Effect of 2-(4”-hydroxybenzyl)-5-2”-dihydroxy-3- methoxystilbene on apoptosis and autophagic cell death of lung cancer cells

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ผลของสาร 2-(4”-hydroxybenzyl)-5-2”-dihydroxy-3- methoxystilbene ต่อการตายแบบอะ-พอพโทซิสและการตายแบบออโทฟาจิกของเซลล์มะเร็งปอด

น.ส.สุชารัศม์ ตั้งสุขฤทัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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สุชารัศมิ์ ตั้งสุขฤทัย : ผลของสาร 2-(4”-hydroxybenzyl)-5-2”-dihydroxy-3-methoxystilbene ต่อการตายแบบอะ-พอพโทซิสและการตายแบบออโทฟาจิกของเซลล์มะเร็งปอด. (Effect of 2-(4”-hydroxybenzyl)-5-2”-dihydroxy-3- methoxystilbene on apoptosis and autophagic cell death of lung cancer cells) อ.ที่ปรึกษาหลัก : ศ. ดร.ปิติ จันทร์วรโชติ, อ.ที่ปรึกษาร่วม : ผศ. ดร.อรภัค เรี่ยมทอง

การเปลี่ยนแปลงของออโทฟาจี ซึ่งเป็นกระบวนการแคแทบอลิซึมเพื่อทำให้เซลล์เกิดความอยู่รอด สามารถทำให้เซลล์เกิดการตายได้ ซึ่งอาจเป็นรูปแบบหนึ่งของการออกฤทธิ์ของยาต้านมะเร็ง นอกจากนี้การตายแบบออโทฟาจิก เป็นกลไกการตายทางเลือกในเซลล์มะเร็งที่ดื้อยา การศึกษานี้แสดงให้เห็นว่า PE5 ซึ่งเป็นสารประกอบสทิลบีนแสดงฤทธิ์ให้เกิดการตายโดยออโทฟาจิกและการตายแบบอะพอพโทซิสในเซลล์มะเร็งปอด ซึ่งอาจเป็นโอกาสในการรักษามะเร็งแบบใหม่ได้ พบว่า PE5 มีฤทธิ์เป็นพิษต่อเซลล์มะเร็งสูงกว่าเมื่อเทียบกับผลต่อเซลล์ปกติ ที่น่าสนใจคือพบว่าการตายของเซลล์ที่เกิดจาก PE5 นั้นเกิดขึ้นพร้อมกันกับการเหนี่ยวนำอย่างมากให้เกิด การย้อมติดสีสภาวะเป็นกรดของเซลล์ ร่วมด้วยกับการพบ ออโทฟาโกโซม และการแปลงของระดับ LC3 ซึ่งได้การยืนยันเพิ่มเติมว่าการเหนี่ยวนำการตายหลักที่เกิดจาก PE5 นั้นเกิดจากการตายแบบออโทฟาจิก เนื่องจากการรักษาร่วมกับตัวยับยั้งของออโทฟาจีสามารถย้อนกลับการตายของเซลล์ได้ นอกจากนี้กลไกการออกฤทธิ์ที่กำหนดสัญญาณกำกับดูแลถูกระบุโดยใช้การวิเคราะห์โปรตีโอมิก การวิเคราะห์โปรตีโอมิกขึ้นอยู่กับเวลาพบว่า PE5 มีผลต่อโปรตีน 2,142 และ 1,996 หลังการรักษา 12 และ 24 ชั่วโมงตามลำดับ ในจำนวนนี้เครือข่าย ซึ่งประกอบด้วยโปรตีน 128 ชนิดที่ควบคุมการตายของเซลล์แบบอะพอพโทซิส และ 25 ชนิดที่เกี่ยวข้องกับออโทฟาจี การวิเคราะห์ปฏิสัมพันธ์ระหว่างโปรตีนกับโปรตีนระบุเพิ่มเติมว่าการเหนี่ยวนำให้เกิดการตายแบบออโทฟาจิกเกิดจากการยับยั้งการทำงานของ AKT/mTOR และ Bcl-2 และการวิเคราะห์โปรตีนด้วย western blot ยืนยันว่ารูปที่ใช้งานของ AKT, mTOR และ Bcl-2 ลดลงอย่างมีนัยสำคัญในเซลล์ที่ได้รับ PE5เมื่อนำผลมารวมกันเราได้แสดงให้เห็นถึงกลไกใหม่ของ PE5 ในการเปลี่ยนออโทฟาจีไปสู่การเหนี่ยวนำการตายของเซลล์ โดยกำหนดเป้าหมายการยับยั้ง AKT/mTOR และ Bcl-2

สาขาวิชา เภสัชวิทยา ลายมือชื่อนิสิต .....

ปีการศึกษา 2563 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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KEYWORD: lung cancer, autophagy, Apoptosis, Akt, mTOR, Bcl-2, proteomic, autophagic cell death

Sucharat Tungsukruthai : Effect of 2-(4”-hydroxybenzyl)-5-2”-dihydroxy-3-methoxystilbene on apoptosis and autophagic cell death of lung cancer cells. Advisor: Prof. PITHI CHANVORACHOTE, Ph.D. Co-advisor: Asst. Prof. Onrapak Reamtong, Ph.D.

The shifting of autophagy, a catabolic process for cell survival, toward death could be a potential mode of anti-cancer drug action. In addition, autophagic cell death (ACD) is an alternative death mechanism in resistant malignant cancer cells. This study, we demonstrated how PE5, a stilbene compound, exhibits potent ACD-promoting activity and apoptosis in lung cancer cells that may offer an opportunity for novel cancer treatment. It was found that PE5 exerted a higher toxic effect toward cancer cells compared with its effect on normal cells. Interestingly, the cell death caused by PE5 was found to be concomitant with dramatic autophagy induction as indicated by acidic vesicle staining, autophagosome and by the LC3 conversion. We further confirmed that the main death induction caused by PE5 was via ACD, since the co-treatment with an autophagy inhibitor could reverse PE5-mediated cell death. Furthermore, the defined mechanism of action and upstream regulatory signals were identified using proteomic analysis. Time-dependent proteomic analysis showed that PE5 affected 2,142 and 1,996 proteins after 12 and 24 h of treatment, respectively. Among these, the crosstalk network comprising 128 proteins that control apoptosis and 25 proteins involved in autophagy was identified. Protein–protein interaction analysis further indicated that the induction of ACD was via AKT/mTOR and Bcl-2 suppression. Western blot analysis confirmed that the active forms of AKT, mTOR and Bcl-2 were significantly decreased in PE5-treated cells. Taken together, we demonstrated the novel mechanism of PE5 in shifting autophagy toward cell death induction by targeting AKT/mTOR and Bcl-2 suppression.

Field of Study:	Pharmacology	Student's Signature .....
Academic Year:	2020	Advisor's Signature .....
		Co-advisor's Signature .....

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Sucharat Tungsukruthai

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1CHAPTER I

INTRODUCTION

1.1 Background and rationale

Lung cancer is the leading cause of morbidity and mortality worldwide [1]. There are several therapies available for lung cancer treatment, including surgery, radiotherapy, and chemotherapy [2]. However, high mortality and low 5-year survival rates have been found due to drug resistance [3,4]. Interestingly, previous research revealed that conventional chemotherapy is unable to induce apoptotic cell death in 60% of NSCLC patients, leading to major hurdles to achieving a positive clinical outcome [5]. Therefore, it is important to develop and find new compounds that can induce apoptotic-independent cell death with only a minor incidence of resistance. Despite the role of autophagy as a catabolic process that enhances cell survival through the recycling of bioenergetics and basic cellular units and by the elimination of damaged organelles and unwanted proteins [6,7], several lines of evidence suggest that autophagy is also somehow critical for cell death, especially when the apoptosis mechanism is inhibited or defective [8]. Autophagic cell death (ACD) has also been shown to be important for cell death in cancer cells that are resistant to chemotherapies [6,9-11]. Certain drugs or compounds that trigger autophagy are believed to be beneficial for killing cancer cells or for improving the response to conventional drugs [12,13].

Autophagy is induced by phosphatidylinositol 3-kinase (PI3K) type III, which interacts and forms a complex with Atg6 (Beclin1), VPS15, VPS34, and Atg14L [14]. Class III PI3Kpromotes activation of the phagophore [15], which then expands via the functions of the LC3/GABARAP and Atg5-Atg12 pathways, resulting in the formation of autophagosomes. LC3s is important for autophagosome formation because a cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine (PE) to form LC3-II, which is recruited to autophagosomal membranes [16]. Importantly, autophagy is strictly controlled by the protein kinase B (AKT)/mammalian target of rapamycin (mTOR) axis, which is responsible for a variety of cellular signals in nutrient sensing, protein synthesis, survival, and cell growth [17]. The inhibition of mTOR by stress or by the deprivation of nutrients has been evidenced to induce autophagosome formation. The well-known classic mTOR inhibitor rapamycin (Sirolimus) has been widely used in studies aiming at autophagy induction [18,19]. Besides, rapamycin and other autophagic inducers were shown to exert beneficial effects in a preclinical evaluation for aging and increasing lifespan [20]. Unlike for classical autophagy, the regulatory mechanism of ACD is largely unknown. Recent information has suggested that ACD and apoptosis are interconnected through many nodes of signal crosstalk, leading to coordinated cellular component degradation and cell death [21]. One important central node of crosstalk between apoptosis and autophagy is the Bcl-2 protein. Bcl-2 has been long known to play an important role in the inhibition of apoptosis at the mitochondria [22] and its functions in regulating autophagy are

recently becoming clearer. The anti-apoptosis activity of Bcl-2 is based on its ability to antagonize pro-apoptotic members of the Bcl-2 family. Likewise, Beclin 1, a Bcl-2 homology 3 (BH3) domain-only protein [23] that functions as a crucial initiator of autophagosome formation, is known to interact with Bcl-2 [24] and such a protein interaction prevents Beclin 1 from carrying out its function [25]. A previous study demonstrated that the suppression of Bcl-2 by siRNA knockdown could induce ACD but not apoptosis in breast cancer cells [21], so the induction of autophagy together with Bcl-2 suppression could offer a better option for cell death induction for cancer treatment.

Numerous bioactive phytochemicals, such as stilbenoids and flavonoids, have been discovered in orchids [26,27], including 2-(4''-hydroxybenzyl)-5-2''-dihydroxy-3-methoxystilbene (PE5), which is a natural compound isolated from the roots of *Phragmipedium sp*. [28]. Previous studies have revealed that compounds in stilbenoids, such as resveratrol, have potential anti-cancer effects [29-31]. Other stilbenes, such as piceatannol, combretastatin A-4, and pterostilbene, have various pharmacological effects against various type of cancers [31-33]. With an aim to investigate the activity of PE5 on ACD induction in lung cancer cells and its regulatory profiles involving the crosstalk between apoptosis and ACD, we utilized molecular pharmacological and proteomic approaches to unravel the full profile of the molecular regulations that

could help us gain a better understanding of ACD control in lung cancer as well as the development of a treatment compound for cancer therapy.

1.2 Objective

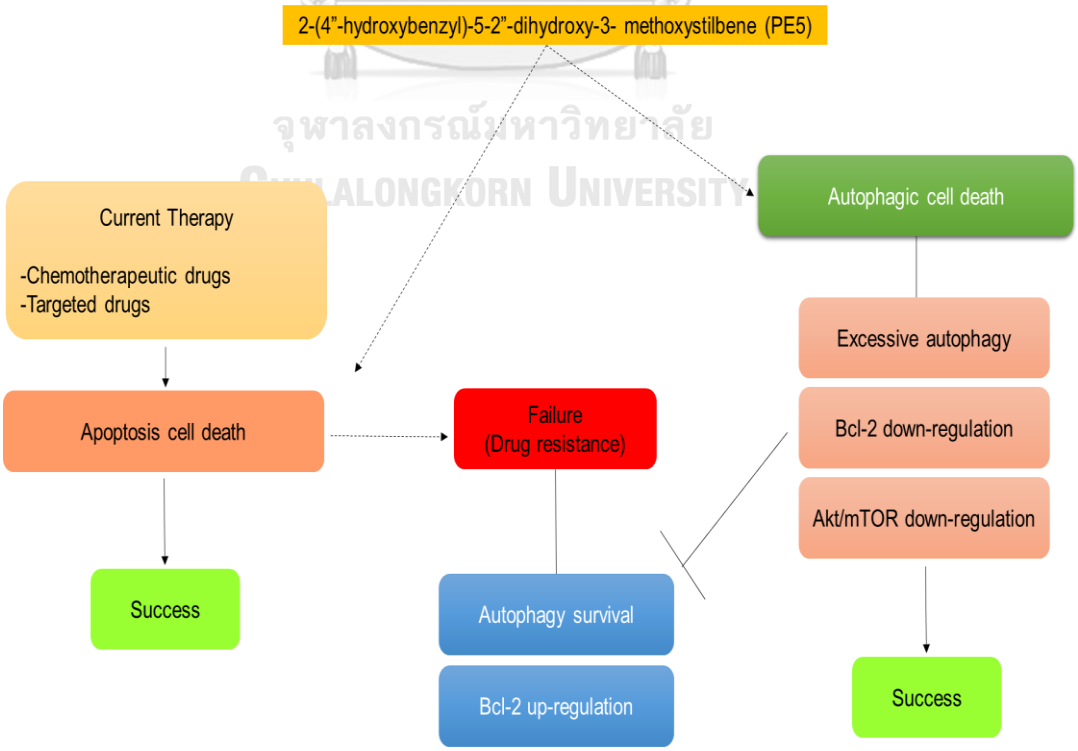
1.2.1 The purpose of this study is to investigate the effect of PE5 on apoptosis and autophagic cell death of lung cancer in vitro.

1.2.2 To elucidate the underlying mechanism(s) of apoptosis and autophagic cell death effects caused by PE5 on lung cancer in vitro.

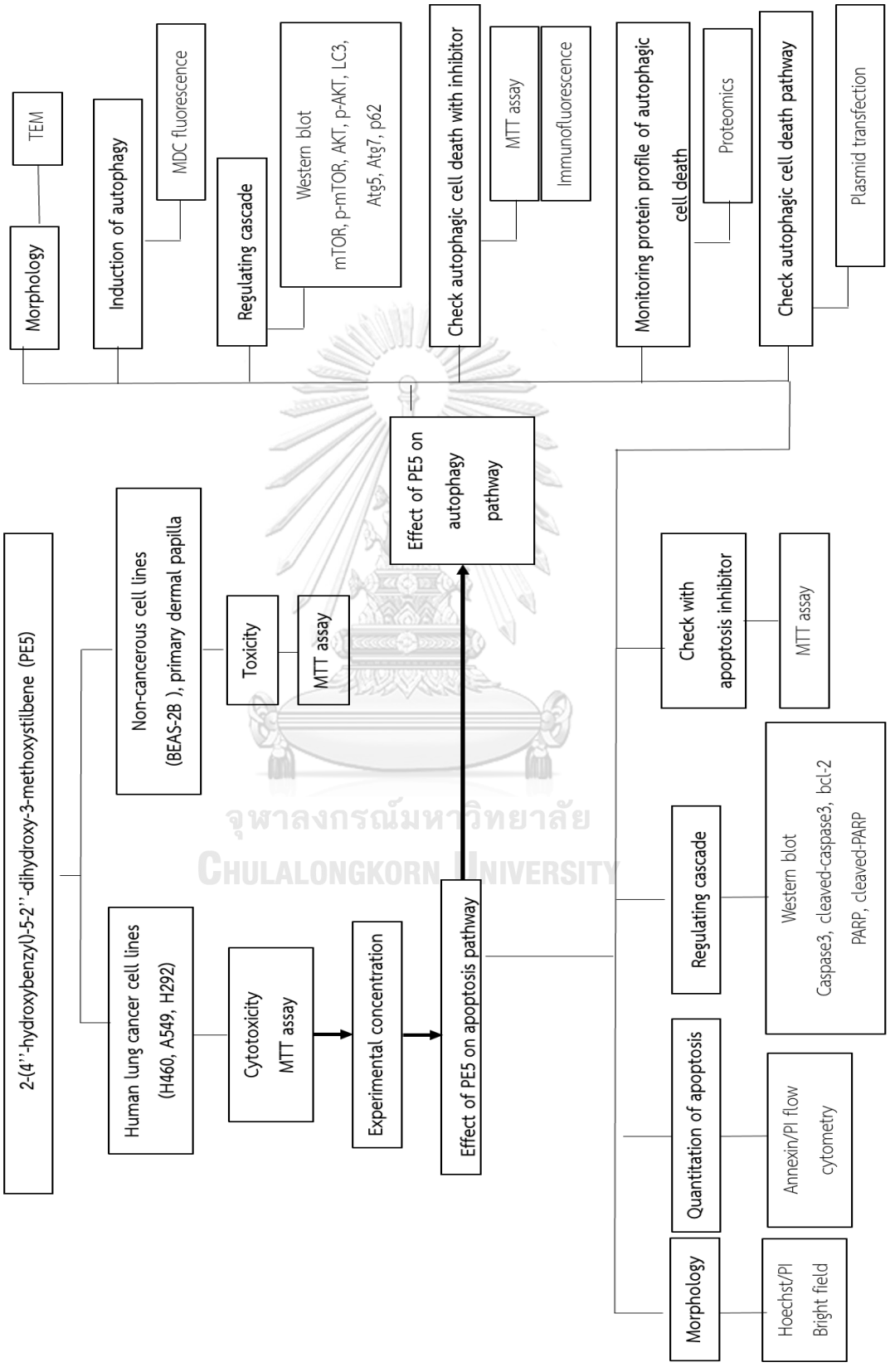
1.3 Hypothesis

PE5 might be able to be induce autophagic cell death and apoptosis by inhibiting AKT/mTOR and Bcl-2 pathway in lung cancer in vitro.

1.4 Conceptual framework



1.5 Research design



CHAPTER II
LITERATURE REVIEWS

2.1 Lung cancer

Lung cancer is a common cause of cancer deaths in the world [34]. In 2018, 234,030 estimated new lung and bronchus cancer cases will be diagnosed; both the incidence of males (121,680 cases) and females (112,350 cases) are the second highest among all cancer types. Most patients with lung cancer have one or more of these symptoms such as; chest discomfort or pain, coughing, trouble breathing, wheezing, blood in sputum (mucus coughed up from the lungs), or hoarseness [35].

2.1.1 Type of lung cancer

Human lung cancers are classified into two major types, small cell lung cancers (SCLC) and non-small cell lung cancers (NSCLC) [34]. Non-small cell lung cancers (NSCLC) represent 75–80% of all lung carcinomas and have an overall 5-year survival rate of patients only 15% [36]. Non-small cell lung cancers (NSCLC) include adenocarcinoma (gland-forming), squamous cell carcinoma and large-cell carcinoma histosubtypes.

2.1.2 Risk factor

Several risk factors of lung cancer such as cigarette smoking, air pollution, and occupational exposure and lung cancer susceptibility genes are associated with the development of lung cancer.

- Cigarette smoking

Cigarette use causes 90% of all lung cancers. In a cohort study of more than 5000 lung cancer patients diagnosed between 1997 and 2002, only 25% were current smokers and more than 60% were former smokers [37]. Interestingly, secondhand smoke or passive smoking is also a significant risk factor of lung cancer. Moreover, children who lived with a smoker have an increased risk of developing lung cancer in adulthood [38].

- Air pollution and Occupational exposure

Exposure to surrounding air pollution can increase the risk of lung cancer. In 2006, it was found that 10.7% of incidents of lung cancer in Europe were related to urban air pollution [39]. Moreover, carcinogens present in indoor air such as radon are responsible for 4.5% of lung cancers [40]. In addition, many workers exposed to carcinogens including crystalline silica and chrysotile asbestos tend to have an increased risk of lung and other cancers [41].

- Lung cancer susceptibility genes

Not only exposure to environmental carcinogens, but also intrinsic factors such as lung cancer susceptibility genes are determined as major factors involved in the pathogenesis of lung cancer. Previous research found that people who have a mutation at p53 are more likely to develop lung cancer [42]. Furthermore, a mutation

in the epidermal growth factor receptor (EGFR) is responsible for 20% of lung adenocarcinomas [43].

2.1.3 Stages of lung cancer

The stage of the lung cancer at the time of diagnosis is an important predictor of survival and treatment options using TNM system, which is based on 3 key pieces of information: the size of the tumor (T), whether the cancer has reached nearby lymph nodes (N) and whether the cancer has metastasized (spread to other parts of the body) (M) [44]. However, the TNM classification appropriated for use in the classification of non-small-cell lung cancers (NSCLC) is used less in the classification of small-cell lung cancer (SCLC) [45]. In the early stages of lung cancer, the tumor is smaller and the cancer has not spread outside the lung, referred to as a carcinoma in situ. Then, the tumor gradually increases in size and begins to metastasize to other organs in the following stages as figure 1 [46].

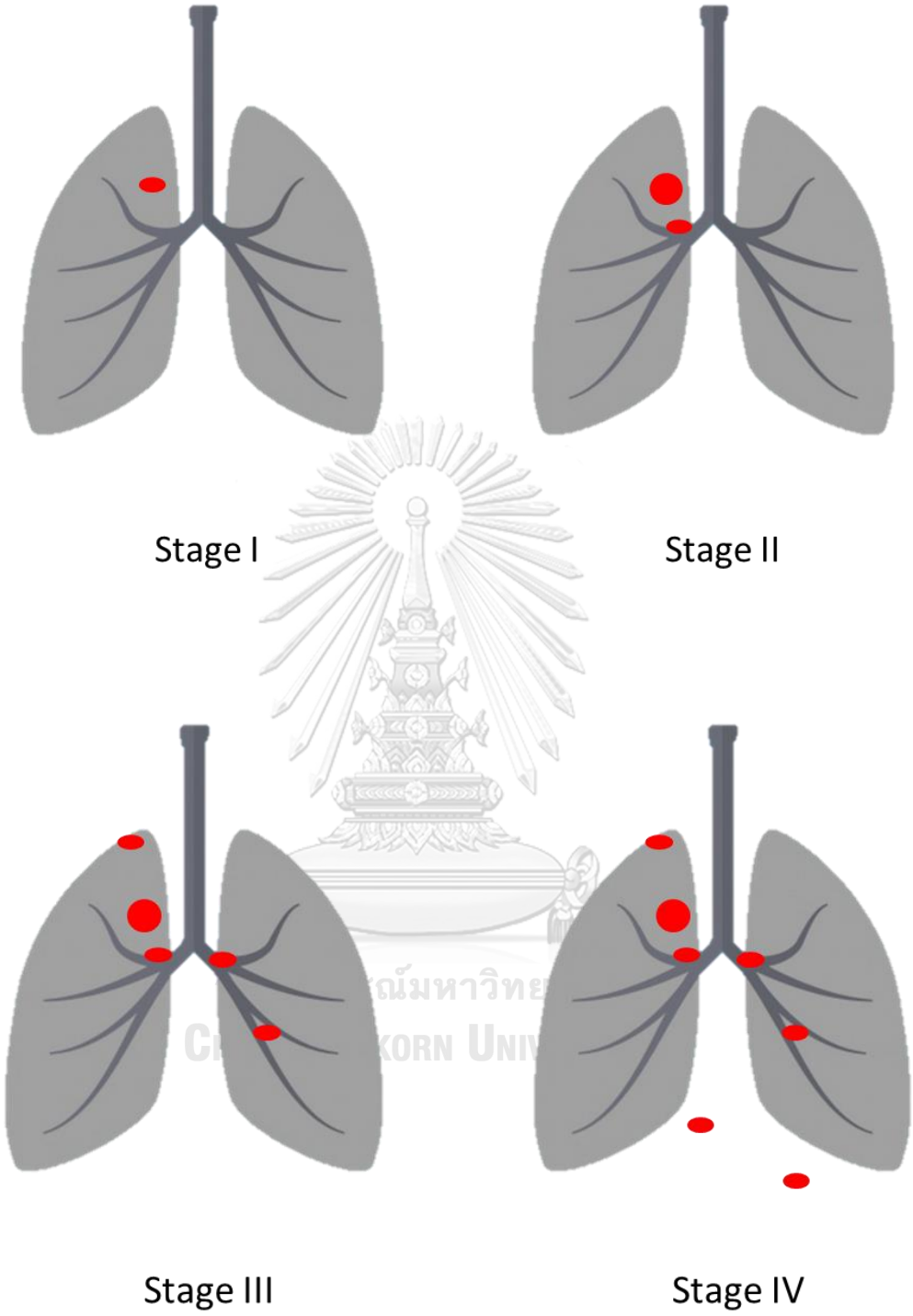


Figure 1 Stages of lung cancer (Sucharat, 2020)

2.1.4 Symptoms of lung cancer

The Symptoms experienced by patients with lung cancer can be caused by the primary tumor and include coughing and hemoptysis, whereas symptoms such as Horner syndrome and superior vena cava obstruction can be caused by intrathoracic spread. Moreover, tumors at distant metastases sites can cause bone pain [47]. Other predictors of lung cancer are weight loss, fatigue, dyspnea and chest or rib pain. Patients of lung cancer usually present more than two symptoms. For example, previous studies have determined that 9. 2% of patients who display the combination of weight loss and hemoptysis develop lung cancer [48].

2.1.5 Treatment for lung cancer

There are many ways to cure lung cancer patients including surgery, radiation therapy, targeted therapy and chemotherapy. Firstly, surgical resection ruins the most regular and successful option, but it still fitting for patients with stage I or II with no evidence of lymph node spread [49]. Secondly, radiation therapy is commonly used in stage III infiltrative NSCLC , is known as intrathoracic cancer [50]. In addition, the first and second methods are usually used with chemotherapy or targeted therapy drugs to promote higher rates of survival than the alone method. However, many studies have revealed that using chemotherapy such as cisplatin, paclitaxel and etoposide result in significant increases in hematologic toxicity, nephrotoxicity, and nausea and vomiting [51,52].

2.2 Program cell death

Cancer is a genetic disease which can be caused by gene mutation in genes such as oncogenes and tumor suppressor genes. Cancer can occur as a result of alterations in various signaling pathways and numerous studies have reported that its development is associated with program cell death [53]. Programmed cell death (PCD) plays important roles many biological events such as morphogenesis, the maintenance of tissue homeostasis, and the elimination of harmful cells. PCD is divided into 3 forms: apoptosis, autophagy and programmed necrosis [54]. However, the main programs that are associated with the treatment of cancer are apoptosis and autophagy [55]. Hence, it is important to concentrate on these two programs.

2.2.1 Apoptosis

Apoptosis is an effective program that eliminates undesirable cells, senescent cells and genetically damage cells. It is characterized by the blebbing of cell membrane, cell shrinkage and the condensation of DNA [56]. This program is called the suicide program and can have a slight effect on surrounding cells with no inflammation. In addition, apoptosis has been classified into 2 types: intrinsic pathway and extrinsic pathway as shown in figure 2.

Briefly, intrinsic pathway or mitochondria-mediated pathway can be altered by varied receptor-independent stimuli such as free radical, ultra violet, chemotherapeutic and hypoxia. After that, the inner mitochondrial membrane permeability is changed and results in the creation of mitochondrial permeability

transition (MPT) pore, leading to the release of group of proteins. The group of proteins comprises pro-apoptotic proteins such as cytochrome c (cyt c) and Smac/DIABLO (second mitochondria-derived activator of caspases). Cytochrome c recruits Apaf-1 induces oligomerization and then activates pro-caspase9 to form apoptosome which triggers caspase 9/3 signaling cascade (caspase dependent pathway) [57].

As for the extrinsic or receptor-mediated pathway, external stimulation is required; this occurs via a death receptor (DR) family member, such as tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) receptor 1 (TRAILR1, also known as DR4 and TNFRSF10A), TRAILR2 (also known as DR5 and TNFRSF10B), FAS (also known as CD95 and APO1) or TNF receptor 1 (TNFR1, also known as TNFRSF1A), located at the plasma membrane. After ligands bind to death receptors, the death signal translocates into the cell, leading to the recruitment of a death-inducing signaling complex (DISC). DISC can trigger procaspase-8, activated caspase 8 and stimulate effector caspase (caspase 3), resulting in the inducement of mitochondrial damage [58].

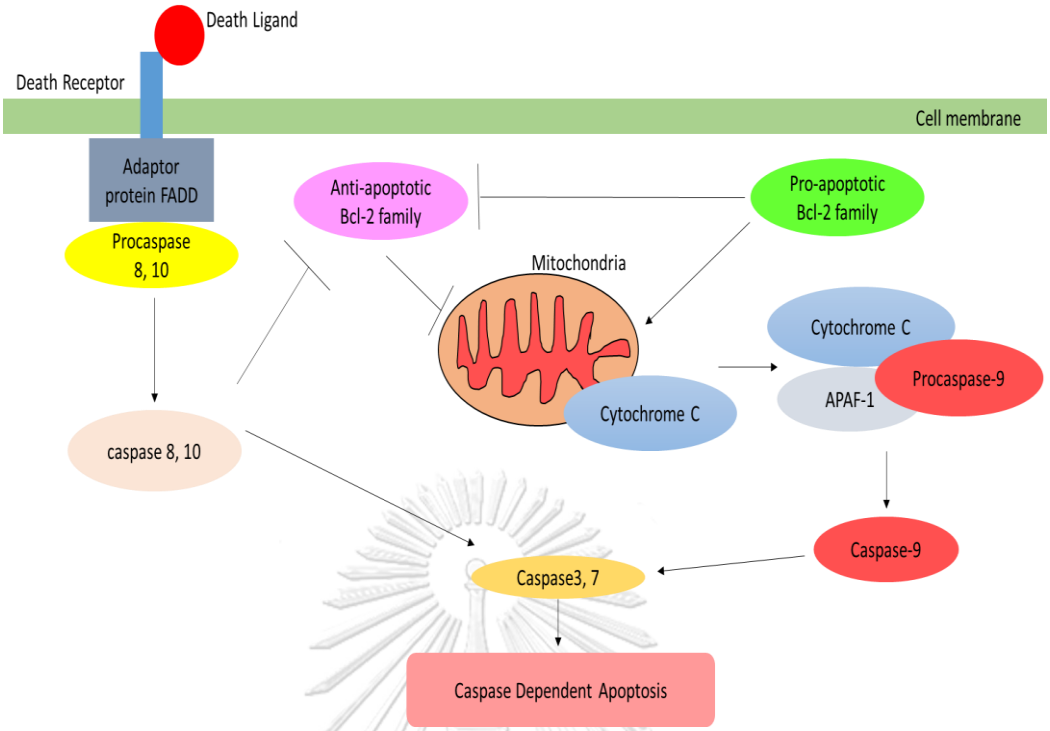


Figure 2 Apoptosis pathway (Sucharat, 2020)

2.2.2 Autophagy

Autophagy is a process that has an important role in the turnover of macromolecule and cellular proteins, such as mitochondria, and misfolded protein via the lysosomal degradative mechanism. Normally, autophagy aims to recycle long-lived proteins by forming double membrane autophagosome which engulfs protein and then fuses it with lysosome, where upon the sequestered material is denatured because of the acidic environment [59]. Based on its mechanisms, autophagy has been classified into three types: macroautophagy, microautophagy, and chaperone mediated autophagy (CMA) [60] (Figure 3).

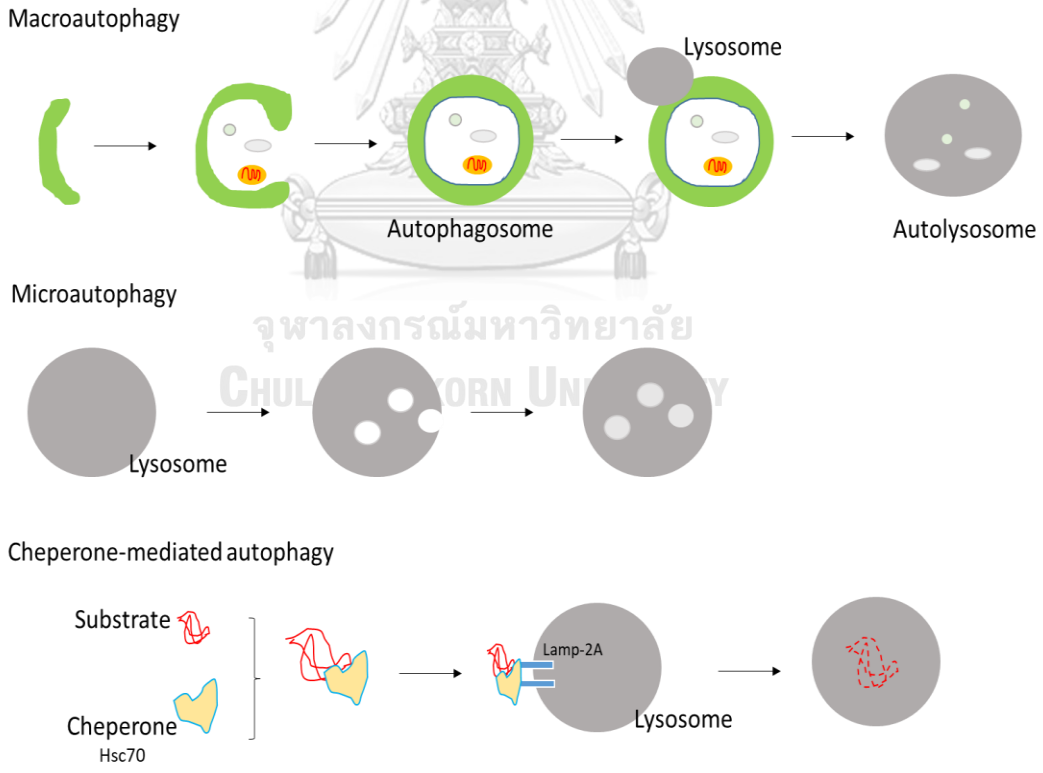


Figure 3 Classification of autophagy (Sucharat, 2020)

The molecular basis of autophagy has been investigated mainly in yeast and mammals. Briefly, when cell have conditions including hypoxia, ER stress and nutrient deprivation, PI3K class 3 mediates nucleation of the phagophore membrane which engulfs cytosolic proteins or organelles. In this step, Bcl-2 (anti-apoptotic protein) inhibits Beclin 1, which is a component in PtdIns3K complex that has a role in the nucleation step. Then, Atg12–Atg5-Atg16, Atg8– phosphatidylethanolamine (PE) conjugates are recruited to the phagophore for inducing phagophore in the expansion step. In addition, in this step, a cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine to form LC3-phosphatidylethanolamine (PE) conjugate (LC3-II), which is recruited to autophagosomal membranes. The next step is called maturation (vesicle completion) , most of the ATG are separated from autophagosome. In the last step, autophagosome fuses with lysosomes to form autolysosomes, and all components and LC3-II in autolysosomal lumen are degraded by lysosomal hydrolases [61] (Figure 4).

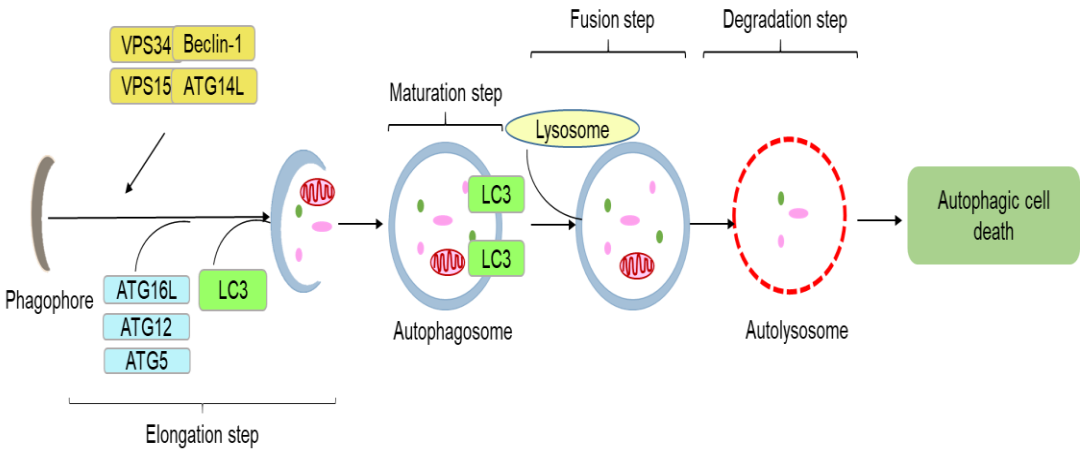


Figure 4 Autophagy pathway (Sucharat, 2020)

Interestingly, autophagy usually acts as a survival mechanism for cancer cells, but it can also act like apoptosis and induce cancer cell death, is termed autophagic cell death [62]. Previous studies have revealed that defective apoptosis cells can undergo autophagic cell death. Moreover, autophagic cell death can occur in cells that are treated with caspase inhibitor [63,64]. Additionally, apoptotic cells quickly show degradation of the cytoskeleton, but preserve organelles until late in the process, whereas autophagic cell death is associated with the accumulation of large numbers of autophagic vesicles, which degrade many organelles early in the process, while the cytoskeleton remains undamaged and functional until late in the process [65].

2.2.3 Crosstalk between autophagy and apoptosis

Numerous studies report that apoptosis and autophagy pathways are linked and share some common molecular signaling pathways [66,67]. The major pathway that mediates between apoptosis and autophagy is PI3K/AKT/mTOR pathway.

The phosphoinositide-3-kinase (PI3K) signaling pathway has a critical role in cell growth and survival. Previous studies have determined that PI3K pathway is activated by receptors tyrosine kinase (RTKs) such as EGFR [68] which commonly cause dysregulation in lung cancer patients [69]. Subsequently, the serine threonine kinase Akt is activated by transduces intracellular signaling. The activated Akt activates downstream targets such as mTOR (Mammalian target of rapamycin) via phosphorylation at serine 473. In addition, mTORC1 is a major negative regulator of autophagy [70], as shown in figure 5. Importantly, the activation of AKT not only effects the autophagy pathway via mTOR pathway but also leads to the inhibition of the apoptosis process by phosphorylating the B-cell CLL/lymphoma (Bcl-2) family BAD [71]. Moreover, Akt also reduces the activity of caspase 9, which is involved in apoptosis pathway [72]. Hence, this effect could promote cell survival [73].

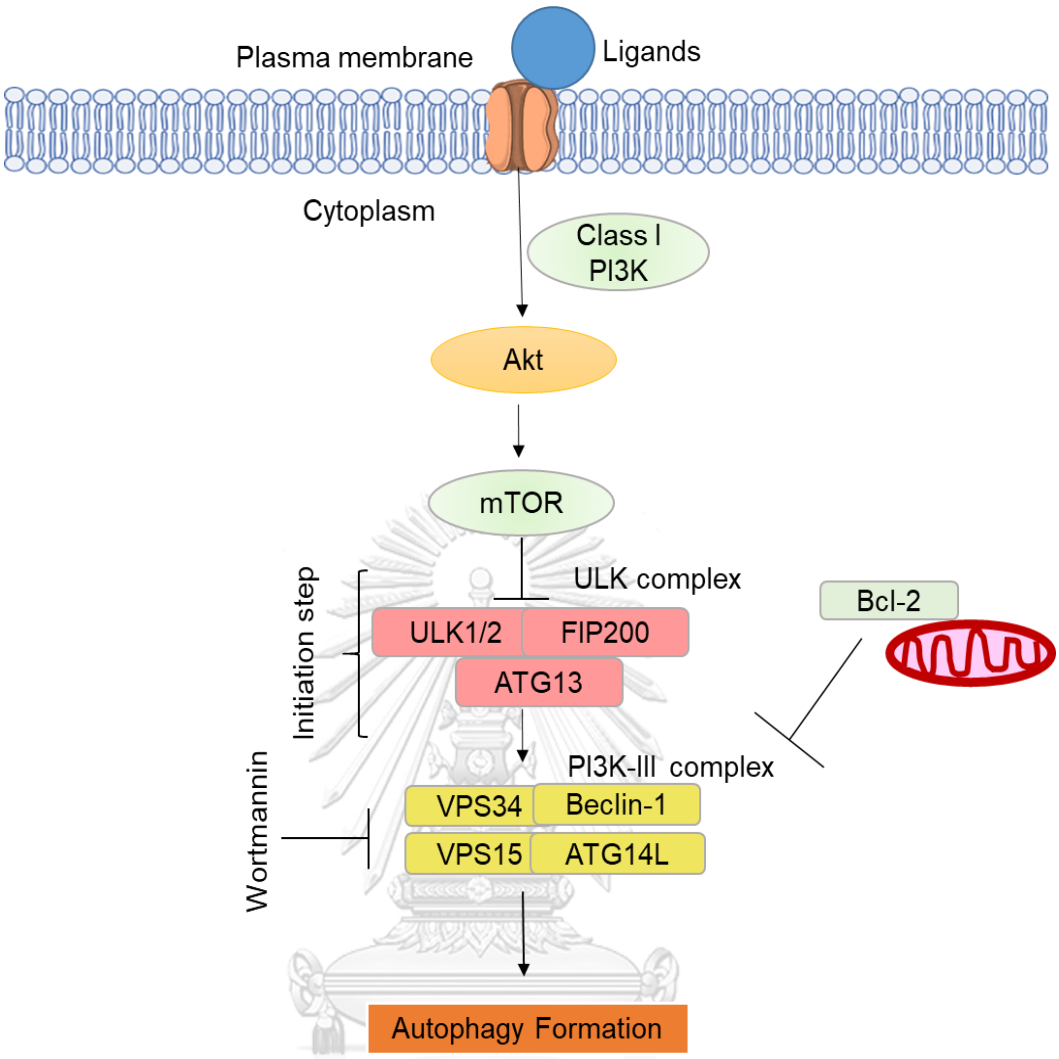


Figure 5 AKT/mTOR pathway (Sucharat, 2020)

Furthermore, current studies demonstrate that natural compounds can trigger both autophagy and apoptosis and so increase success to eliminate cancer [74]. For example, natural compound, apigenin can inhibit PI3K/ Akt/ mTOR pathway in hepatocellular carcinoma cells which leading to induce apoptosis and autophagy [75]. Moreover, Afrocyclamin A, an oleanane-type triterpene saponin, can induce both apoptosis and autophagic cell death via the PI3K/ Akt/ mTOR pathway in human

prostate cancer cells [76]. In addition, recent studies have found that cancer cells which are less sensitive to apoptosis inducing drugs can be killed by autophagy pathway [13]. Thus, the promotion of autophagic cell death could be a viable option for treatment under such circumstances.

2.3 Phytochemicals and anti-cancer activities

2-(4''-hydroxybenzyl)-5-2''-dihydroxy-3-methoxystilbene (PE5) (Figure 6) is a natural compound which is extracted from the roots of Paphiopedilum exul. (A terrestrial lady's slipper orchid endemic to southern Thailand) and is also found in other orchids [28]. Previous studies have revealed that compounds in stilbenoids such as resveratrol have a role in mediating inflammation, tumorigenesis, and cardioprotective effects [77]. For instance, this compound is used to induce apoptosis in many lung cancer cell lines such as A549, SPC-A-1/CDDP and H23 [78,79]. In addition, resveratrol is used to induce autophagy mediated cell death in PC3 and DU145 cells [80]. Furthermore, the derivative of resveratrol has been revealed to be able to induce autophagy in lung cancer cell lines [81]. Furthermore, other stilbene such as Piceatannol, Combretastatin A-4, and Pterostilbene have also shown various pharmacological effects against various type of cancers [31].

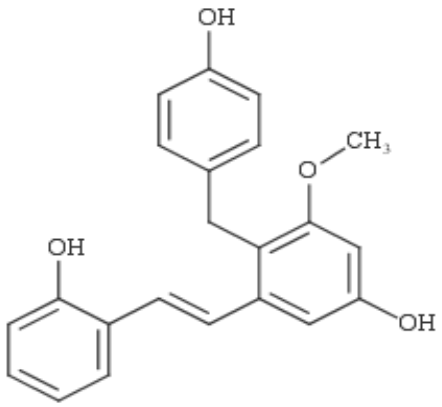


Figure 6 Structure of 2-(4’’-hydroxybenzyl)-5-2’’-dihydroxy-3-methoxystilbene (PE5)

CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals

- Cis-diamminedichloroplatinum II (cisplatin, CDDP) (St. Louis, MO)
- 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (St. Louis, MO)
- Mono-Dansylcadaverine will be obtained from Sigma Chemical, Inc. (St. Louis, MO).
- Propidium iodide (PI) (Molecular Probes, Inc. (Eugene, OR))
- Hoechst 33342 (Molecular Probes, Inc. (Eugene, OR))
- Primary antibodies (Cell Signaling Technology, USA)
    - AKT rabbit monoclonal antibody
    - p473-Akt rabbit monoclonal antibody
    - mTOR rabbit monoclonal antibody
    - p2448-mTOR rabbit monoclonal antibody
    - BAX rabbit monoclonal antibody
    - Bcl-2 rabbit monoclonal antibody
    - LC3B rabbit monoclonal antibody
    - PARP rabbit monoclonal antibody
    - caspase3 rabbit monoclonal antibody
    - ATG5 rabbit monoclonal antibody

- ATG7 rabbit monoclonal antibody
- p62 rabbit monoclonal antibody
- GAPDH rabbit monoclonal antibody

- peroxidase-conjugated secondary antibodies (Cell Signaling Technology, USA)
- RIPA buffer (Cell Signaling Technology, USA)
- Chloroquine (Cell Signaling Technology, USA)
- Z-vad-(OME)-FMK (pan-caspase inhibitor) (Cell Signaling Technology, USA)
- Wortmannin (Cell Signaling Technology, USA)

3.2 Test compound

Whole plants of P. exul were collected from the southern region of Thailand and identified by Associate Professor Thatree Phadungcharoen at the Department of Pharmacognosy and Pharmaceutical Botany, Chulalongkorn University, Thailand. The fresh roots were separated from the plants, cleaned and dried at a temperature of 50°C. Voucher specimens (RS15011) were deposited at the Herbarium of Natural Medicine, Chulalongkorn University, Thailand. The dried roots of P. exul (380 g) were powdered and macerated three times with methanol at room temperature. The methanol extract was concentrated under reduced pressure and combined to yield 100 g of crude methanol extract.

The methanol extract (50 g) was separated on a silica gel column (1.25 kg, 10 × 40 cm) and washed down with n-hexane/acetone (3:1) to yield eight fractions (A-H). These fractions were assayed by cytotoxicity activity. Fraction E (9.9 g), the most active fraction, was subjected to further separation. Fraction E (9.9 g), was separated on a silica gel column (300 g, 4.5 × 40 cm) and washed down with n-hexane/acetone (2:1) to provide six subfractions (E1-E6). Size exclusion chromatography of fraction E5 (780 mg) on a Sephadex LH-20 column eluted with methanol yielded four subfractions (E51-E54). Separation of subfraction E53 (290 mg) using a silica gel column (15 g, 2 × 14 cm), eluted with CH2Cl2/acetone (20:1), gave five subfractions (E531-E535). PE5 (58.0 mg) was obtained from subfraction E534. PE5 was be dissolved in DMSO and media to achieve the desired concentrations, containing less than 0.5% DMSO at final dilution.

3.3 Cell culture

Human lung cancer H460, H292, A549, BEAS-2B (a normal human bronchial epithelium) and HCT116 (a human colorectal carcinoma cell line) were obtained from the American Type Culture Collection (Manassas, VA, USA). Primary dermal papilla (DP) was purchased from Celprogen (Benelux, Netherlands). H460, H292 and HCT116 were cultured in RPMI 1640, whereas BEAS-2B, A549 and DP were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin/streptomycin and 2 mM L-glutamine. The cells were incubated in a 5% CO_2 environment at 37°C.

3.4 Methods

3.4.1 Cytotoxicity assays

Cytotoxicity was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) colorimetric assay. Cells in 96-well plates were treated with various concentrations of PE5 (0-100 μM) for 24-48h. Then, cells were incubated with 0.4 μg/mL of MTT for 4 hours at 37°C. The intensity of the MTT product were measured at 570 nm using a microplate reader. The relative percentage of cell survival was calculated by dividing the absorbance of treated cells by that of the control in each experiment.

3.4.2 Cell death assay

Apoptosis cells were analyzed using a fluorescent DNA staining assay with Hoechst 33342. (Molecular Probes). The cells were seeded at density 1 x 10^4 cells/well in 96 well plates for 24 hours. The cells were treated with PE5 at concentration of 0-100 μM for 24 and 48 hours. Then, cells were incubated with 10 μg/mL Hoechst 33342 for 30 min. Then, cells were visualized using fluorescence microscopy (Olympus IX51 with DP70).

3.4.3 Flow cytometry for apoptosis

To investigate cell apoptosis, cells in 96-well plates were treated with PE5 at concentration 50 and 100 μM for 24 h. Then, cancer cells were washed with PBS and then were be re-suspended in a binding buffer, followed by 20 μg/ml Annexin V-FITC (KeyGENE BioTECH) staining and 20 μg/ml propidium iodide (PI, Thermo Fisher

Scientific, MA, USA) incubation for 15 min at the room temperature in the dark, based on the manufacturer's instruction. Finally, 500 µl binding buffer was added into cells and flow cytometry (BD Bioscience, CA, USA) was used to assess the cells.

3.4.4 Western Blot Analysis

Cells were seeded at density 3 x 10^5 in 6 well plate for 24 hours. The cells were treated with PE5 at concentration 0-100 µM for 24h (concentration dependent) and were treated with PE5 at 50 µM for 6, 12, 24, 48 h (time dependent). Then, cells were incubated on ice for 40 min with RIPA buffer, 1% Triton X-100, 100mM PMSF, and a protease inhibitor. Cell lysates were analyzed for protein content using the BCA protein assay kit from Pierce Biotechnology (Rockford, IL). Equal amounts of denatured protein samples (80 µg) were loaded onto 10%-15% SDS-PAGE for various proteins. Then, the gels were transferred to 0.2 µm PVDF membranes (Bio-Rad, Hercules, CA). The transferred membranes were blocked with medium (25mM Tris-HCl (pH 7.5), 125mM NaCl, and 0.05% Tween20 (TBST)) containing 5% non-fat dry milk powder for 1 hour and were incubated overnight with specific primary antibodies. After the overnight block, the membranes were washed three times with TBST and were incubated with the following appropriate horseradish peroxidase-labelled secondary antibodies: anti-rabbit IgG or anti-mouse, for 2 h at room temperature. The immune complexes were detected by SuperSignalWest Pico chemiluminescent substrate (Pierce Biotechnology) and were exposed to the film.

3.4.5 Transmission electron microscopy

Cultured H460 cells were treated with PE5 at concentration of 50 μM for 24 h. Cells were harvested, wash with PBS and then were fixed in 2.5% glutaraldehyde for 4 h at 4 °C. The samples were washed with PBS and then were treated with 1% osmium tetroxide for 1 h. After the samples have been washed, they were dehydrated in a graded series of ethanol (50%, 70%, and 90%) and acetone and then were embedded in durcupan resin. Thin sections (120 μM) were post-stained with uranyl acetate and lead citrate before examination under JEM-1011 transmission electronic microscope (TEM) (Japan).

3.4.6 Monodansylcadaverine staining

Cultured H460 cells were treated with PE5 at concentration of 50 μM for 24h in 96 well plates. After 24 h, cells were stained with MDC (50 μmol/l) for 30 min at 37°C and then cells were visualized using fluorescence microscopy (Olympus IX51 with DP70).

3.4.7 Immunofluorescence

H460 cells were seeded at a density of 1 × 105 cells/well onto coverslips in six-well plate and incubated overnight. After the treatment, the cells on coverslips were fixed with 4 % paraformaldehyde for 30 min and permeabilized with 0.1 % Triton-X for 20 min. Then, the cells were incubated with 3 % bovine serum albumin (BSA) for 30 min to prevent nonspecific binding. Next, the cells were washed and incubated with rabbit anti-LC3B antibody for 1 h at room temperature. Primary

antibody was removed, and the cells were washed and subsequently incubated with Alexa Fluor 488 (Invitrogen) conjugated goat anti-rabbit IgG (H + L) secondary antibody for 1 h at room temperature. Samples were washed with PBS 3 times then visualized and imaged by fluorescence microscope (Olympus IX 51 with DP70, Olympus America Inc., Center valley, PA).

3.4.8 Mass spectrometry-based proteomics

H460 cells were seeded at a density of 1×106 cells in a 10 cm2 dish for 24h. Then, the cells were treated with PE5 at concentration of 50 μM for 12h and 24h. After being treated, cells were washed with PBS and incubated with lysis buffer (1% SDS, 1% NaCl, 1% triton-X and protease inhibitor). The lysates were disrupted by an ultrasound sonicator (Sonics & Materials, USA) on ice for 5s × 10 with an interval of 2 min. The protein lysates were centrifuged at 12,000 × g for 5 min at 4°C to remove cell debris. Cell lysates were analysed for protein content using Quick Start™ Bradford Protein Assay (Bio-Rad, USA). The protein samples (30 μg) were loaded onto 12% SDS-PAGE. To detect the protein bands, the SDS-polyacrylamide gels were stained with Coomassie blue G-250 (Biorad, CA, USA). After that, the gel was de-stained, until clear bands were visible. Each gel lane was cut into small pieces and were kept at −20 °C until use. Gel pieces were de-stained by 50% acetonitrile in 50 mM ammonium bicarbonate. The colourless gel pieces were reduced and alkylated by dithiothreitol and iodoacetamide then, dehydrated by acetonitrile and were allowed to dry completely in a fume hood. To rehydrate the gel pieces, trypsin

solution at a concentration of 0.02 mg/mL in 50 mM ammonium bicarbonate (Sigma, USA) were added to the tubes and each gel piece were incubated at 37 ℃ overnight. Peptides were extracted by addition of acetonitrile and the solution were shaken for 15 min. The supernatant was collected, and the peptide mixtures were completely dried by a speed-vac (Eppendorf, Hamburg, Germany). The samples were resuspended in 0.1% formic acid and subjected to an Ultimate® 3000 Nano-LC system analysis (Thermo Scientific, USA coupled with a microTOF-Q II (Bruker, Bremen, Germany). The mass spectrometric data was converted to .mgf file using DataAnalysis™ software version 3.4 (Bruker, Germany). Mascot server (Matrix Science, Boston, MA, USA) against human NCBI database. The significance threshold for protein identification was set at 95%. Three biological replications were performed. The gene ontology classification and protein-protein interaction were performed using Panther and STITCH software, respectively.

3.4.9 Small interfering RNA (siRNA) transfection

H460 cells were seeded into a 24-well plate at a density of 1.5×10^5 cells per well and allowed to reach approximately 70% confluence on the day of transfection. The siRNA including siRNA control and siRNA against ATG7 were obtained from Cell Signaling Technology, Inc. (Danvers, MA). Cells were transfected with 100 nM siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

3.4.10 Statistical analysis

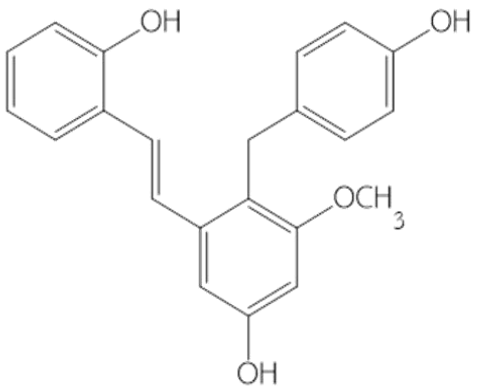
Data was given as the means ± standard deviation of at least 3 independent replicated samples procedures composed with at least 3 independent replicated samples. Multiple comparisons for statistically significant differences between multiple groups (ANOVA) was calculated by using SPSS software program version 16 (SPSS Inc., Chicago, IL, USA), followed by individual comparisons with Scheffe's post-hoc test. For 2-group comparisons, t-test was calculated by using the program same as previous calculation. Differences were be considered statistically significant for (*) $P < 0.05$ and (**) $P < 0.01$. GraphPad prism 5 was used for creating graphs in all experiments (GraphPad Software, San Diego, CA, USA).

CHAPTER IV
RESULTS

4.1 Anti-cancer activities of PE5 on lung cancer cells

To elucidate the anti-cancer potential of PE5 (Figure 7A-B), we first determined the cytotoxic profile of PE5 in several non-small cell lung cancer (NSCLC) cells, including the H460, H292, and A549 cell lines. In addition, we also determined the cytotoxic effect of the compound in other cell lines, including the colon cancer cell line (HCT116), primary dermal papilla (DP), and human bronchial epithelial cells (BEAS-8B), for comparison. Cell viability was evaluated by the MTT assay. Cells were incubated with various concentrations of PE5 (0–100 μM) for 24–48 h. The results showed that PE5 significantly reduced cell viability in all NSCLCs and colon cancer cells (Figure 8), while it had a slightly toxic effect on non-cancerous DP and BEAS-2B cells. In addition, we also calculated the selectivity index (SI), which represents the IC50 value for the normal cell line/IC50 value for the cancerous cell line after 24–48 h of PE5 treatment. The results showed that the SI index for H460 and H292 cells was higher than that for A549 cells (Figure 9).

A



2,5'-dihydroxy-2'-(4-hydroxybenzyl)-3'-methoxystilbene (PE5)

B

Paphiopedilum exul.

Figure 7 PE5 structure and the plant specimen of Paphiopedilum exul. (A-B)

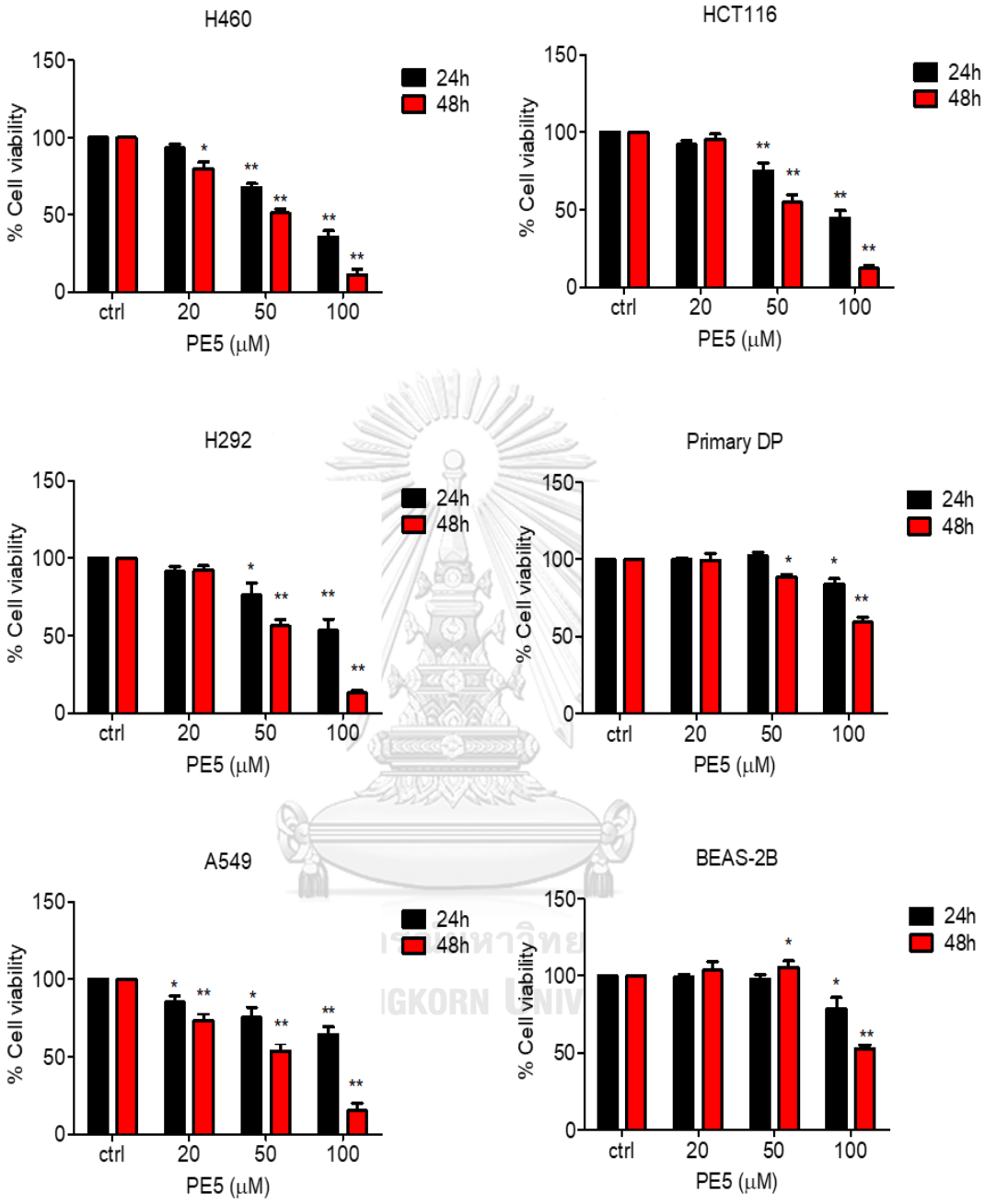


Figure 8 Cytotoxic effects of PE5 on lung cancer cell, colorectal cancer cell and normal cell. All cells were treated for 24–48 h and analyzed by MTT assay. Normal cells were similarly treated for 24–48 h and analyzed for cell viability. Data represent the mean ± SD (n = 3), (*$p < 0.05$, ** $p < 0.01$, compared with the untreated control).

Cells	IC50 values (μM) 24h	IC50 values (μM) 48h		
BEAS-2B	>130	99.61±0.12	**SI INDEX 24h**	**SI INDEX 48h**
H460	73.81±0.36	55.14±0.53	1.76	1.80
H292	76.22±0.95	53.67±1.10	1.70	1.85
A549	>120	58.45±0.28	1.08	1.70

Figure 9 Selective Index (SI) of PE5 in different cells. SI, which represents the IC50 value for the normal cell line/IC50 value for the cancerous cell line after 24–48 h of PE5 treatment.

Apoptosis is an effective program that eliminates undesirable cells, senescent cells, and genetically damaged cells [82]. It is characterized by blebbing of the cell membrane, cell shrinkage, and the condensation of DNA. Therefore, we examined whether the cytotoxic effect of PE5 was involved in apoptosis. Hoechst 33342 staining was used to evaluate the nucleus morphology of the PE5-treated cells. The NSCLC cells were treated with PE5 at the concentrations of 0–100 μM for 24–48 h. The results revealed that PE5 caused an increase in apoptosis in a concentration- and time-dependent manner in the H460 and H292 cells (Figures 10A-B).

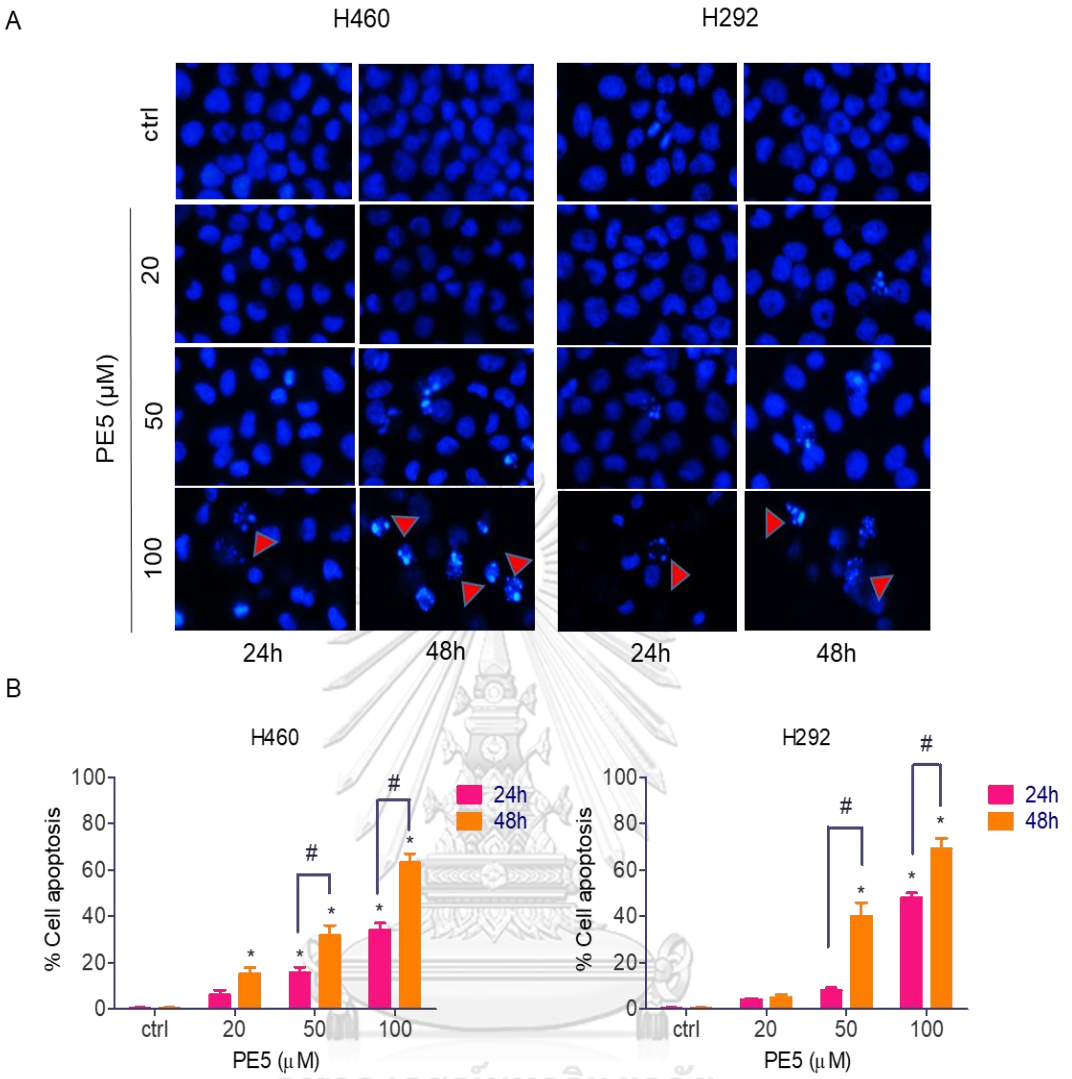


Figure 10 Apoptosis effect of PE5 on lung cancer. (A-B) Apoptotic nuclei in the cells treated with PE5, determined by Hoechst 33342 staining and visualized by fluorescence microscopy. Data represent the mean ± SD (n = 3), (*p < 0.05, compared with the untreated control, # p < 0.05, compared with different time)

To confirm this, flow cytometric analysis of apoptosis using annexin V-FITC/PI staining was utilized. H460 cells were similarly treated with PE5 (0–100 μM) for 24 h. The results showed that PE5 slightly induced apoptosis in a concentration-dependent manner (Figures 11 A-B). As shown in Figure 11B, the percentages of early apoptotic cells in response to 50 and 100 μM of PE5 were 14.45%, and 30.81%, respectively.

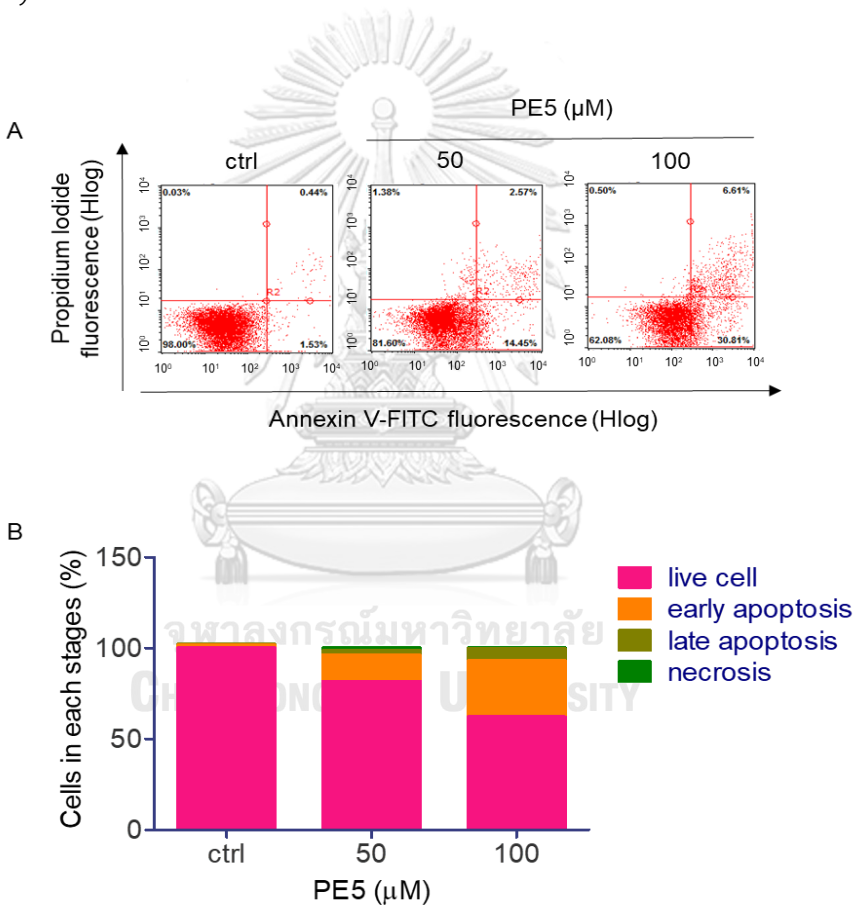


Figure 11 Effect of PE5 on apoptosis by flow cytometry assay. (A-B) Cells were treated with PE5 (0-100 μM) for 24h. Apoptotic and necrotic cells were determined using annexin V-FITC/PI staining with flow cytometry in H460 cells. Data represent the mean ± SD (n = 3), (*p < 0.05, compared with the untreated control)

The apoptotic protein markers, including caspase-3, poly (ADP-ribose) polymerase (PARP), and their cleaved forms, were determined by western blot analysis. We treated H460 and H292 cells with various concentrations of PE5 for 24 h and used cisplatin, a standard treatment drug for lung cancer that is known for apoptosis induction, as an internal control (at its IC50 concentration). Western blotting revealed that, in response to PE5 treatment, the activated caspase-3 was significantly increased compared with the untreated control. Likewise, the cleaved form of PARP was up-regulated simultaneously (Figures 12, 13). In addition, the activated caspase-3 and the cleaved form of PARP when treated with cisplatin were significantly increased compared with treatment with 100 μM of PE5 in all the NSCLC cell lines.

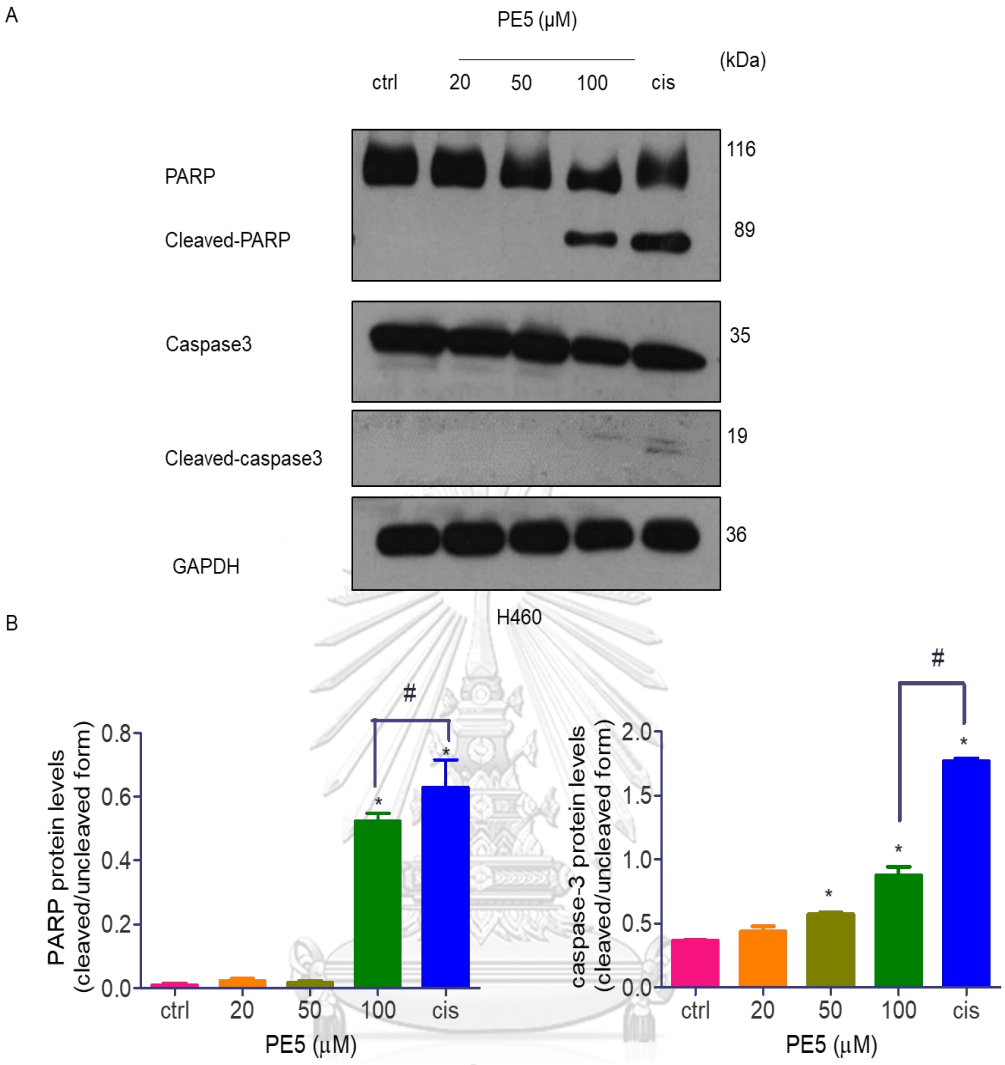


Figure 12 Effect of PE5 on apoptosis markers in H460 cell. (A-B) Cells were treated with PE5 (0-100 μM) for 24h. Apoptosis-related proteins were measured by western blot analysis in H460 cells. The blots were re-probed with GAPDH to confirm equal loading of the protein samples. The relative protein levels were calculated by densitometry. Data represent the mean ± SD (n = 3), (*p < 0.05 compared with the untreated control), and (# p < 0.05, compared with cisplatin).

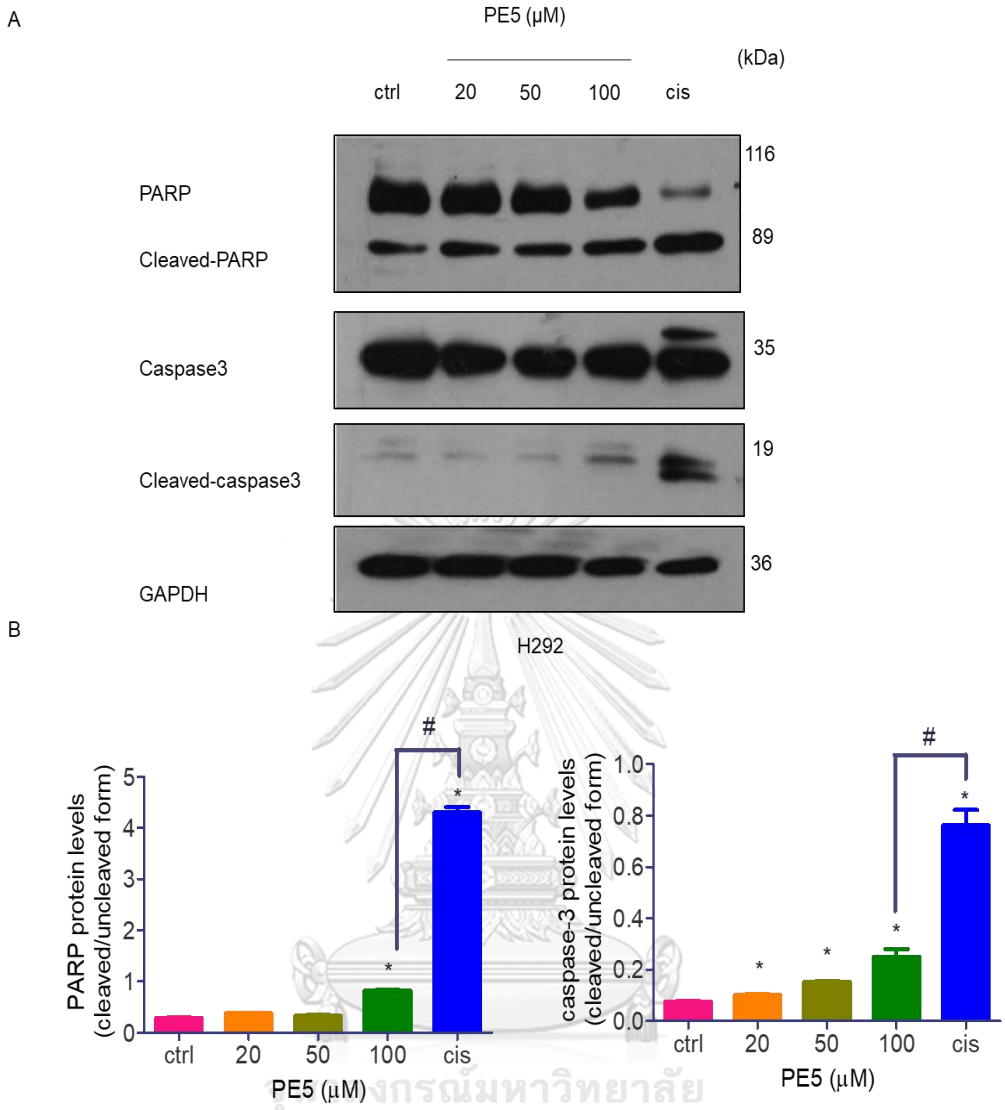


Figure 13 Effect of PE5 on apoptosis markers in H292 cell. (A-B) Cells were treated with PE5 (0-100 μM) for 24h. Apoptosis-related proteins were measured by western blot analysis in H292 cells. The blots were re-probed with GAPDH to confirm equal loading of the protein samples. The relative protein levels were calculated by densitometry. Data represent the mean ± SD (n = 3), (*$p < 0.05$ compared with the untreated control), and (# $p < 0.05$, compared with cisplatin).

4.2 PE5-induced autophagy in lung cancer cells

Autophagy has been shown to mediate cell death in malignant cells and has been proposed as a possible means of achieving cancer elimination [6]. We next observed NSCLC cells after treatment with various concentrations of PE5 for 24–48 h and found that cytoplasmic vacuoles were clearly noticeable after treatment with 50–100 μM of PE5 in a time- and concentration-dependent manner (Figures 14, 15). In the cells treated with 100 μM PE5, cytoplasmic contraction, a morphological feature of typical apoptosis, was observed and most of the cells were detached at 48 h.

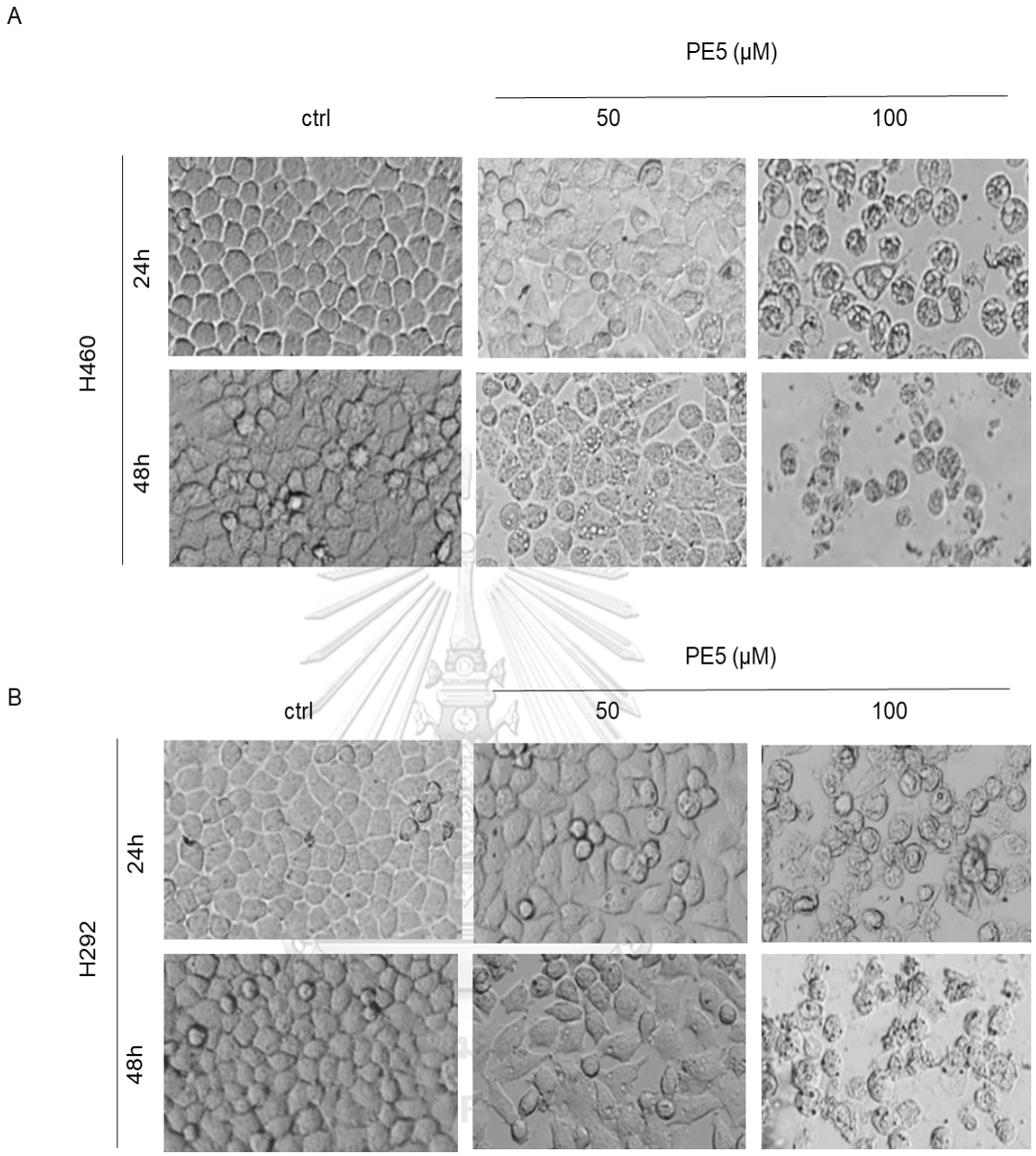
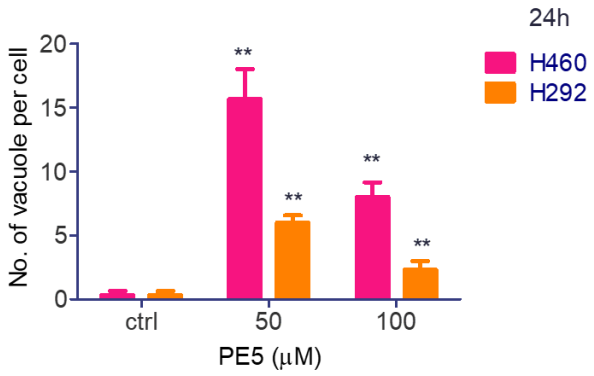


Figure 14 Effect of PE5 on cell morphology. H460 and H292 cells were treated with PE5, the morphological changes of the cells were observed by phase-contrast microscope; scale bar = 100 μm.

A



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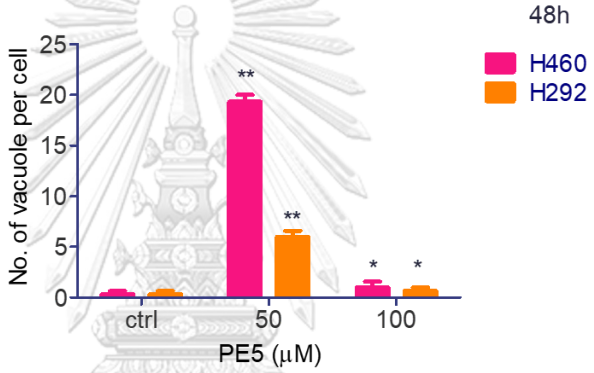


Figure 15 Effect of PE5 on number of vacuole. H460 and H292 cells were treated with PE5, the number of vacuoles per cells was calculated. Data represent the mean ± SD (n = 3) and (*$p < 0.05$, ** $p < 0.01$, compared with the untreated control)

Next, we treated the cells with PE5 (50 μM) for 24 h and stained them for autolysosome using monodansylcadaverine (50 μmol/l). The results indicated that the vacuoles observed in the PE5-treated cells were mostly stained with the monodansylcadaverine compared with those of the non-treated control cells (Figure 16). Furthermore, the cytoplasmic vacuoles were observed by transmission electron microscopy (TEM) (Figure 17). In the PE5-treated cells, we found degraded cell materials inside the double membrane vesicles, which appeared to be autophagosomes (Figure 17, indicated with arrowhead). The vacuoles in which all their contents were degraded (empty vesicles) were likely to be autophagosomes fused with lysosomes, representing active autolysosomes (Figure 17, indicated with arrows).

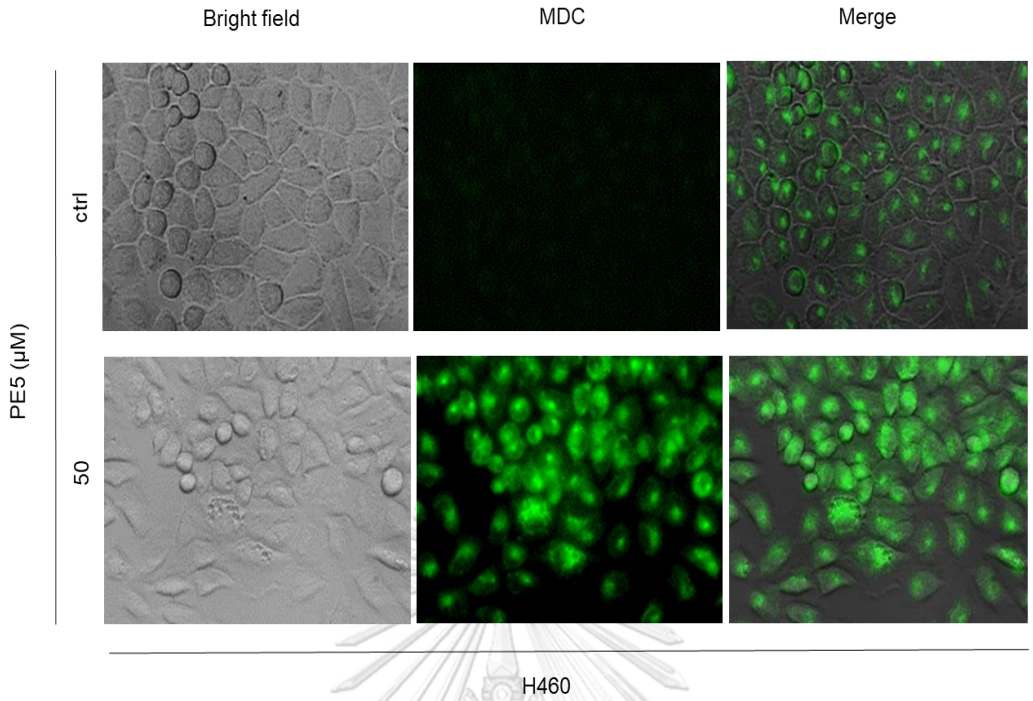


Figure 16 Effect of PE5 on autolysosome in H460 cell. (A) H460 cells were treated with PE5 (50 μM) and stained with monodansylcadaverine (50 μmol/l) and visualized by fluorescence microscopy (Olympus IX51 with DP70).

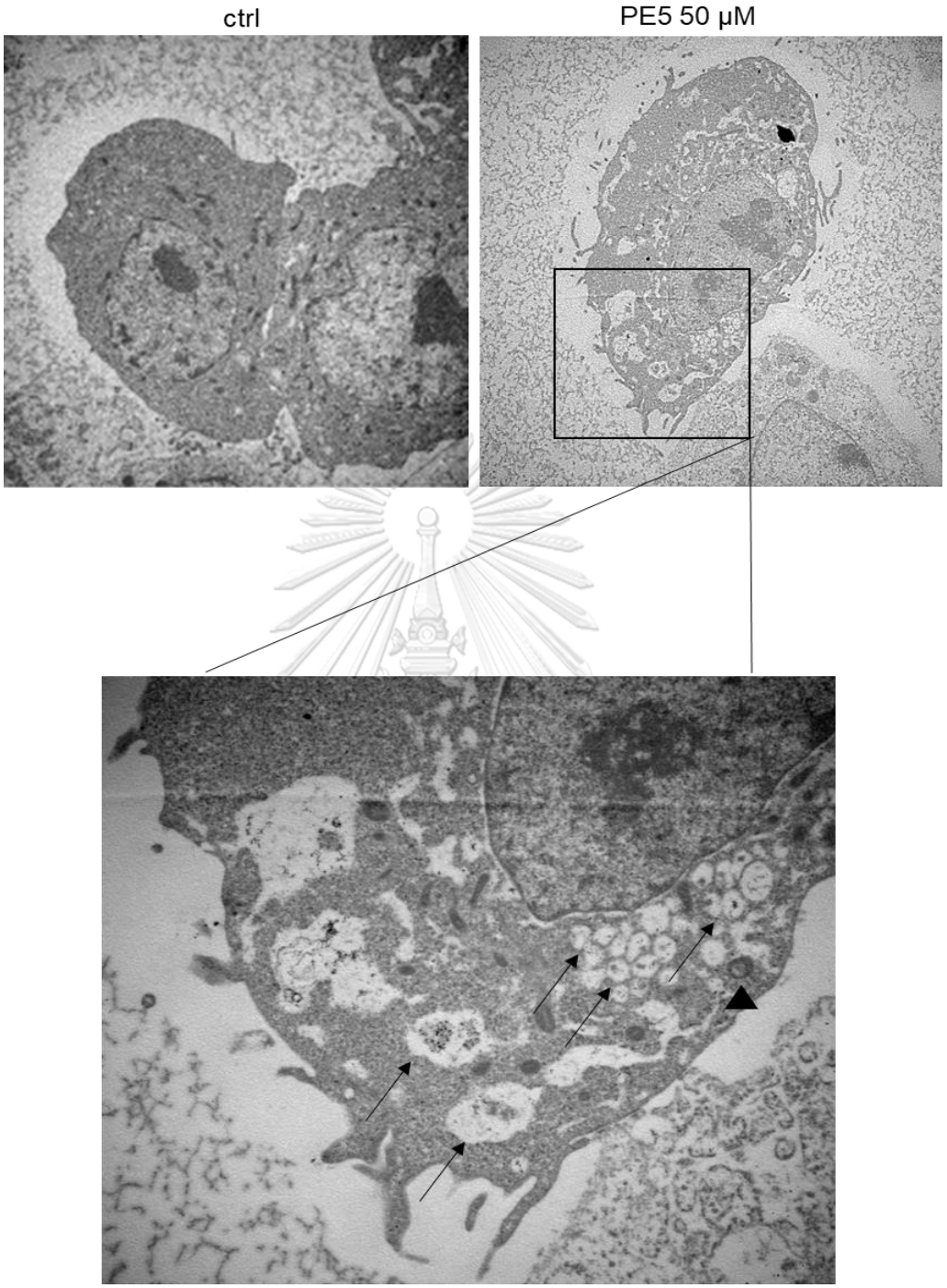


Figure 17 Effect of PE5 on autophagy morphology. Cells were treated with 50 μM PE5 for 24 h and observed by transmission electron microscopy. Arrowheads indicate the autophagosomes and the arrows show the vacuoles.

Western blot analysis was carried out to confirm the expression of autophagy-related marker proteins, such as LC3-I to II conversion. The protein analysis further confirmed autophagy induction in the PE5-treated cells, as the increasing of LC3-II could be clearly observed in a time-dependent manner after PE5 treatment (Figures 18). Moreover, CQ increased LC3II upon co-treatment with PE5 compared to treatment with PE5 alone. Therefore, PE5 induces autophagic flux in H460 cells (Figure 19).

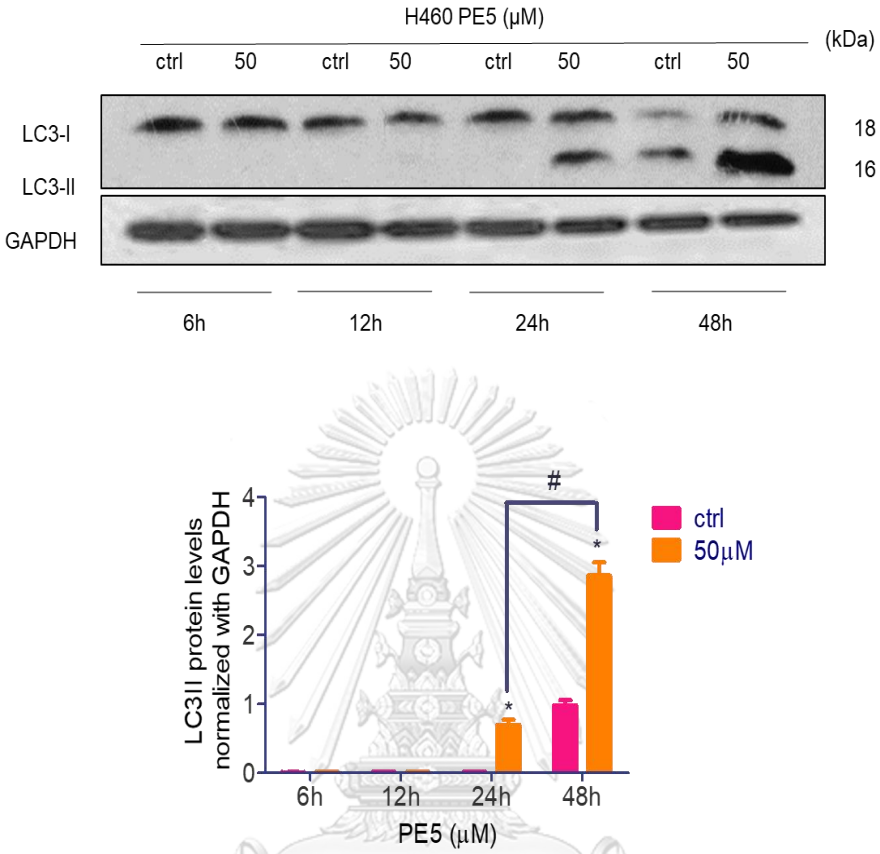


Figure 18 Effect of PE5 on LC3-II levels in H460 cells. Cells were treated with 50 μM PE5 for 6–48 h before western blot analysis for LC3 conversion. Data represent the mean ± SD (n = 3) and (*p < 0.05, ** p < 0.01, compared with the untreated control) (# p < 0.05, compared with PE5-treated alone or different time).

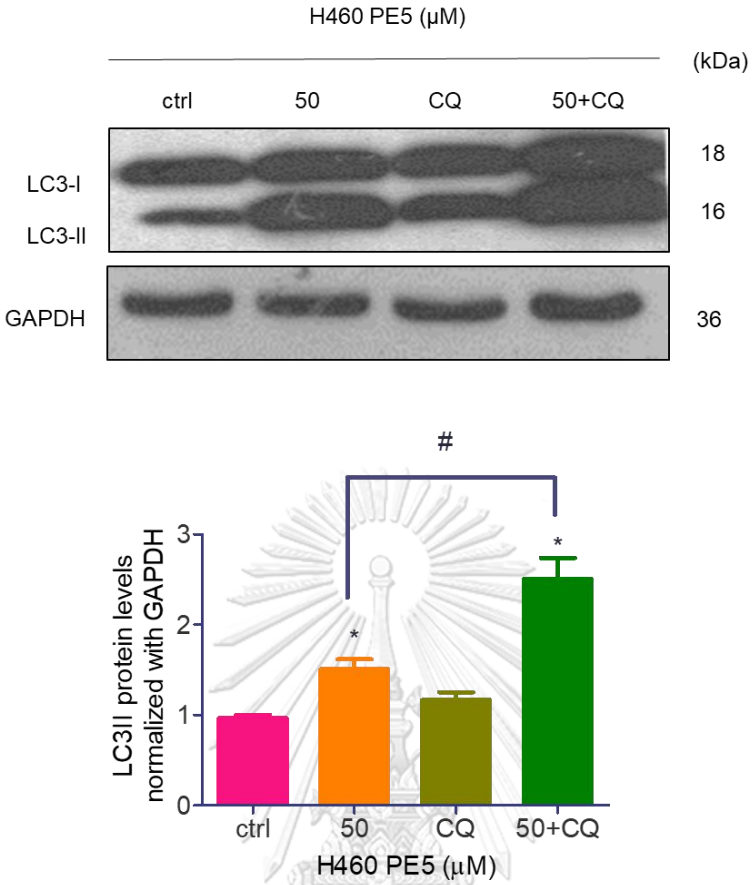


Figure 19 Effect of PE5 on autophagic flux. Autophagic flux was detected in chloroquine (10 μM) in treated cells with 50 μM PE5. Cells were lysed and the level of LC3 protein was analyzed by western blot. Data represent the mean ± SD (n = 3) and (*p < 0.05 compared with the untreated control) (# p < 0.05, compared with PE5-treated alone)

In the autophagy process, LC3, SQSTM1/p62, and many ATG proteins are recruited to phagophores for inducing the phagophores to undergo an expansion step. Therefore, we determined the expression of LC3, ATG5, ATG7, and p62 by western blot analysis. In response to PE5 treatment, the protein analysis showed the sufficient conversion of LC3-I to LC3-II along with the increase in p62 and ATG7; however, ATG5 was found to have slightly decreased (Figures 20).

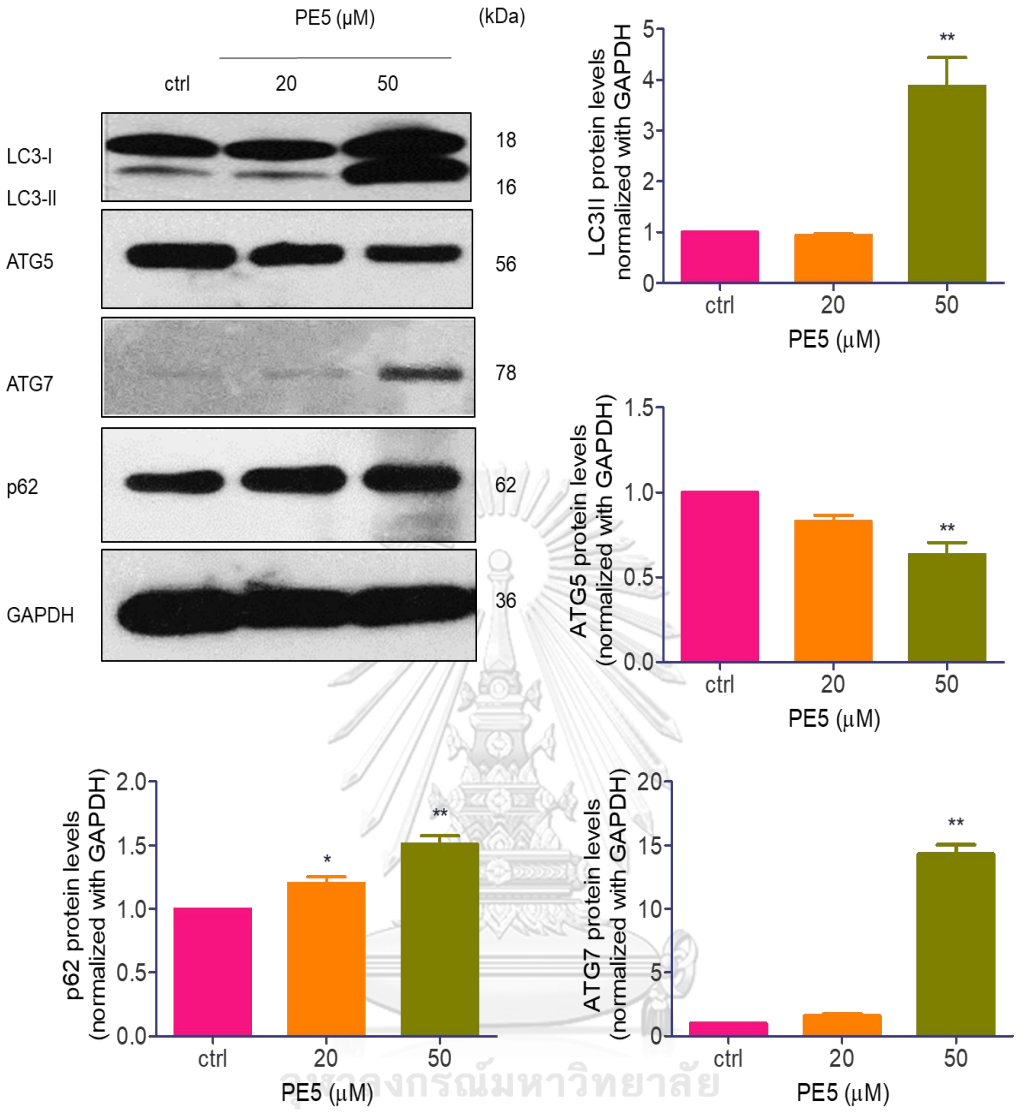


Figure 20 Effect of PE5 on autophagy markers. H460 cells were treated with PE5 and detected for LC3, p62, ATG5, and ATG7 proteins by western blotting. Data represent the mean ± SD (n = 3) and (*p < 0.05 compared with the untreated control)

Having shown the cell killing as well as the potent autophagic-induction activities of the compounds, the next question was whether the autophagy induced by PE5 in our system was associated with a cytotoxic action. Consequently, we co-treated the cells with PE5 and wortmannin (an autophagy inhibitor) and analyzed the autophagosomes and cell viability. Figure 21 shows that the addition of the autophagy inhibitor completely abolished the formation of autophagosomes induced by PE5, as indicated by the absence of LC3 puncta in the autophagosomes in comparison to those of the PE5-treated cells.

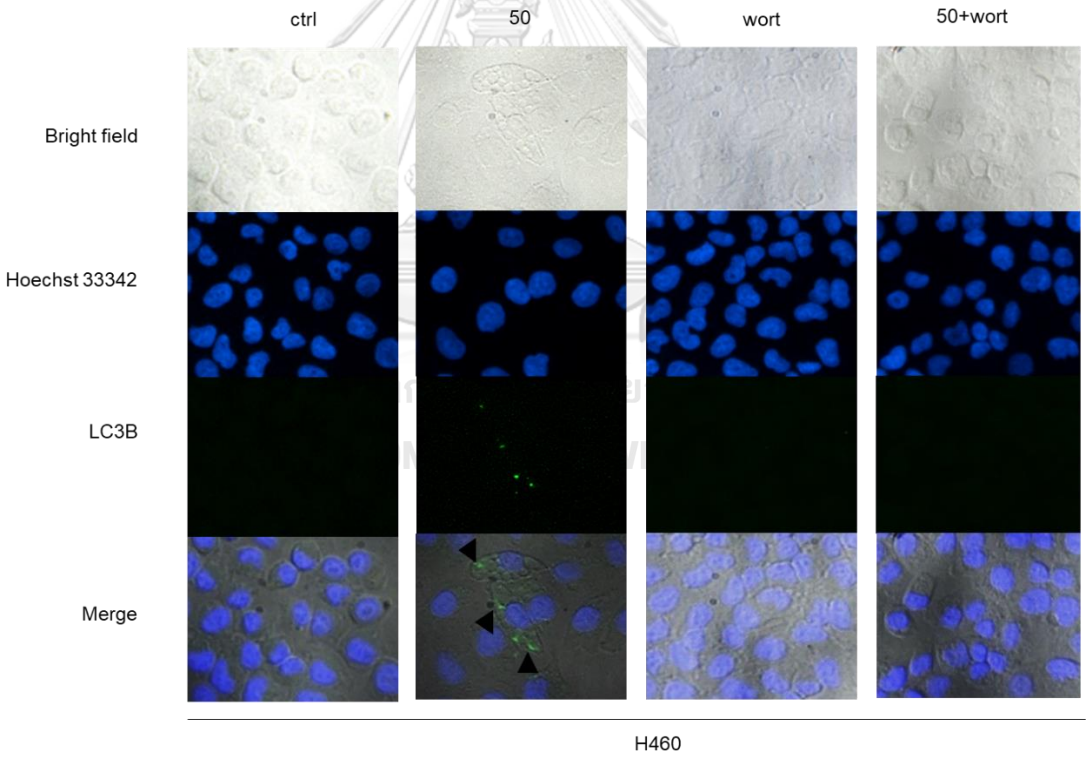


Figure 21 Effect of PE5 combined with autophagic inhibitor on autophagy induction in H460 cell. H460 cells were pre-treated with wortmannin (1 μM) (an autophagic inhibitor) and treated with PE5 for 24 h. Expression of LC3 was analyzed by immunofluorescence staining.

In addition, Figure 22 shows that wortmannin could reverse the cytotoxic effect induced by PE5. In contrast, rapamycin (autophagy inducer) significantly enhanced cytotoxic effect of PE5.

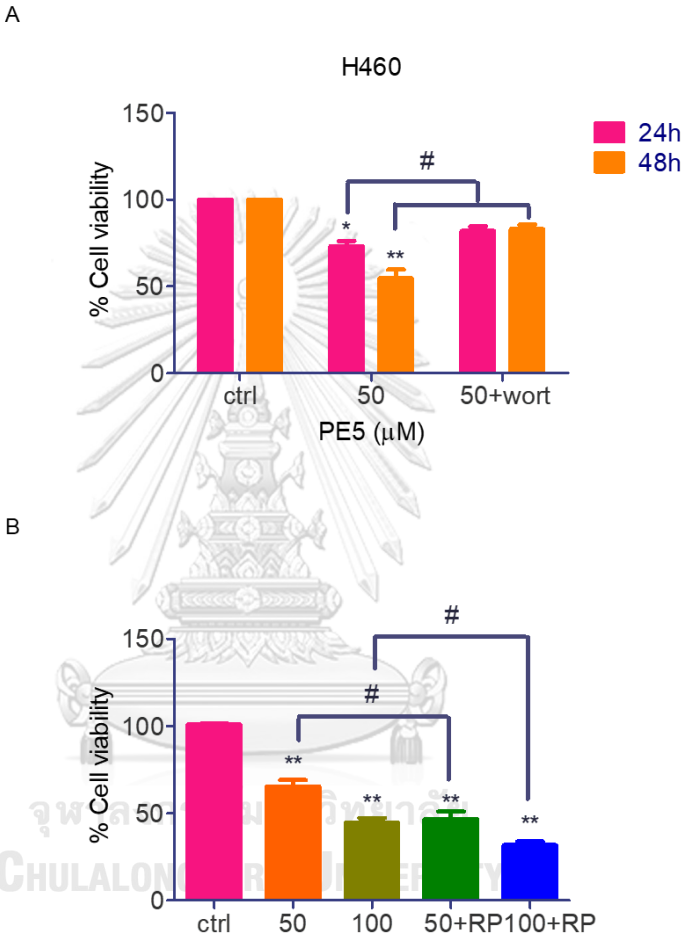


Figure 22 Effect of PE5 combined with autophagic inhibitor and inducer on cell viability in H460 cells. H460 cells were treated with PE5 in the presence of wortmannin (1 μM) or rapamycin (200 nM). Cell viability was analyzed by MTT assay. Data represent the mean ± SD (n = 3) and (*p < 0.05, ** p < 0.01, compared with the untreated control) (# p < 0.05, compared with PE5-treated alone)

To confirm, the precise suppression of autophagy was conducted using siRNA approach. Result indicated that siATG7 transfection caused a dramatic depletion of ATG7 and effectively attenuated the PE5-induced conversion of LC3-I to II as shown in figures 23. Importantly, siATG7 significantly decreased PE5-induced cell death.

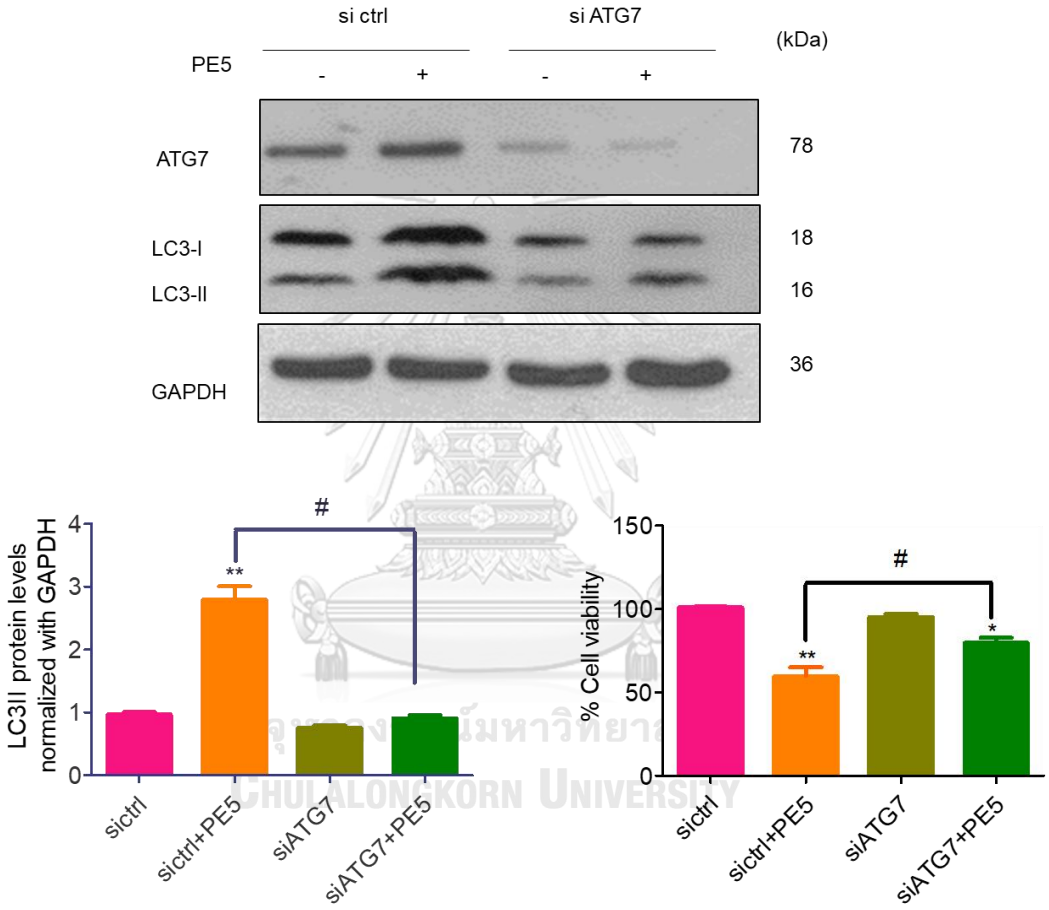


Figure 23 Effect of siATG7 on autophagy marker and cell viability in H460 cell.
Cells were transfected with siATG7 and treated with 50 μM PE5 for 24 h. Expression levels of each ATG, and LC3 were assessed by western blot analysis. Cell viability was assessed using the MTT assay at 48 h. Data represent the mean ± SD (n = 3) and (*p < 0.05, ** p < 0.01, compared with the untreated control) (# p < 0.05, significantly different from siATG-transfected cells).

4.3 Molecular functions and biological processes of the proteins in PE5-treated cells

It is essential to determine the full profiles of the proteins and signals affected by a pharmacological compound in order to define its major mechanism of action. Proteomic studies have generated numerous datasets of therapeutic significance in cancer [83]. In this study, we used proteomics to identify the proteins that control ACD as induced by PE5. Here, cells were seeded in a 10 cm^2 dish and treated with PE5 at a concentration of 50 μM for 12 and 24 h. Next, we separated the proteins by SDS-PAGE and analyzed the proteins using mass spectrometric analysis (Figure 24). In our setting, the proteins affected by the treatment were determined at 2 different time points (12 and 24 h) for determining the time-dependent mechanisms and for defining the crosstalk between apoptosis and ACD. The total number of proteins identified from the control non-treated cells was 2,240 proteins, while from the PE5-treated cells there were 2,142 and 1998 proteins at 12 and 24 h, respectively. The protein lists from the control and treated cells were input to a Venn diagram. The results showed that 69 proteins were uniquely found in the PE5-treated cells at 12 h, and 46 proteins were specifically found in the PE5-treated cells at 24 h (Figure 24).

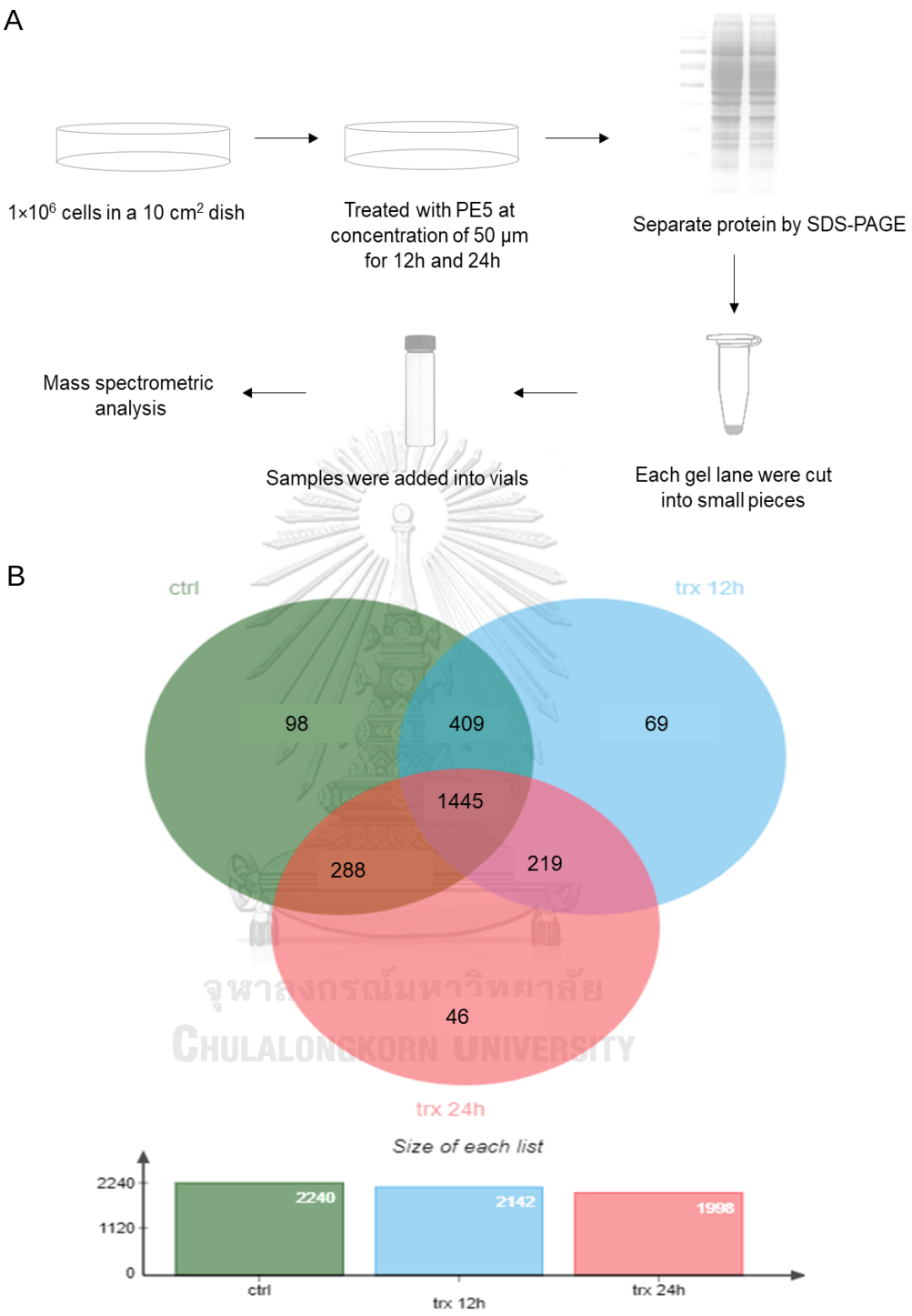
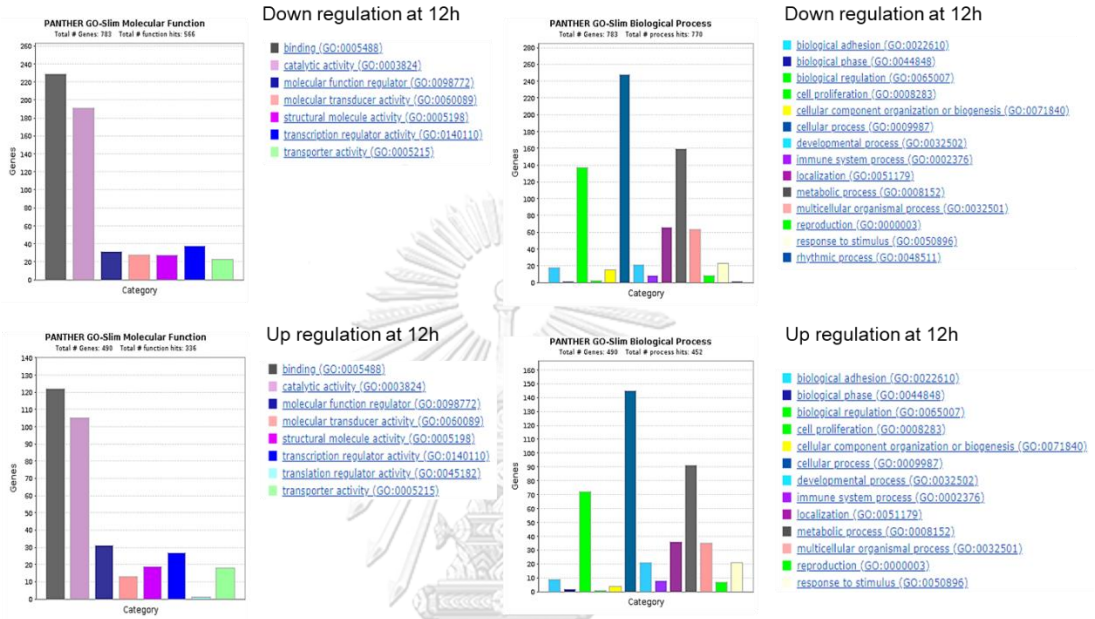


Figure 24 Proteomic analysis of PE5-treated cells. (A) Brief method involving proteomic analysis and mass spectrometric analysis. (B) Venn diagram showing the different proteins between the control and PE5-treated cells at 12 and 24 h.

Furthermore, the proteins that were significantly altered at 12 and 24 h were subjected to further molecular function and biological process analysis using Panther software (conducted on December 1, 2019) (Figure 25).



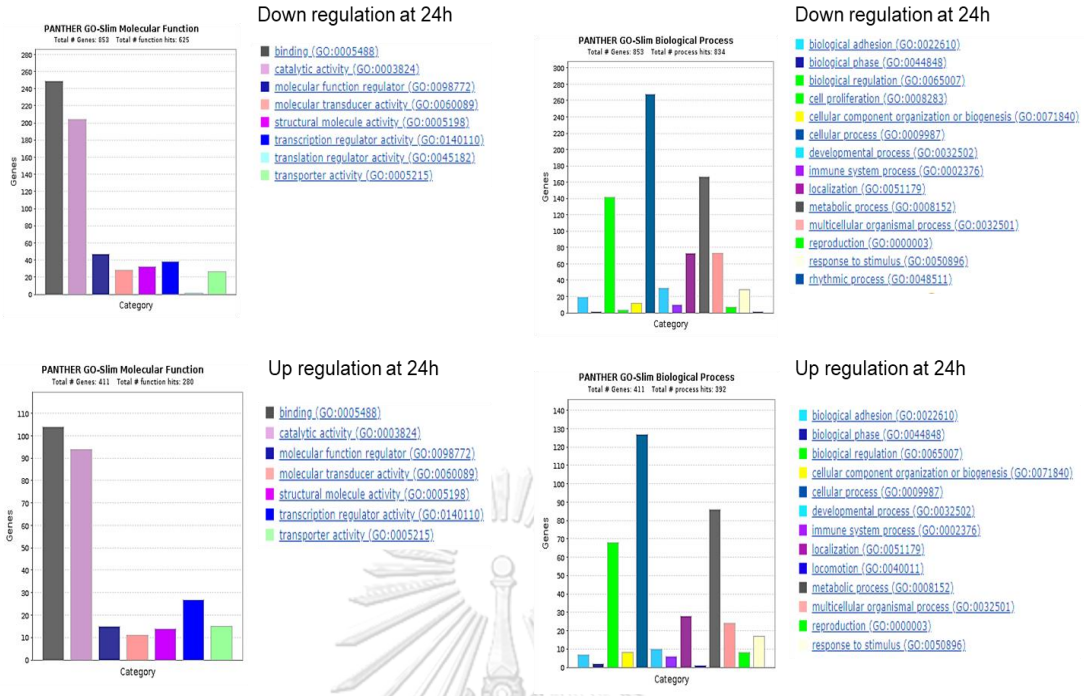


Figure 25 Effect of PE5 on gene oncology classification. Gene ontology classification according to the biological process and molecular function terms of the upregulated and downregulated proteins using Panther software.

From gene ontology (biological process) and gene ontology (molecular function) analysis, our proteomic analysis evidenced that PE5 directly or indirectly modulated the expression of proteins that regulate apoptosis and the autophagy process (Figure 26). We identified that PE5 affected 128 apoptosis-related proteins as well as 25 proteins of autophagy regulation. In addition, 8 proteins altered by PE5 were found to be involved in both apoptosis and autophagy.

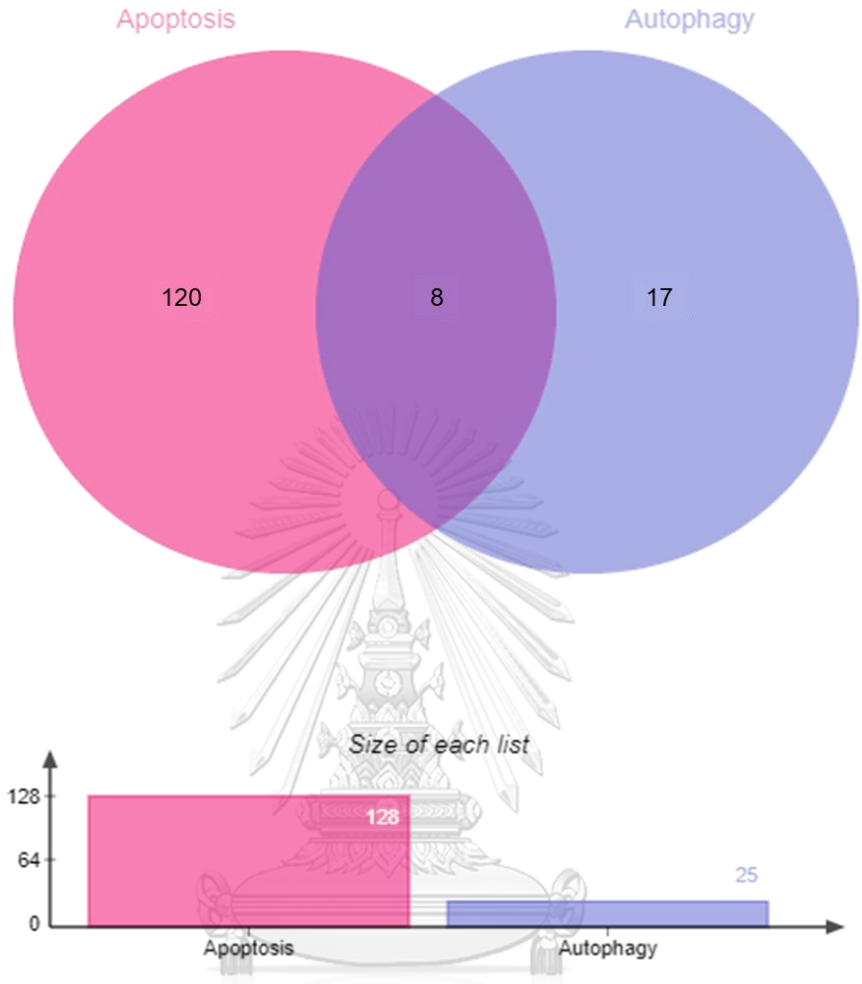


Figure 26 Apoptosis and autophagy protein alteration by PE5. Venn diagram showing the different apoptosis and autophagy proteins affected by PE5 from Enrichr software.

As shown in Figure 27, the proteins involved in apoptosis processes (Figure 27A) and the autophagy proteins (Figure 27B) were altered in a time-dependent manner. It was noteworthy that the apoptosis proteins were upregulated at 12 h with some of them, such as NISCH, CCAR1, RBM5, MSH6, BBC3, TRAF3, PIP5KL1, and MYOCD, maintained at a high level until 24 h, while most of the autophagy proteins were upregulated at 12 h but then dropped afterward. For autophagy, the level of the mTOR protein was found to be critically decreased in a time-dependent manner. The proteins involved in the apoptosis pathway, such as INPP5D, CASP8AP2, EEF2K, DICER1, EIF2AK4, TXNIP, SLK, NCF1, USP17L7, C5AR1, XDH, ITCH, TMEM117, USP53, TOP2A, RB1CC1, DHODH, DIDO1, and TNFRSF10B, were significantly downregulated in a time-dependent manner (Figure 27B).

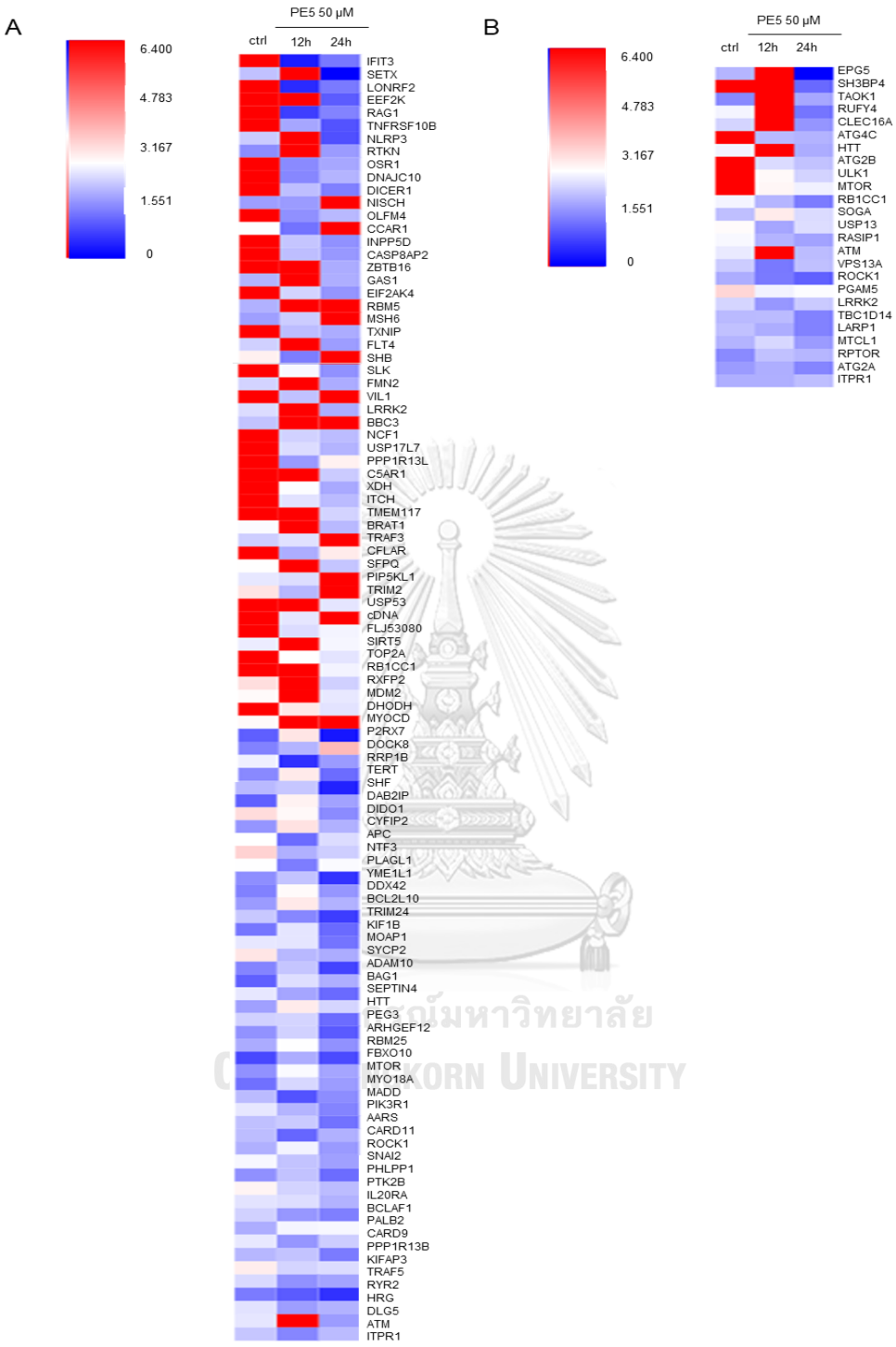


Figure 27 Effect of PE5 on alteration of protein in apoptosis and autophagy pathway. (A) The pathways regulating apoptosis in the control and PE5-treated H460 cells at 12 and 24 h. (B) Heatmap representing the levels of 25 proteins associated with autophagy in the control and PE5-treated H460 cells at 12 and 24 h.

4.4 Mechanisms of action of PE5 analyzed by the protein–protein interaction networks and signaling pathways

The hyperactivity or overexpression of some groups of proteins, such as the PI3K/AKT/mTOR and Bcl-2 family proteins, can be found in lung cancer [84]. In order to identify the major contributing mechanism of action of PE5, the proteins in PE5-treated cells at different times were subjected to protein–protein interaction network analysis with the STITCH database and with interactions contained in the STRING database in order to determine the significant kinase pathways. The resulting networks at 12 and 24 h are presented in Figures 28 and 29, respectively. The top 20 most downregulated proteins were subjected to STITCH for network node evaluation and for the prediction of their molecular function. The results revealed that proteins such as RTKN and PRKAA2 interacted with PI3K/AKT and the mTOR pathway at 12 h (Figure 28). In addition, the BBC3 protein was found to link with the anti-apoptotic Bcl-2 and Mcl-1 proteins. Regarding the proteins involving malignant phenotypes, the FN1 protein was found to be associated with PI3K/AKT and its pathway in cancer (Figure 29). These results suggested that the major mechanisms by which PE5 mediated ACD was through the potent and sustained suppression of the PI3K/AKT/mTOR axis as well as by the depletion of Bcl-2 at 24 h.

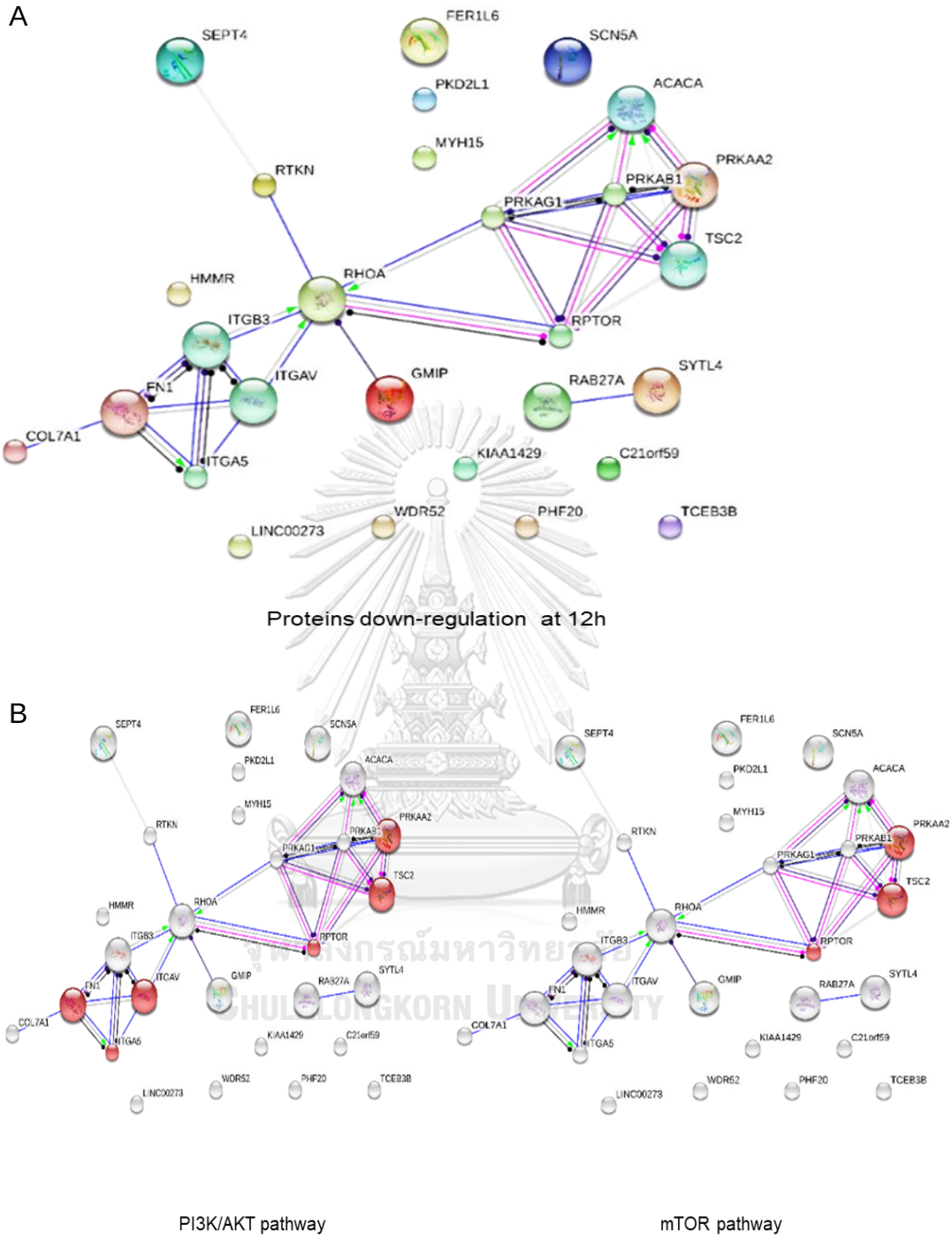


Figure 28 Effect of PE5 on protein-protein interaction at 12h. (A)The functional protein–protein interactions of the top 20 down-regulated proteins at 12h. (B) The significant nodes of each network were identified and rebuilt as a network of the signaling pathway in cancer.

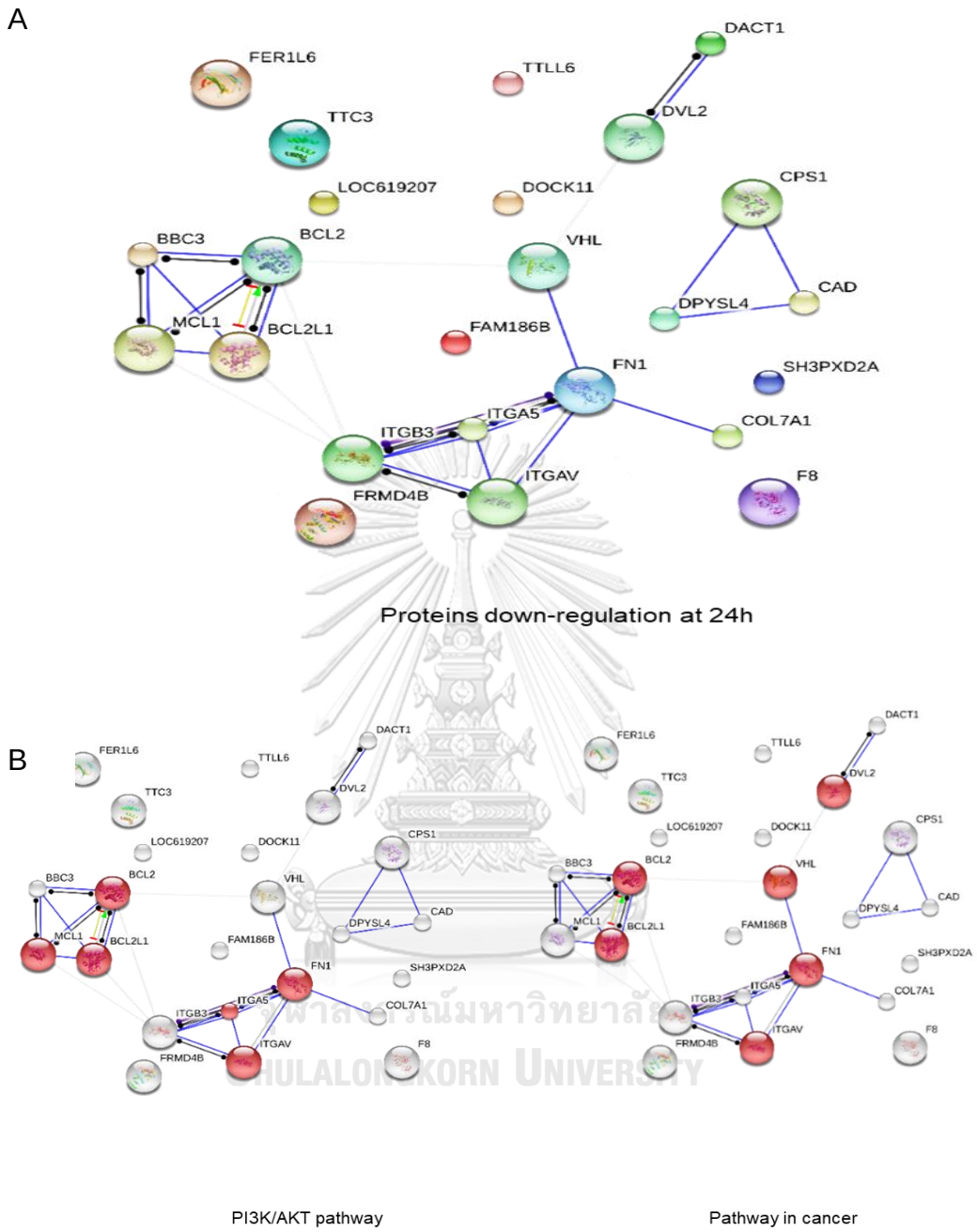


Figure 29 Effect of PE5 on protein-protein interaction at 24h. (A)The functional protein–protein interactions of the top 20 down-regulated proteins at 24h. (B) The significant nodes of each network were identified and rebuilt as a network of the signaling pathway in cancer.

Having shown the node of protein interaction as well as the dominant mechanisms of PE5, we further confirmed the significance of the proteomics finding with western blot protein analysis using specific phosphorylated antibodies against the active forms of AKT and mTOR proteins. In addition, we validated the effect of the compound on Bcl-2. The results indicated that treatment of the cells with PE5 significantly decreased the level of active p-AKT and p-mTOR proteins in comparison to those of the non-treated control. Moreover, PE5 significantly decreased the level of Bcl-2 proteins.

In order to distinguish the ACD mechanism by PE5 with apoptosis, we utilized cisplatin. We found that treatment of the cells with 100 μM PE5 caused a greater effect on p-AKT reduction than that of cisplatin treatment at the IC50 concentration. Interestingly, while PE5 selectively decreased the anti-apoptotic Bcl-2 protein with no significant effect on the pro-apoptotic Bax protein, cisplatin depleted the Bcl-2 protein concomitantly with Bax induction (Figures 30).

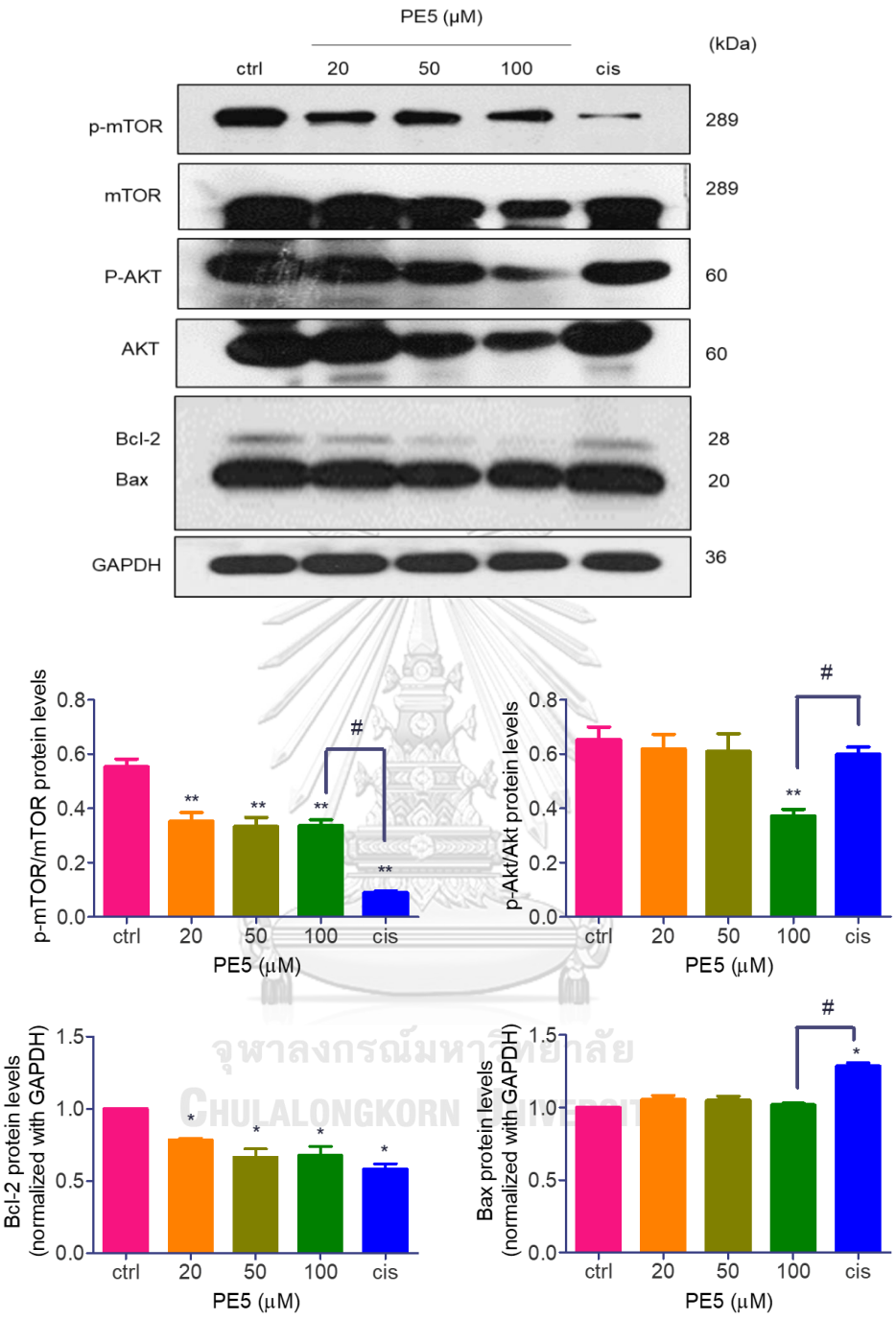


Figure 30 Effect of PE5 on key molecular protein of AKT/mTOR and down-regulation pathway. The key proteins mTOR, p-mTOR, AKT, p-AKT, Bcl-2, and Bax were determined by western blotting in H460 cells and the immunoblot signal intensities were quantified by densitometry. Data represent the mean ± SD (n = 3), (*$p < 0.05$, ** $p < 0.01$, compared with the untreated control), and (# $p < 0.05$, compared with cisplatin).

CHAPTER V
DISCUSSION AND CONCLUSION

The incidence of lung cancer is continuing to increase worldwide. At present, platinum-based drugs, such as cisplatin and paclitaxel, are commonly used for the treatment of lung cancer patients in the clinic [85]. Unfortunately, it is common for these patients to develop drug resistance. Resistance to platinum-based drugs among these patients mostly arises from a deficiency of the apoptotic pathways in their lung cancer cells [86]. Accordingly, it is important to discover novel drugs with the ability to enhance lung cancer cell death via another type of programmed cell death.

Generally, autophagy is a cellular process that is responsible for stress management and nutrient deprivation to ensure an adequate supply of the cellular basic units and bioenergy. In such a way, autophagy is recognized as a pro-survival mechanism. Previous evidence suggests that combination of autophagic inhibitor (wortmannin and chloroquine) and compound can use to sensitize cancer cell to chemotherapeutic drugs [87]. On the contrary, several evidences have shown that a long period or excessive activation of autophagy can lead to cell destruction, termed “autophagic cell death (ACD)” or “type-II cell death” [88]. However, the conclusive mechanistic pathways involved in controlling ACD as well as the cellular decision for survival or death are largely unknown and, in most cases, they rely on several

factors, including the duration and potency of autophagy induction and the cellular signaling condition [14,89] . As ACD is frequently observed as a backup cell death mechanism in drug-resistant or apoptosis-defective cancers [13,90], it may offer a new avenue for therapeutic exploitation. ACD has been shown to have significant value as a novel mechanism for cancer cell death induced by chemotherapeutics or natural compounds [89,91]. Previous studies have revealed that cellular pathways, including the adenosine monophosphate-activated protein kinase (AMPK) pathway and the phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR pathway, function in the processes of autophagy and ACD[17,92]. A recent study found that inhibition of the AKT/mTOR axis is critical for the regulation of ACD in human NSCLCs [19]. Consistently, we found that PE5 exerts its ACD induction via suppression of the AKT/mTOR pathways (Figure 27-30).

PE5 induces ACD as a major mechanism of action through the suppression of AKT-mTOR and the anti-apoptotic members of the Bcl-2 family protein. In addition, the morphology of the PE5-treated cells displayed apoptosis characteristic including membrane blebbing and DNA condensation at 50-100 μM PE5, while the cells contained excessive vacuoles (Figures 10, 14). Autophagosomes induced by PE5 exhibited a clear double membrane as observed by an electron microscope (Figures 17), with evidence from protein analysis showing the conversion of LC3-I to LC3-II (Figures 18, 20). In addition, PE5 exhibited autophagic flux in H460 cells (Figure 19).

We confirmed the involvement of autophagy in death induction by co-treatment of the compound with wortmannin, and the results indicated that when autophagy was suppressed, the cell death caused by PE5 was interrupted (Figures 21, 22). Consistent with our data, previous research found that autophagic inhibition by wortmannin could decrease autophagy-dependent cell death in Iso-GNA treated cells. Previous research found that the level of Atg7 were increased in concentration dependent manner during autophagy induction [9]. Consistent with our data, we found that PE5 increased Atg7 in H460 cells. Moreover, we confirmed that autophagy-related (ATG) proteins are involved in autophagy induced by PE5 by using siRNA (Figure 23). Consistent with our data, previous research found that siATG proteins reduced the conversion of LC3 and attenuated colon cancer cell death [10].

Proteomics is the use of quantitative protein analysis to illustrate biological processes, including drug effects and disease processes [83]. In this study, the key proteins altered by the treatment at different times were monitored in order to verify the time-dependent cellular profiles in response to cellular events at 12 and 24 h after treatment. We observed autophagy induction effect of PE5 at 12 h, while the cell viability remained 100% versus the control, suggesting that the results at this time point may reflect mainly autophagy induction or upstream regulation of the cells. However, at 24 h, when cell death was significantly found, this point may reflect mainly the mechanism of cell death. Our analysis showed that PE5 directly or

indirectly modulated several proteins functioning in apoptosis and the autophagy pathway (Figure 24-27). A previous study also used proteomics profiling to determine the effect of resveratrol, a stilbene compound, in combination with doxorubicin, showing that resveratrol effectively sensitized MCF-7 cells to cytotoxic therapy and that HSP27 inhibition enhanced the cytotoxicity of doxorubicin [93]. Among these PE5-modulated proteins, we also found proteins associated with several cellular events described in cancer cells, including apoptosis inhibition and drug resistance [94].

The mTOR protein acts as a central regulator of cell growth, proliferation, and survival [95]. Importantly, mTOR has been recognized as a key modulator of autophagy [17], and inhibition of this protein has been demonstrated to cause autophagy [19], while the induction of mTOR could reduce autophagy [96]. In terms of its mechanism, mTOR controls autophagy through the regulation of a protein complex composed of unc-51-like kinase 1 (ULK1), autophagy-related gene 13 (ATG13), and the focal adhesion kinase family-interacting protein of 200 kDa (FIP200) through phosphorylating and inhibiting this kinase complex to initiate autophagy [97,98]. ULK1 is also known to directly phosphorylate the autophagy-related gene 14 (ATG14) in an mTOR-dependent manner, thus regulating ATG14 and the class-III PI3K vacuolar protein sorting 34 (VPS34) lipid kinase activity to control the level of autophagy [99]. Hence, these kinases represent attractive targets for therapeutic treatment involving autophagy.

From the STITCH database [100], the top 20 downregulated proteins in response to PE5 that had the most protein interactions were involved in the PI3K/AKT/mTOR pathway at 12 and 24 h (Figures 28-29). Furthermore, we confirmed the proteomics results by using western blot analysis, which indicated the main mechanism of PE5 in mediating autophagy was though mTOR suppression. For cell death, we analyzed the crosstalk between apoptosis and autophagy to assess their co-relation. We found that the suppression of AKT-dependent proteins was observed at 12 h and sustained until 24 h of treatment, while the downregulation of mTOR-related proteins was found only at 12 h, suggesting that the suppression of AKT was responsible for mTOR inhibition at 12 h and may be involved in cell death at 24 h. Interestingly at 24 h, the depletion of Bcl-2 and Mcl-1 was noted, suggesting that ACD induced by PE5 may at least in part be related to Bcl-2 and Mcl-1 suppression. Regarding the effect of Bcl-2 on ACD, a previous study reported that the degradation of Bcl-2 was related to ACD and apoptosis [101]. Beclin-1 functioning in the process of autophagy was shown to be antagonized by Bcl-2 and other Bcl-2 family members, including Bcl-XL, and Mcl-1 [102,103]. The role of Bcl-2 depletion on ACD induction was previously demonstrated in breast cancer cell lines. SiRNA-mediated Bcl-2 decrease was shown to induce ACD in MCF-7 cells [21]. Likewise, Mcl-1 depletion was shown to involve the induction of ACD by SC-59, a novel sorafenib derivative, in hepatocellular carcinoma cells [104].

Previous research has shown that natural compounds from the orchid exert potential pharmacological activities [26,28]. In this study, we expanded such knowledge toward their novel activity in ACD regulation. PE5 isolated from the roots of Paphiopedilum exul was demonstrated to facilitate ACD and apoptosis in lung cancer cells, with the underlying mechanisms presented in Figure 31.

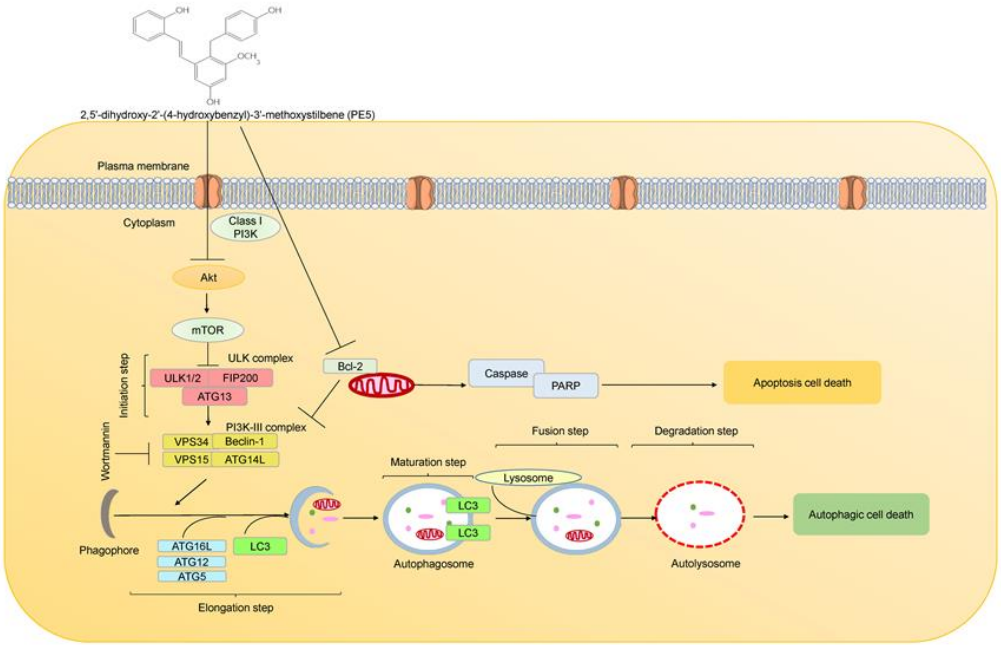


Figure 31 Scheme of PE5 induce apoptosis and autophagic cell death

These data propose the potential of PE5 as an anti-cancer approach and lead to a better understanding of ACD regulation, revealing AKT/mTOR as well as Bcl-2 suppression as therapeutic targets for driving cancer cells to autophagy-dependent cell death.

In conclusion, data from this study established that PE5, stilbene compound can induce autophagic cell death and apoptosis in NSCLCs. This evidence may value and encourage the further investigation of this useful compound to be used for anti-cancer approaches and beneficial for improving the response to conventional drugs.

APPENDIX A

PREPARATION OF REAGENTS

1. DMEM stock solution (1 L)

DMEM powder	10.4 g
NaHCO3	3.7 g
ddH2O	900 mL

Adjust pH to 7.4 with 1 N HCl or 1 N NaOH

Add ddH2O to 1 L and sterilized by filtering through a 0.2 sterile membrane filter

2. RPMI 1640 stock solution (1 L)

RPMI powder	10.4 g
NaHCO3	1.5 g
Glucose	4.5 g
Sodium pyruvate	0.11 g
HEPES (1M)	10 mL
ddH2O	900 mL

Adjust pH to 7.2 with 1 N HCl and 1 N NaOH

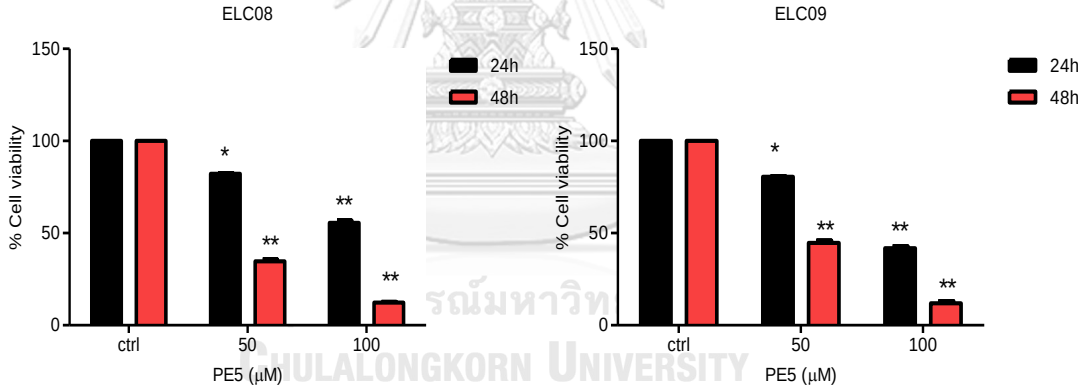
Add ddH2O to 1 L and sterilized by filtering through a 0.2 sterile membrane filter

APPENDIX B

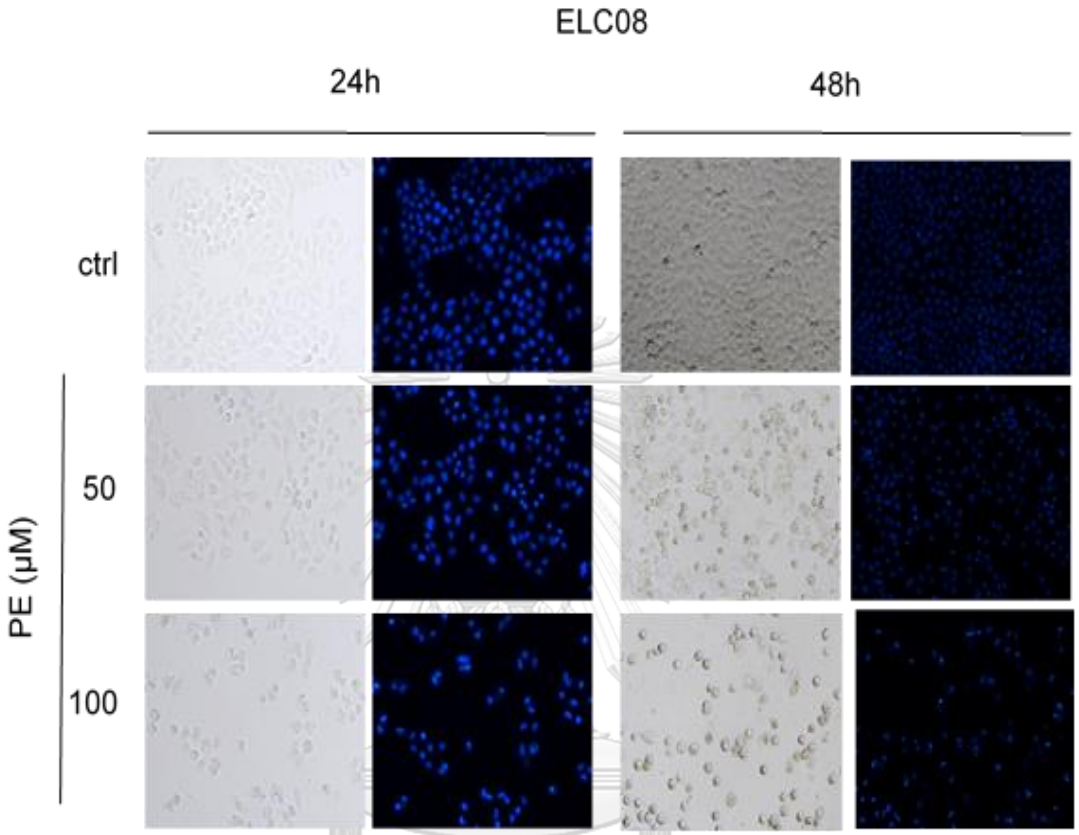
RESULTS

Appendix B-1: Cytotoxic effect of PE5 on primary lung cancer derived from lung cancer patients.

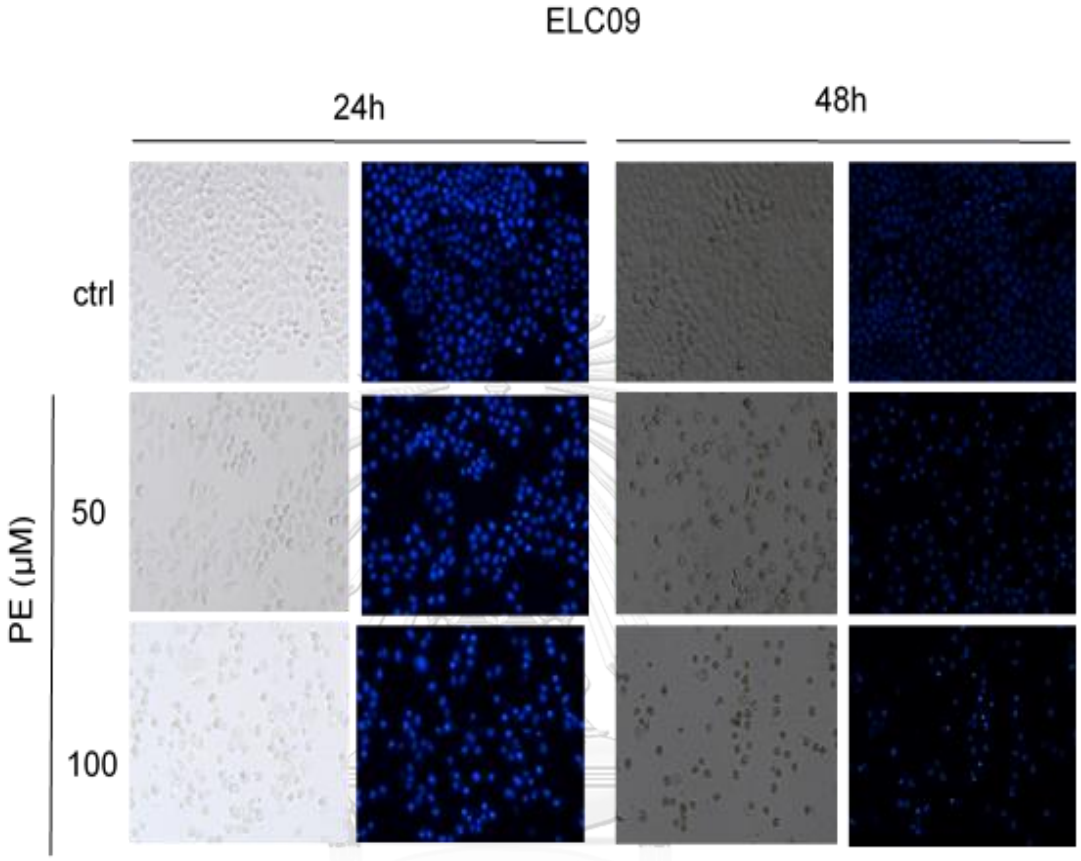
Cell	Clinical Data
ELC08	An 82-year-old male for whom pleural effusion had been collected after 3L erlotinib. In vitro drug sensitivity testing revealed pan-resistance to all potential chemotherapy.
ELC09	Naïve to chemotherapy



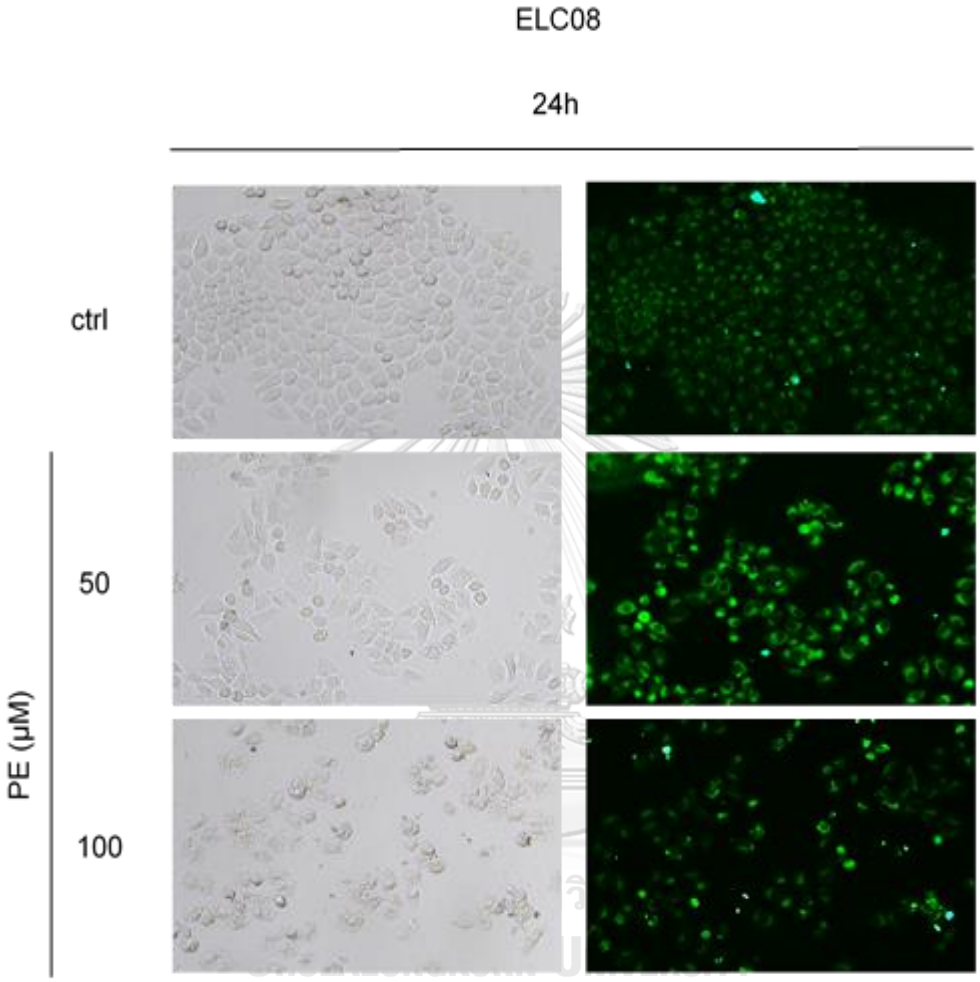
Appendix B-2: Hoechst33342 staining of PE5 on primary lung cancer derived from lung cancer patients ELC08.



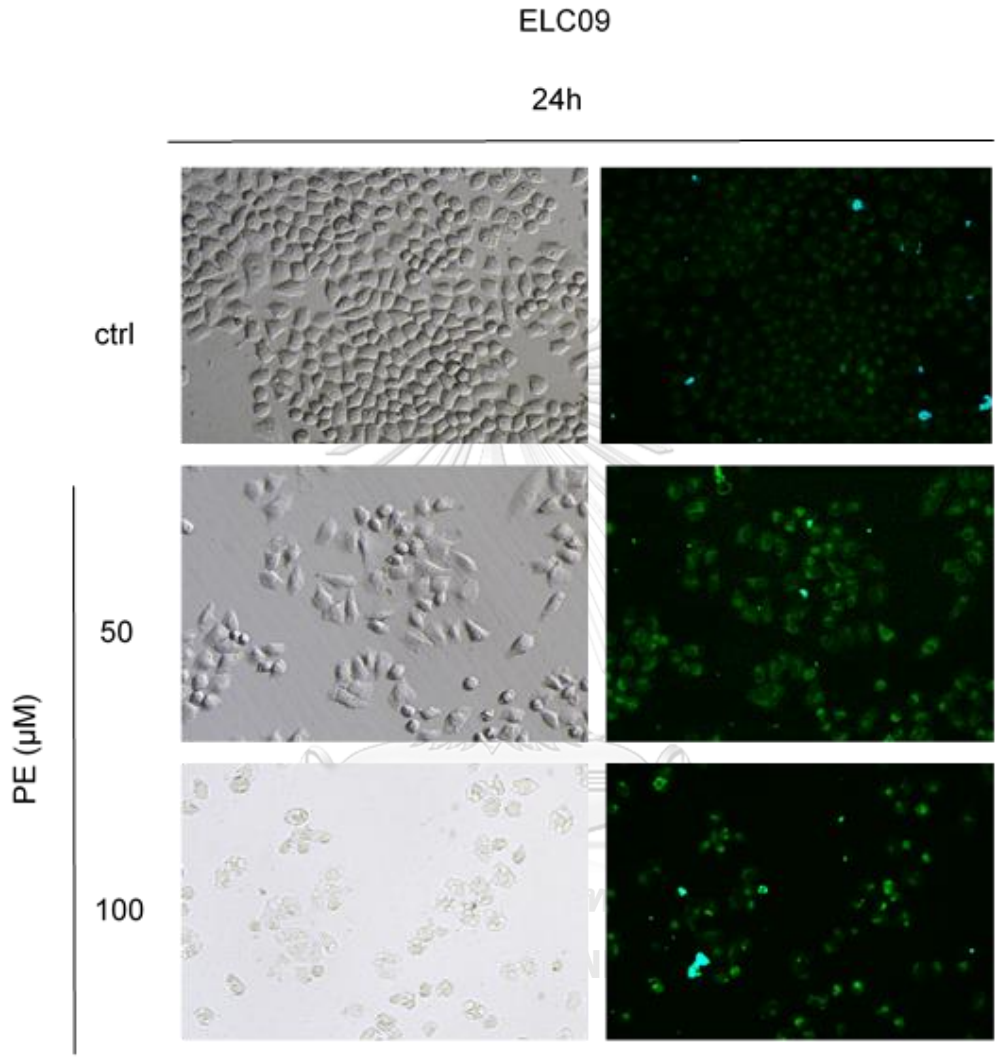
Appendix B-3: Hoechst33342 staining of PE5 on primary lung cancer derived from lung cancer patients ELC09.



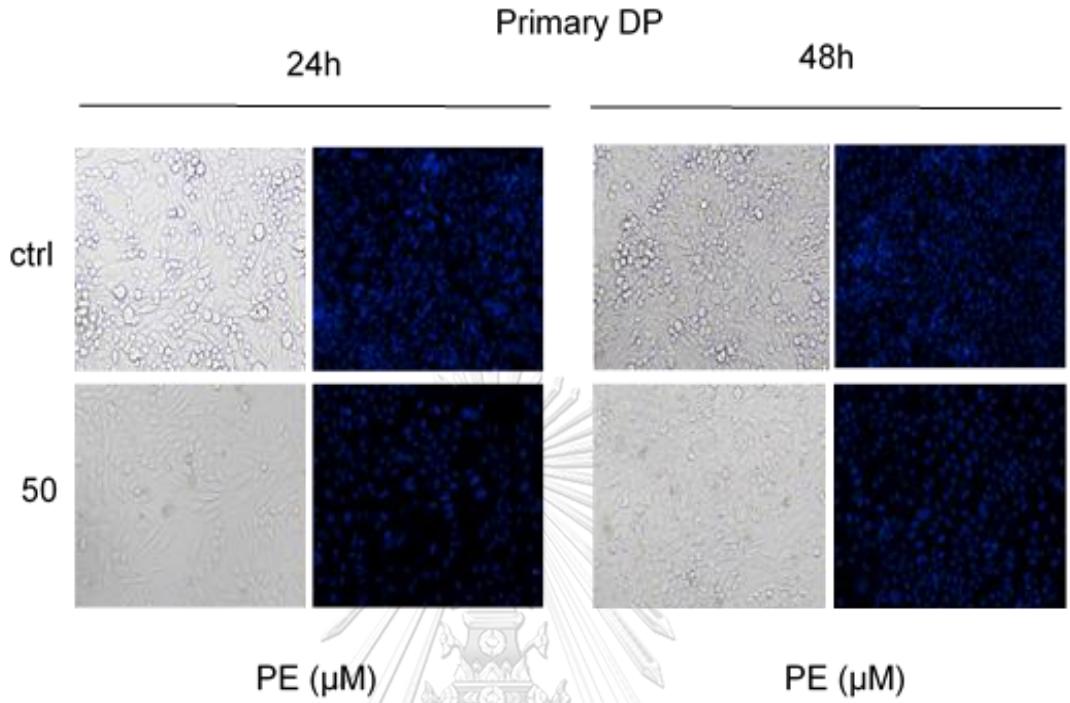
Appendix B-4: MDC staining of PE5 on primary lung cancer derived from lung cancer patients ELC 08.



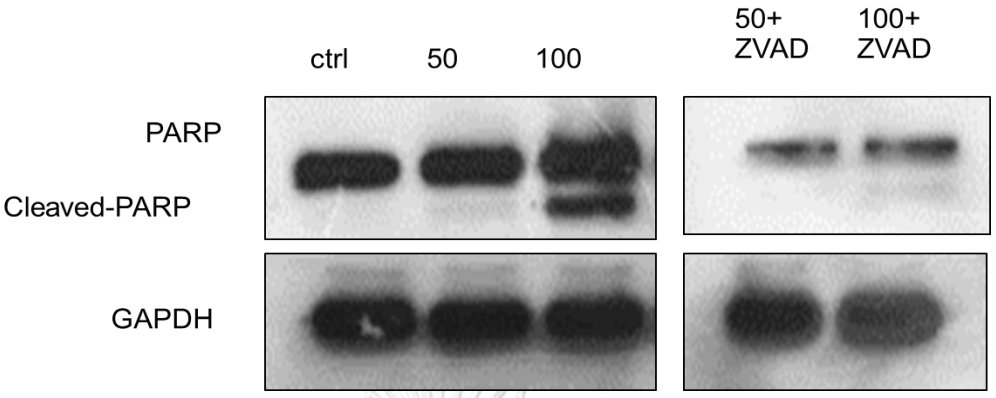
Appendix B-5: MDC staining of PE5 on primary lung cancer derived from lung cancer patients ELC 09.

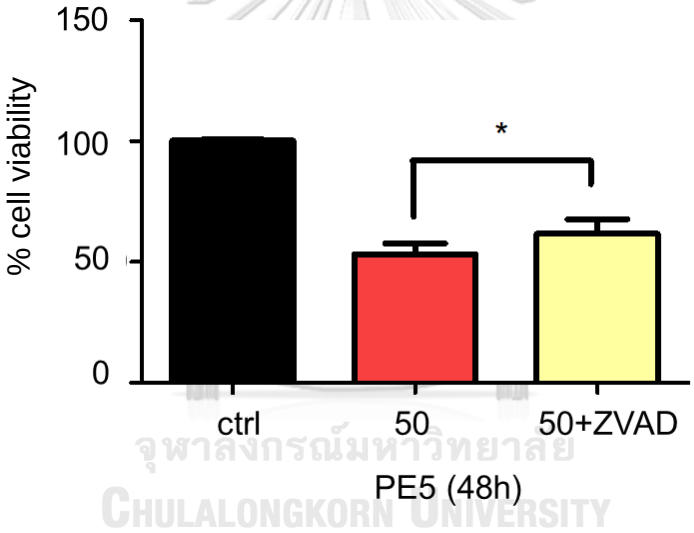


Appendix B-6: Hoechst33342 staining of PE5 on primary dermal papilla.



Appendix B-7 Effect of PE5 on apoptosis pathway with apoptosis inhibitor





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