995). It has also been reported that the presence of γ-H2AX is linked to caspase-controlled DNA fragmentation during apoptosis (Rogakou et al., 2000). We found that PT-262 increased the phosphorylated p38 and γ-H2AX proteins in human lung cancer cells. Therefore, we suggest that p38 and γ-H2AX may be involved in the regulation of apoptosis after treatment with PT-262.

E-cadherin is proposed as a tumor suppressor protein that plays a major role in epithelial cell adhesion and connects the extracellular environment to the contractile cytoskeleton leading to the activation of certain nuclear responses (Aberle et al., 1996; Takeichi, 1993; Witta et al., 2006). Analysis of many tumors derived from epithelia suggests that loss of E-cadherin correlates with tumor cell invasion (Chen et al., 1997;

Rasbridge et al., 1993; Schipper et al., 1991; Shiozaki et al., 1991). The loss of E-cadherin appears to be a key event in acquisition of invasive capacity, because re-expression of E-cadherin suppresses the invasion of tumor cells (Frixen et al., 1991). Also, it has been shown that induction of E-cadherin protein expression in gefitinib-resistant cells leads to apoptotic effect of gefitinib (Witta et al., 2006). Moreover, E-cadherin renders cells more sensitive to the effect of taxol (Ferreira et al., 2005). In this study, PT-262 induced E-cadherin protein expression in human lung cancer cells. In addition, the E-cadherin expression was located on the membrane of lung cancer cells following PT-262 treatment. Thus, we suggest that the induction of E-cadherin protein expression and membranous localization might be involved in the effect of PT-262 on the cancer cell migration inhibition and apoptosis induction. However, the precise mechanism of E-cadherin on regulation of migration inhibition and apoptosis by PT-262 need to be further investigated.

Many anticancer agents exert anti-tumorigenic activity via apoptosis induction (Pan et al., 1999; Shin et al., 2004; Yang et al., 2005). Due to the PT-262-induced apoptosis in human lung cancer cells, we have further demonstrated that the anticancer ability of PT-262 by animal model. Both pre-treatment and post-treatment strategies showed that PT-262 has the ability to reduce tumor size in xenograft lung

tumor of SCID mice. Accordingly, our findings indicate that PT-262 can display anticancer ability in both in vitro and in vivo tests. Nevertheless, we found that PT-262 did not completely inhibited tumor formation. Thus, the treated dosage and time of PT-262 should be further studied for improving the efficiency of tumorigenesis inhibition.

5. Conclusion

In this study, we provide a proposed model that PT-262 exerts apoptosis induction and tumorigenesis inhibition in human lung cancer cells (Fig. 14). PT-262 induces apoptotic signal proteins (eg. p38 and γ-H2AX) and reduces antiapoptotic proteins (eg. survivin and XIAP) for apoptosis in cancer cells. We demonstrate that PT-262 has ability to reduce tumorigenesis in mice. We propose that PT-262 is a novel and potential chemical for developing cancer therapy in lung cancer.

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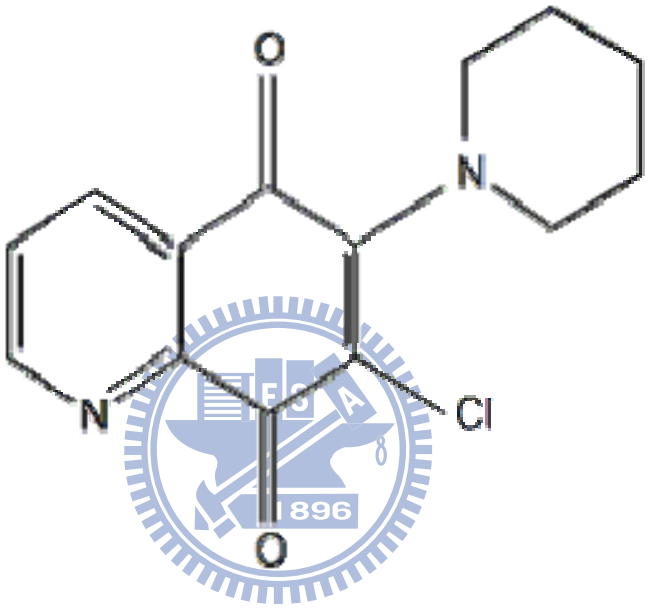

Fig. 1. Chemical structure of 7-chloro-6-piperidin-1-yl-quinoline-5, 8-dione (PT-262)

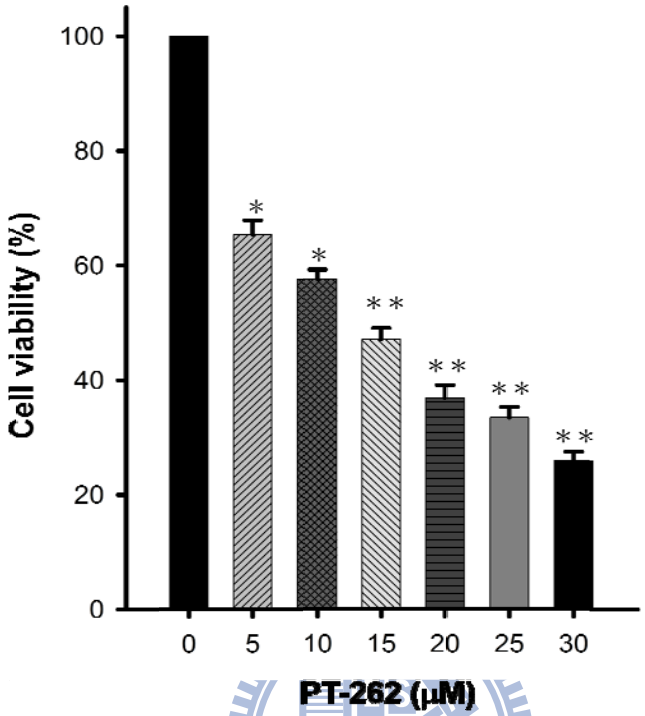

Fig. 2. The effect of PT-262 on the cell viability in A549 cells. After treatment with 0-30 μM PT-262 for 24 h, the cells were washed with PBS once and then the cells were re-cultured in fresh medium for 48 h. The cell viability was determined by MTT assay. The results were from 6 independent experiments and the bars represented mean ± S.E. $p < 0.05$ (*) and $p < 0.01$ (**), indicate significant difference between the control and PT-262 treated samples.

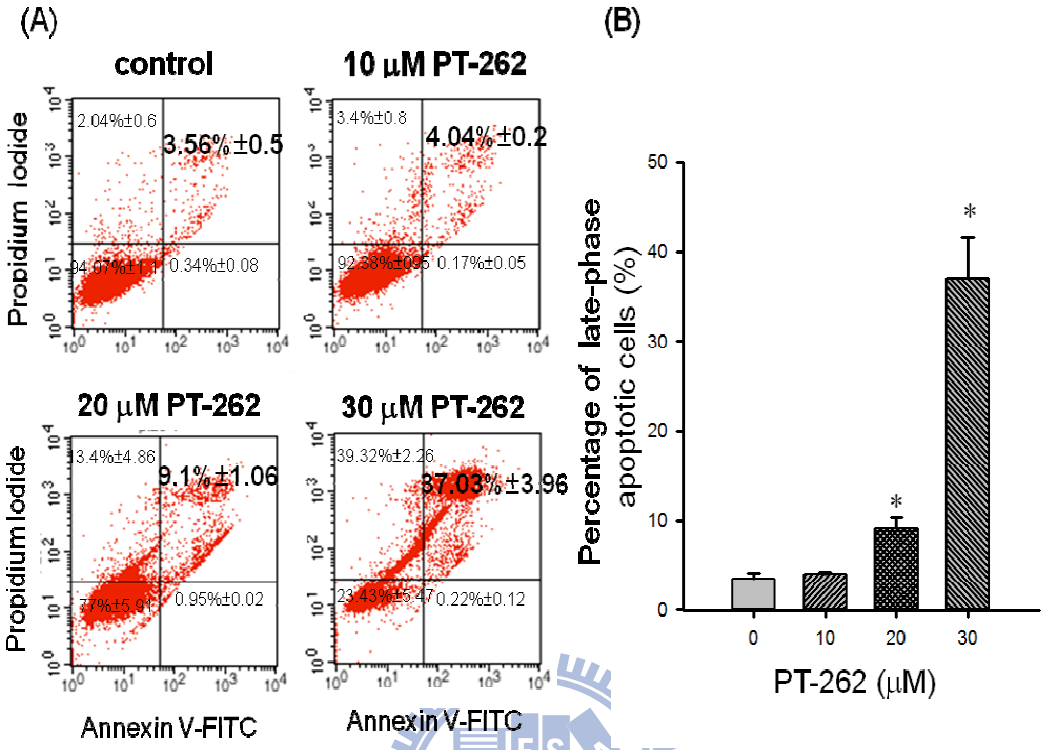

Fig. 3. The effect of PT-262 on apoptosis in A549 lung cancer cells (A) A549 cells were treated with 10-30 μM of PT-262 for 24 h. Apoptosis was determined by Annexin V-PI staining using flow cytometry analysis. The population of Annexin V^+/PI^- cells represents cells undergoing early apoptosis (lower right), whereas the fraction of Annexin V^+/PI^+ cells are those undergoing late apoptosis (upper right). Populations of apoptotic cells were quantified using CellQuest software. Data are shown from one of three separate experiments with similar findings. (B) The percentage of late-apoptotic cells was quantified from average of the three individual experiments. The bar represented the mean ± S.E. $p < 0.05$ (*) indicates significance between control and PT-262-treated samples.

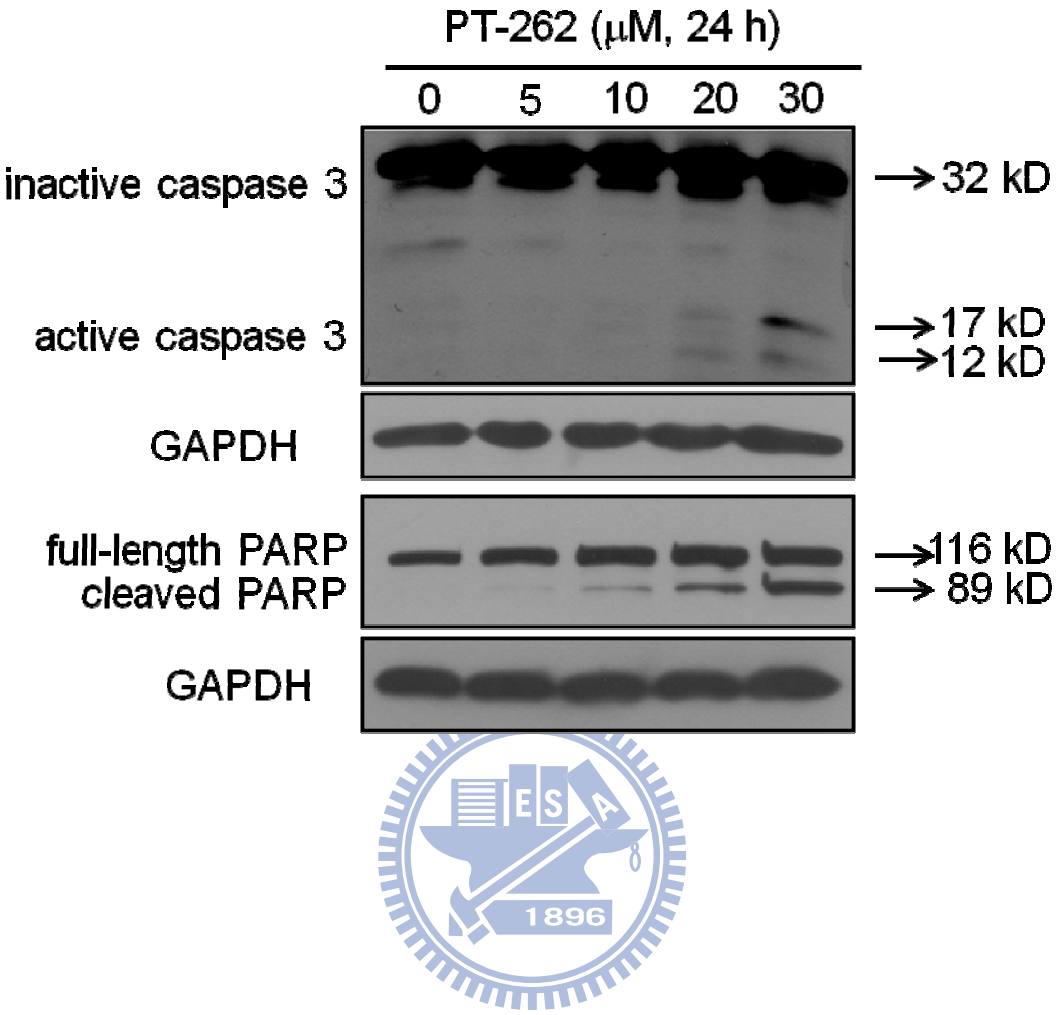

Fig. 4. The effect of PT-262 on the activation of caspase 3 and the cleavage of PARP protein in A549 cells. After treatment with PT-262 (5-30 μM for 24 h), the total cellular protein extracts were collected and subjected to Western blot analysis using anti-caspase-3, anti-PARP and anti-GAPDH antibodies. Representative Western blot results were shown from one of three separate experiments with similar findings.

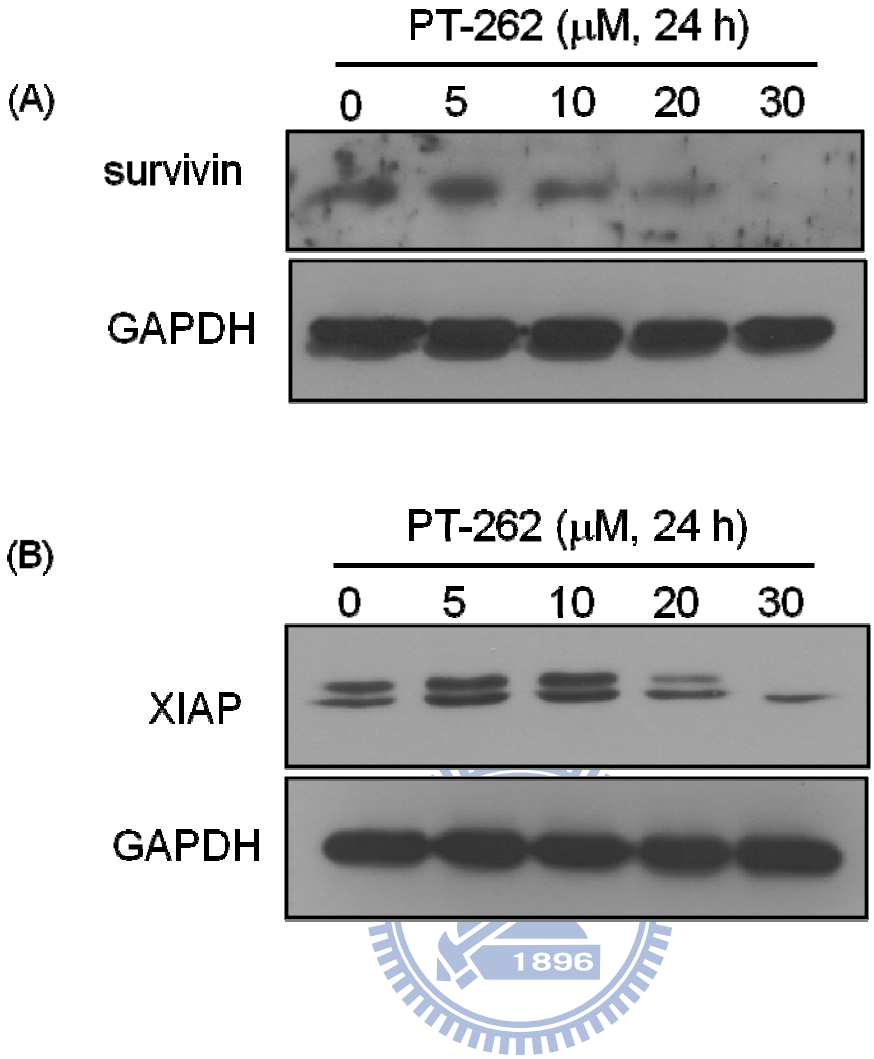

Fig. 5. Effects of PT-262 on survivin and XIAP protein expression. A549 cells were treated with 0 to 30 μM PT-262 for 24 h. The total cellular protein extracts were subjected to Western blot analysis using anti-survivin (A) and anti-XIAP (B) antibodies. GAPDH protein was the internal control. Representative Western blot results were shown from one of three separate experiments with similar findings.

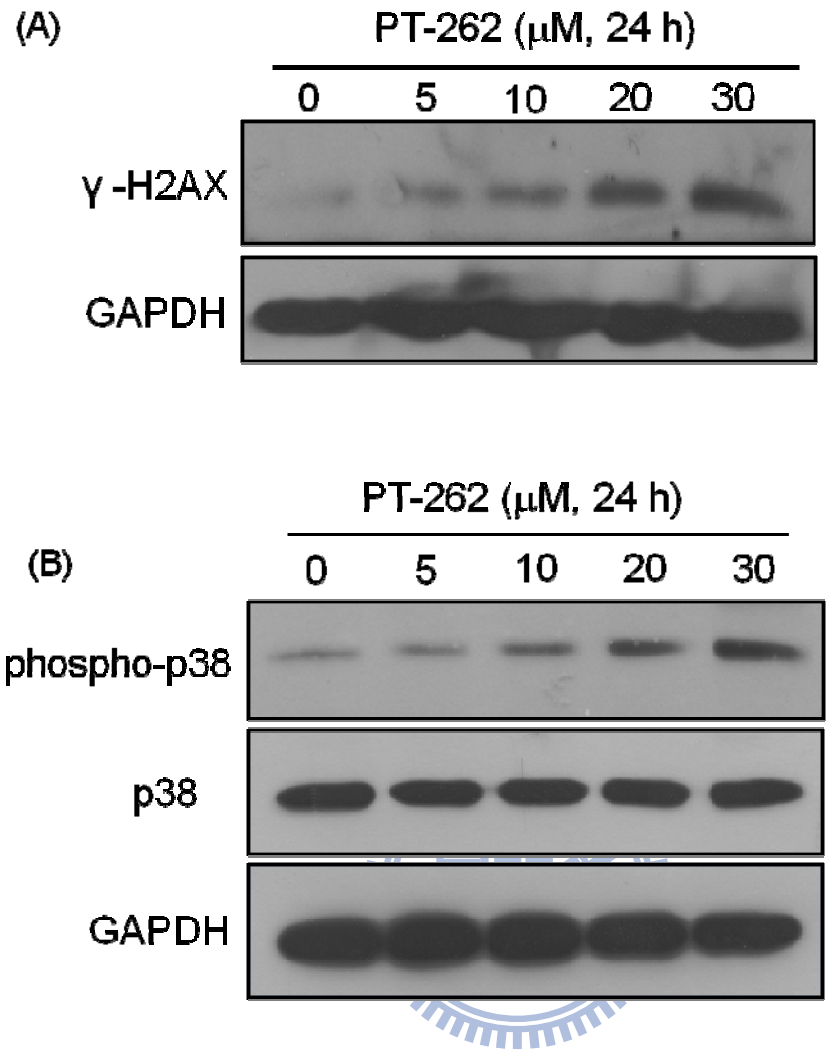

Fig. 6. The effect of PT-262 on the phosphorylation of H2AX and p38 MAP Kinase. A549 cells were treated with 0 to 30 μM PT-262 for 24 h. (A) Anti-phospho-H2AX (Ser139) and anti-GAPDH antibodies were used for Western blot. (B) The total cellular protein extracts were subjected to Western blot analysis using anti-phospho-p38 and anti-p38 where GAPDH was the internal control. Representative Western blot results were shown from one of three separate experiments with similar findings.

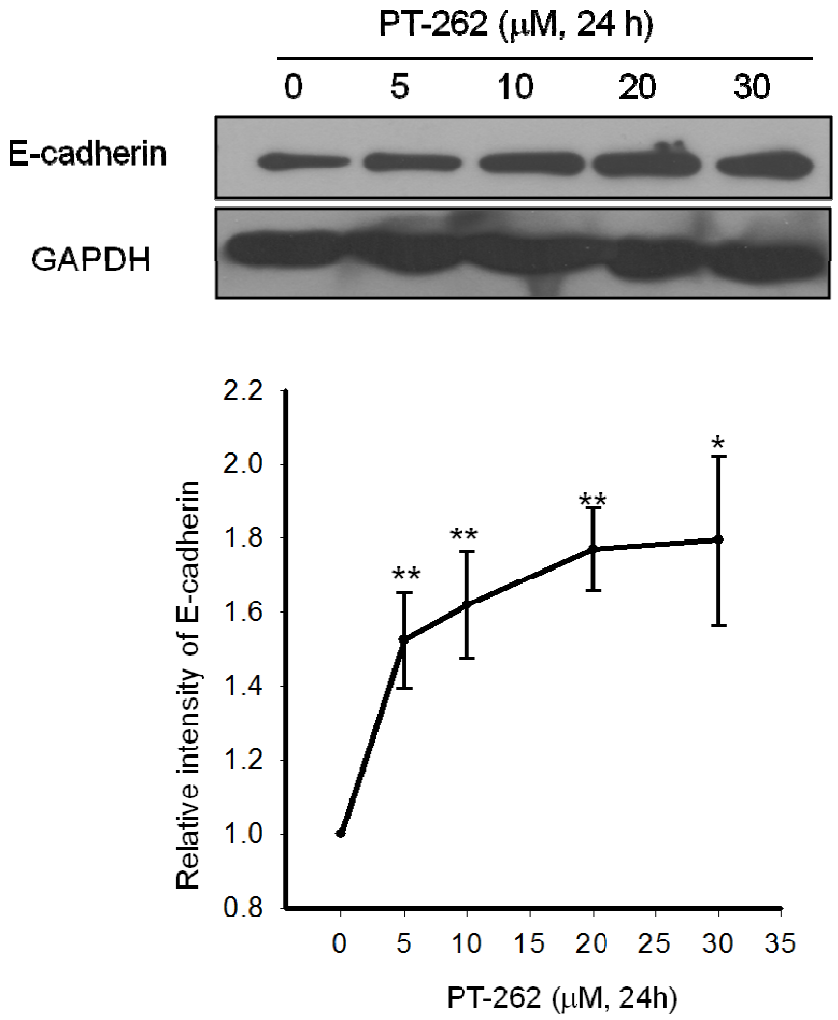

Fig. 7. Effect of PT-262 on E-cadherin protein expression in A549 cells. After PT-262 treatment, total protein extracts were prepared for Western blot analysis using specific anti-E-cadherin and anti-GAPDH antibodies. Representative Western blot data are shown from one of three experiments with similar findings and the bars represented mean ± S.E. $p < 0.05$ (*), indicate significant difference between the control and PT-262 treated samples.

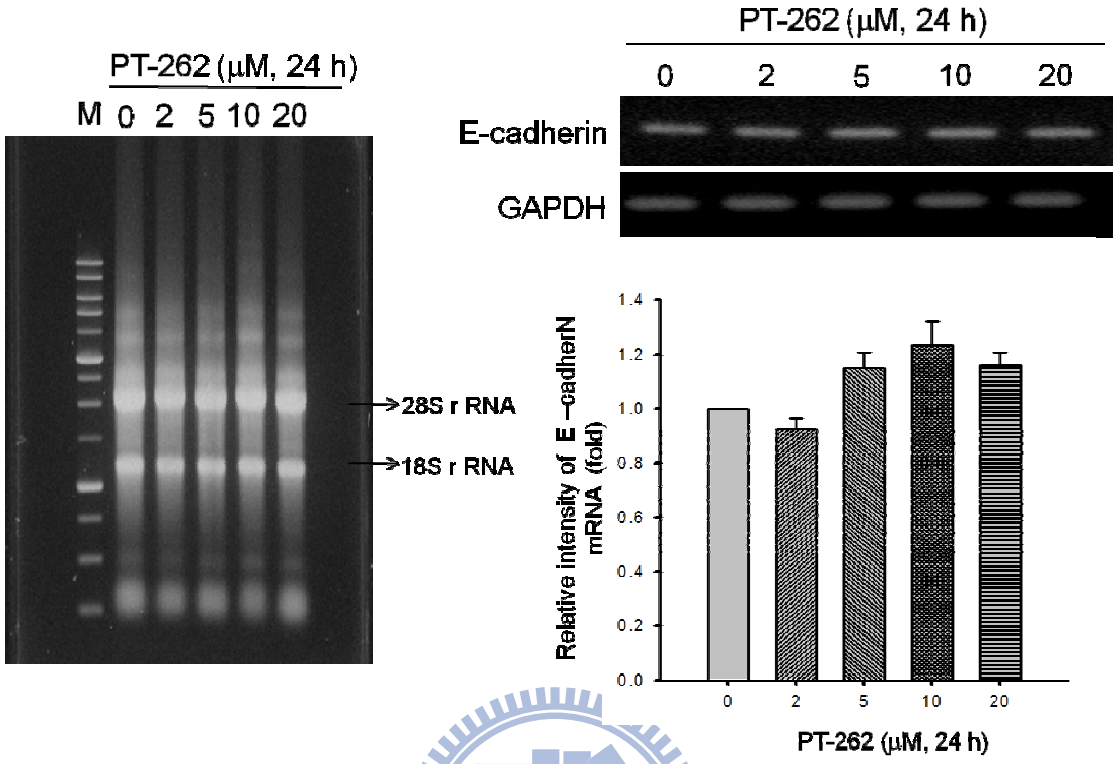

Fig. 8. The effect of PT-262 on E-cadherin gene expression in lung cancer cells. (A) The total RNA was extracted from A549 cells according to the manufacturer's protocol. The bands of 28S and 18S rRNAs were indicated on the gel electrophoresis that was theoretically 2:1 of relative intensity when RNA integrity of the samples was reliable. (B) mRNA expression was analyzed by semi-quantitative reverse transcription-PCR. GAPDH was the internal control. (C) The bands of RT-PCR were quantified by UN-SCAN-IT. Results were from three independent experiments. E-cadherin mRNA expression was normalized to GAPDH and all values were related to control values derived from untreated cells.

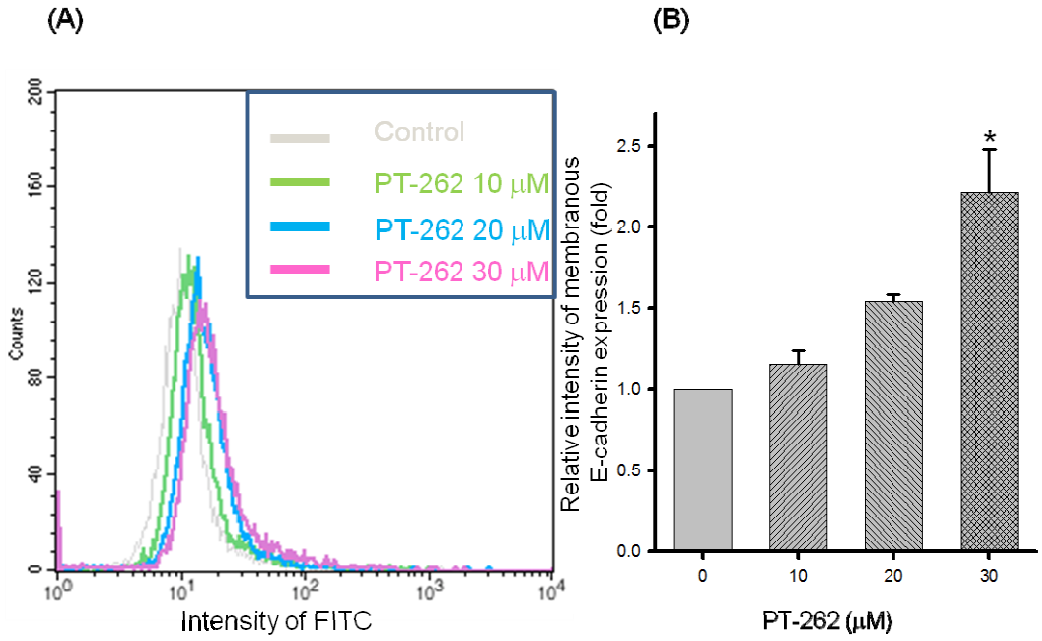

Fig. 9. The effect of PT-262 on the membranous E-cadherin protein level in A549 cells. A representative example of E-cadherin membrane expression in A549 cells. Membranous E-cadherin expression of treated samples were detected as an increased fluorescence intensity of the whole cell population and resulted in peaks shift to the right. The results were obtained from 3 experiments and the bar represents the mean ± S.E. $p < 0.05$ (*) indicates significance between the control and PT-262 treated samples.

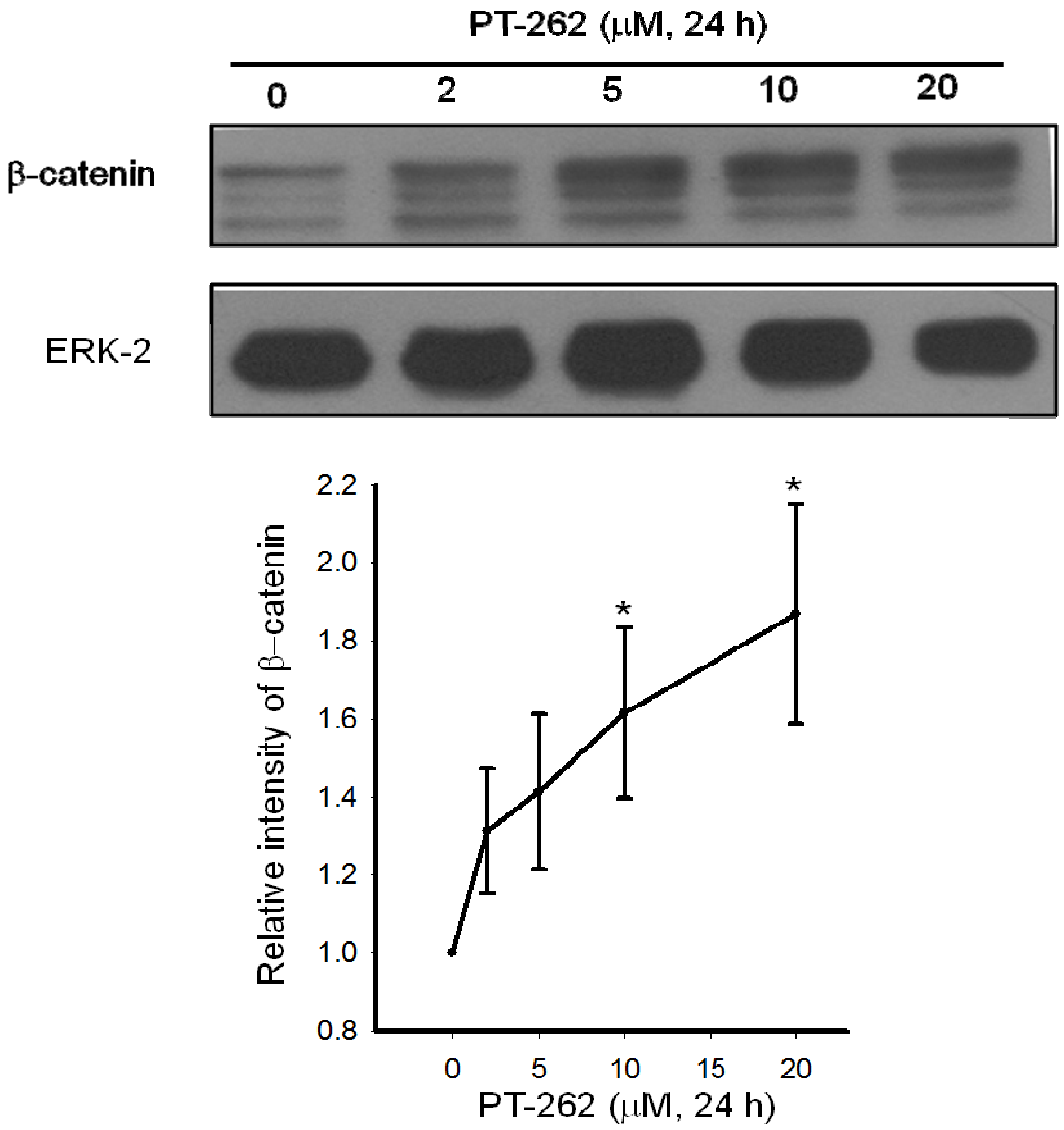

Fig. 10. The effect of PT-262 on the β-catenin protein expression in A549 cells. The total protein extract was collected after 0-30 μM PT-262 treatment for 24 h, and subjected to Western blot analysis by using anti-β-catenin and anti-ERK-2 antibodies The relative protein intensity was quantified Western blot. The results were obtained from 3 experiments and the bar represents the mean ± S.E. $p < 0.05$ *(*)* indicates significant difference between the control and PT-262 treated samples.

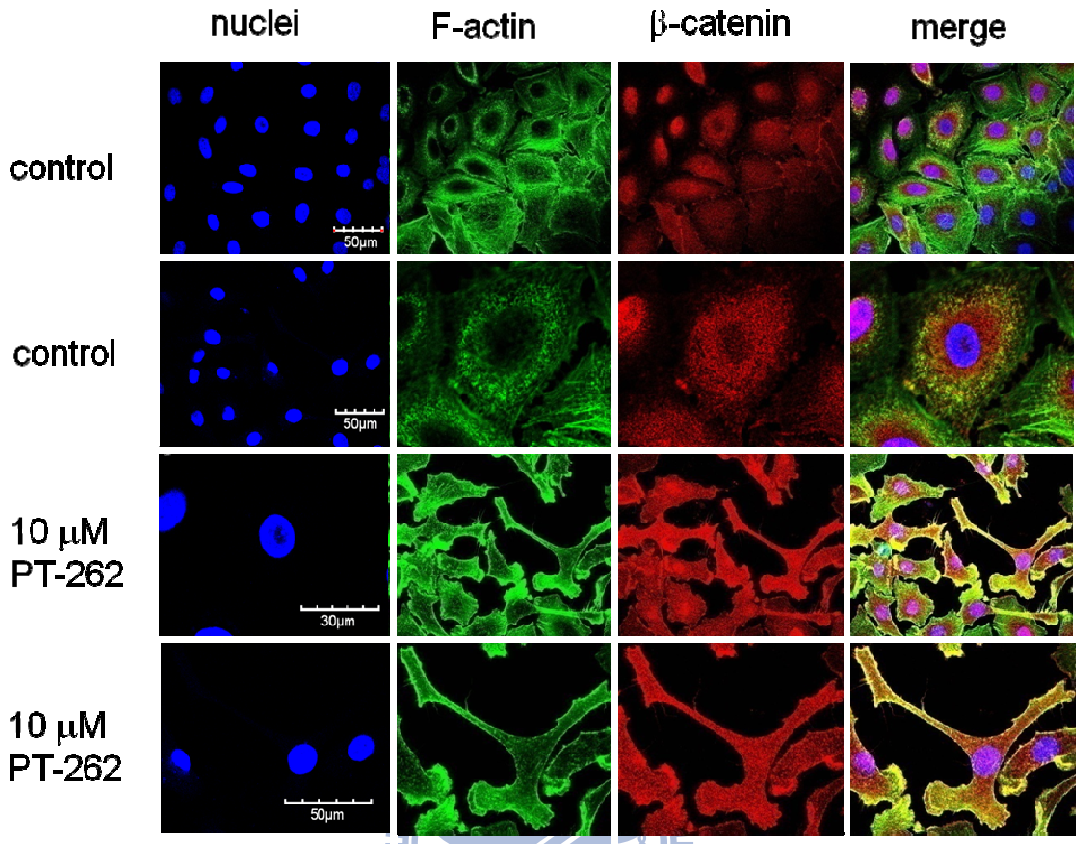

Fig. 11. The effect of PT-262 on the location of β-catenin. A549 cells were treated with or without 10 μM PT-262 for 24 h. The β-catenin was stained with anti-β-catenin-Cy3 which displayed red fluorescence. The actin of the cytoskeleton was stained with phallacidin, which displayed red fluorescence. The nuclei were stained with Hoechst 33258 that displayed blue fluorescence. The yellow color in the merged pictures indicated the co-localization of actin and β-catenin.

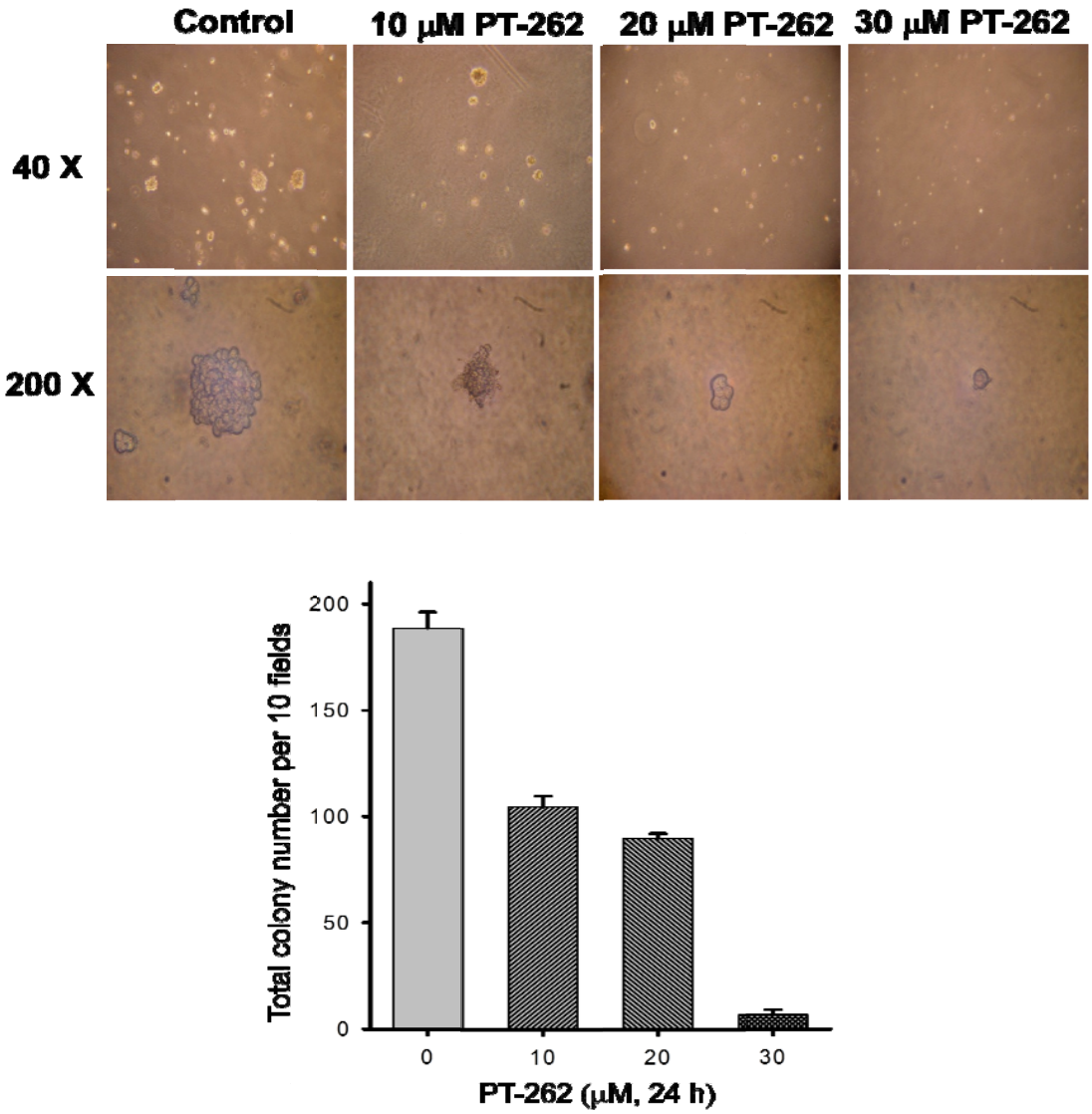

Fig. 12. The effect of PT-262 on the colony formation ability in soft agar. (A) A549 cells were pre-treated with or without PT-262 and then seeded in 0.35 % agarose. Representative phase-contrast photomicrographs (40× and 200× magnification) depicting colony formation of A549 cells in soft agar. The colony numbers were counted after 3 weeks culture. Total soft agar colony counts were done by three independent microscopically visualizing individual colonies (clusters of 10 or more cells) from 10 random microscopic fields. The bars represented mean ± S.E. $p < 0.01$ *(**)*, indicates significant difference between the control and PT-262 treated samples.

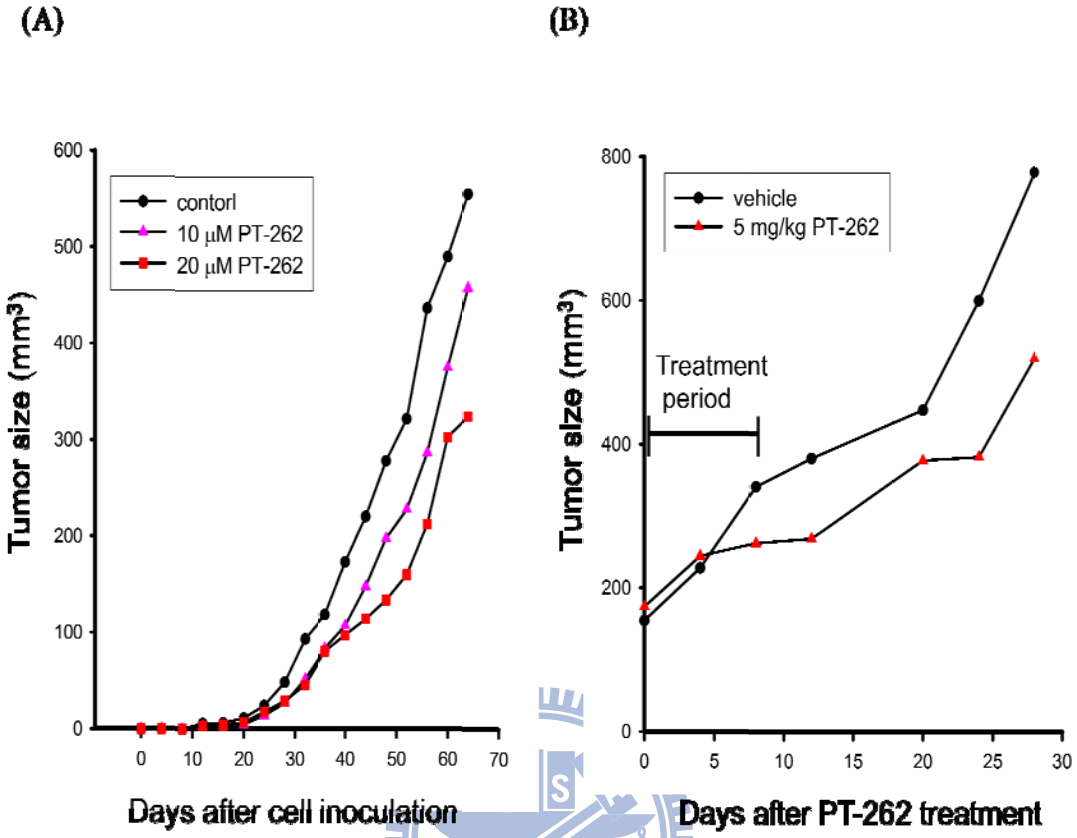

Fig. 13. Inhibition of tumorigenesis by PT-262 in mouse xenograft model. (A) A549 cancer cells were pre-treated with or without PT-262 prior to inoculation into back flank of mice (5 mice for each group). The tumor volume was measured every four days until day 64. The points are the mean tumor volume of 5 mice for each group. (B) A549 human lung cancer cells were s.c. implanted in SCID mice as described in Material and Methods. When the tumor volume had reached above 100 mm^3, the mice bearing A549 xenografts were treated with vehicle control (Tween 80/PBS) or 5 mg/kg of PT-262 intratumorally once every four days starting from day 0 to day 8. The tumor volumes were measured every four days. The points are the mean tumor volume from 3 mice of the PT-262-treated group (triangular points) and 4 of the control group (circular points).

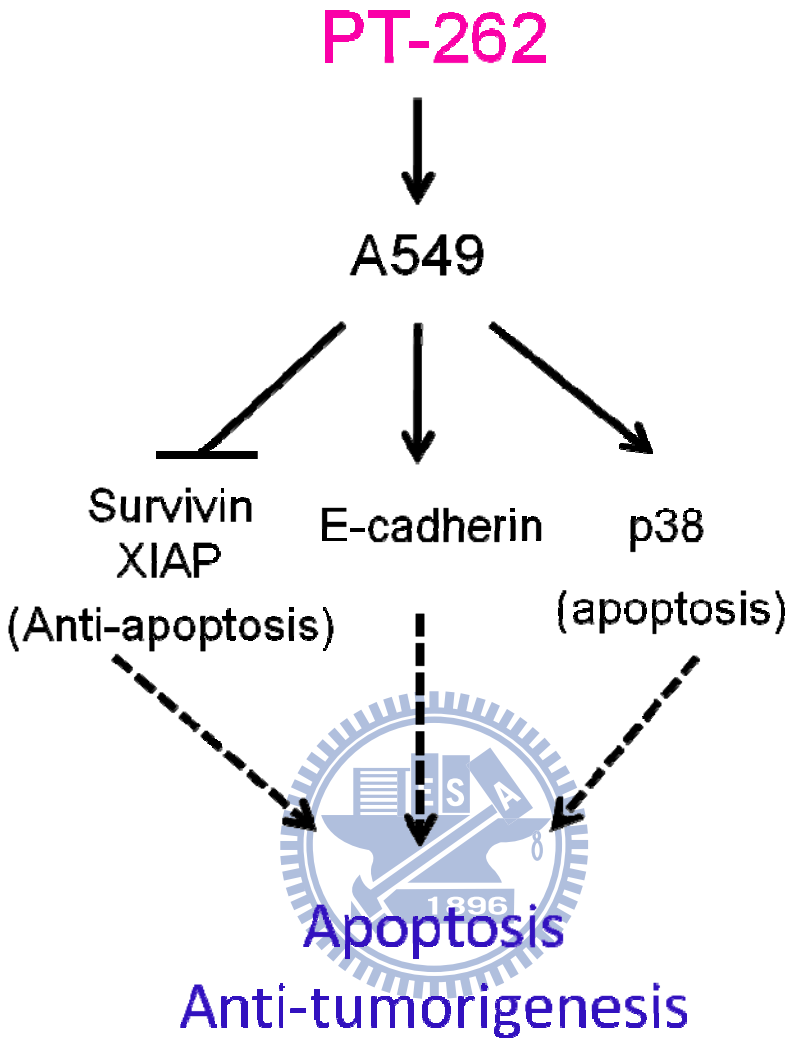

Fig. 14. Proposed model of apoptosis induction and anti-tumorigenesis by PT-262