

Anticancer activity of cepharanthine on non-small cell lung cancer cells



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จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

ฤทธิ์ต้านมะเร็งของเซฟราเรนที่ต่อเซลล์มะเร็งปอดชนิดที่ไม่ใช่เซลล์เล็ก



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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ยewearลักษณะณ์ เดชพิชัย : ฤทธิ์ต้านมะเร็งของเซฟราเรนทีนต่อเซลล์มะเร็งปอดชนิดที่ไม่ใช่เซลล์เล็ก. (Anticancer activity of cepharanthine on non-small cell lung cancer cells) อ.ที่ปรึกษาหลัก : ผศ. ดร.ปิยนุช วงศ์อนันต์

Cepharanthine (CEP) เป็นสารในกลุ่มอนุพันธ์ของอัลลาคอยด์ ที่สกัดจากรากของต้น *Stephania cepharantha* Hayata ซึ่งมีฤทธิ์ต้านมะเร็งหลายชนิด เช่น มะเร็งตับ, มะเร็งลำไส้ใหญ่และลำไส้ตรง มะเร็งเม็ดเลือดขาว และมะเร็งรังไข่ อย่างไรก็ตาม รายงานฤทธิ์ต้านมะเร็งปอดชนิดที่ไม่ใช่เซลล์เล็กของสาร CEP ยังมีอยู่อย่างจำกัด ดังนั้นในการวิจัยครั้งนี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ต้านมะเร็งของสาร CEP และกลไกการออกฤทธิ์ต่อเซลล์มะเร็งปอดชนิดที่ไม่ใช่เซลล์เล็ก ได้แก่ เซลล์ A549 ที่มี p53 และ EGFR ปกติ และเซลล์ H1975 ที่มีการกลายพันธุ์ของ p53 และ EGFR จากการศึกษาพบว่าสาร CEP สามารถยับยั้งการรอดชีวิตของเซลล์ A549 และเซลล์ H1975 ในลักษณะแปรผันตามความเข้มข้น มีค่า IC₅₀ เท่ากับ 4.31±0.52 และ 11.81±2.07 ไมโครโมลาร์ ตามลำดับ นอกจากนี้สาร CEP สามารถเหนี่ยวนำให้เซลล์ A549 และเซลล์ H1975 เกิดการตายแบบอะพอพโทซิส โดยสาร CEP มีผลเพิ่มการแสดงออกของโปรตีน Bax และ Bak ลดการแสดงออกของโปรตีน Bcl-2 และ Bcl-X_L ในเซลล์ A549 และมีผลลดการแสดงออกของโปรตีน Bcl-2 ในเซลล์ H1975 อีกทั้งสาร CEP ยังกระตุ้นให้เกิดการแตกหักของ PARP ได้ทั้งในเซลล์ A549 และเซลล์ H1975 ทั้งนี้พบว่า NAC สามารถลดผลของสาร CEP ต่อการตายแบบอะพอพโทซิส แสดงให้เห็นว่าสาร CEP สามารถเหนี่ยวนำให้เซลล์ A549 และ H1975 เกิดการตายแบบอะพอพโทซิสผ่านการสร้างอนุมูลอิสระ นอกจากนี้สาร CEP ยังสามารถยับยั้งวิถีสัญญาณ ERK ในเซลล์ A549 แต่มีผลกระตุ้นวิถีสัญญาณ ERK ในเซลล์ H1975 ยิ่งไปกว่านั้นพบว่าสาร CEP สามารถยับยั้งวิถีสัญญาณ STAT3 และ Akt ได้ทั้งในเซลล์ A549 และเซลล์ H1975 เนื่องจาก NAC สามารถป้องกันผลของสาร CEP ในการยับยั้งวิถีสัญญาณ STAT ในเซลล์ A549 และเซลล์ H1975 อีกทั้ง NAC ยังสามารถลดผลของสาร CEP ในการยับยั้งวิถีสัญญาณ Akt ในเซลล์ H1975 แสดงให้เห็นว่า ROS มีความเกี่ยวข้องกับผลของสาร CEP ในการยับยั้งวิถีสัญญาณดังกล่าว โดยสรุปจากผลการศึกษากครั้งนี้ แสดงให้เห็นว่าสาร CEP สามารถยับยั้งเซลล์มะเร็งปอดชนิดที่ไม่ใช่เซลล์เล็ก โดยชักนำให้เกิดการตายแบบอะพอพโทซิส ผ่านการสร้างอนุมูลอิสระ และไปมีผลปรับเปลี่ยนวิถีสัญญาณ STAT3, PI3K/Akt และ MAPK/ERK ได้ ซึ่งชี้ให้เห็นว่าสาร CEP อาจจะสามารถนำมาพัฒนาเป็นยารักษา มะเร็งปอดชนิดที่ไม่ใช่เซลล์เล็กได้



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Yaowaluck Detpichai : Anticancer activity of cepharanthine on non-small cell lung cancer cells. Advisor:

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Cepharanthine (CEP) is a biscochlorine alkaloid, isolated from the roots of *Stephania cepharanthine* Hayata that possesses potent anticancer properties against several types of cancer such as hepatocellular carcinoma, colorectal cancer, leukemia and ovarian cancer. However, there is still limited information regarding to its effects on non-small cell lung cancer cells. Therefore, the objectives of the present study were to determine the cytotoxicity of CEP and its underlying mechanisms in non-small cell lung cancer cells expressing wild-type p53 and EGFR (A549) and non-small cell lung cancer cells expressing mutant p53 and EGFR (H1975). The results indicated that CEP significantly inhibited the viability of A549 and H1975 cells in a concentration-dependent manner, with the inhibitory concentration at 50% cell growth (IC_{50}) values of 4.31 ± 0.52 and 11.81 ± 2.07 μ M, respectively. Moreover, apoptosis-inducing effect of CEP were detected in both A549 and H1975 cells. Western blot results demonstrated that CEP could upregulate Bax and Bak as well as downregulate of Bcl-2 and Bcl-X_L in A549 cells and downregulate Bcl-2 in H1975 cells. Also, CEP was able to induce the cleavage of poly-(ADP-ribose) polymerase (PARP) in both A549 and H1975 cells. It should be noted that apoptosis-inducing effect of CEP were diminished when the cells were pretreated with *N*-acetylcysteine (NAC), a ROS scavenger, suggesting that CEP-induced apoptosis was mediated through ROS generation. In addition, CEP inhibited ERK signaling pathway in A549 cells but activated ERK signaling pathway in H1975 cells. Moreover, CEP could inhibit STAT3 and Akt signaling pathways in both NSCLC cell lines. Remarkably, NAC could prevent CEP-mediated inhibition of STAT3 in both A549 and H1975 cells and CEP-mediated Akt inhibition in H1975 cells, indicating that ROS is involved in the inhibitory effects of CEP on STAT3 and Akt signaling pathways. Taken together, these results suggest that CEP exhibited potent cytotoxicity through induction of apoptosis, generation of ROS and modulation of STAT3, PI3K/Akt and MAPK/ERK signaling pathways in NSCLC cells. Our findings demonstrated that CEP may have a potential to be develop as a novel anticancer drug for lung cancer.

Field of Study: Pharmacology

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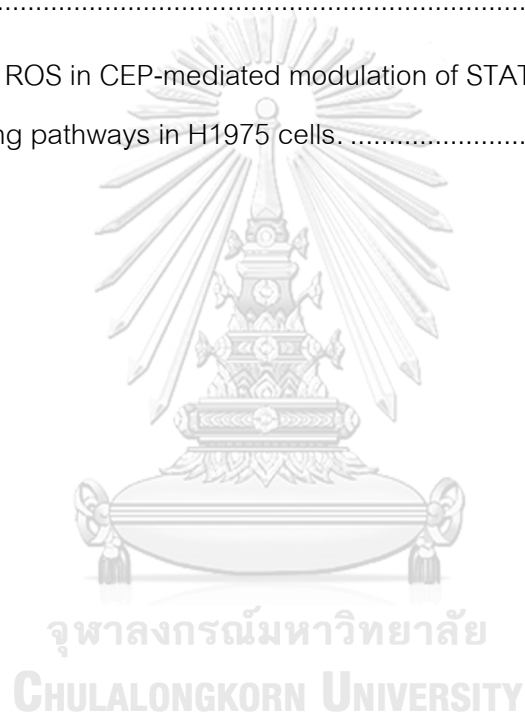
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LIST OF ABBREVIATIONS

%	Percentage
µg	Microgram
µl	Microliter
µM	Micromolar
ANOVA	Analysis of variance
APAF-1	Apoptotic protease-activating factor-1
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BAD	BCL-2 antagonist of cell death
BAK	Bcl-2 antagonist killer
BAX	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma-2
Bcl-X _L	B-cell lymphoma-extra large
BH domain	Bcl-2 homology domain
Bid	BH3-interacting domain death agonist
Bim	BCL-2-like-protein 11
Caspase	Cysteine aspartic acid specific protease
CEP	Cephranthine
CDDP	Cisplatin
c-Myc	Cellular myelocytomatosis oncogene
CO ₂	Carbon dioxide
COPD	Chronic obstructive pulmonary disease
cPARP	Cleaved poly (ADP-ribose) polymerase
DCF	Dichlorofluorescein
DCFH	Dichlorodihydrofluorescein
H ₂ DCF-DA	Dichloro-dihydro-fluorescein diacetate
DIABLO	Direct inhibitor of apoptosis-binding protein binding protein with a low isoelectric point

DISC	Death-inducing signaling complex
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FITC	Fluorescein conjugate
g/mol	Gram per mole
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Guanosine diphosphate
Grb2	Growth factor receptor-bound protein 2
GSH	Glutathione
GTP	Guanosine-5'-triphosphate
h	Hour
H ₂ O ₂	Hydrogen peroxide
HBSS	Hank's buffered salt solution
HO [•]	Hydroxyl radical
HRP	Horseradish peroxidase
IARC	International Agency for Research on Cancer
IC ₅₀	Half inhibitory concentration
LSD	Least square difference
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
mL	Milliliter
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC	N-acetylcystein

NaOH	Sodium hydroxide
NCI	The National Cancer Institute
NFDM	Non-fat dry milk
nm	nanometer
NSCLC	Non-small cell lung cancer
p53	Tumor suppressor protein 53
p-AKT	phospho-AKT
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffer saline
PDK1	Phosphoinositide-dependent kinase 1
p-ERK	phospho-ERK
PH	Pleckstrin-homology
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-trisphosphate
PKB	Protein kinase B
PS	Phosphatidyl serine
p-STAT3	phospho-STAT3
RAF	Rapidly accelerates fibrosarcoma
RAS	Rat sarcoma viral oncogene homolog
RIPA	Radioimmunoprecipitation assay
RNase	Ribonuclease
ROS	Reactive oxygen species
rpm	Round per minutes
RPMI	Roswell Park Memorial Institute
SCLC	Small cell lung cancer
SDS	Sodium dodecyl sulfate

SEM	Standard error of mean
SI	Selective index
SMAC	Second mitochondria-derived activator of caspases
SOD	Superoxide dismutase
SOS	Son of sevenless
SPSS	Statistical package for the social sciences
STAT3	Signal transducer and activator of transcription 3
TEMED	<i>N,N,N,N</i> -tetramethyl ethylenediamine
TKI	Tyrosine kinase inhibitor



CHAPTER I

INTRODUCTION

1.1 Background and rationale

Lung cancer is the leading cause of cancer-related death for both men and women [1]. In Thailand, lung cancer has been the second most leading cause of death after liver cancer [2]. The most common cause of lung cancer is long-term cigarette smoking [3]. Lung cancer is divided into two main types: small cell lung cancer (SCLC), accounting for about 15% of all lung cancer patients and non-small cell lung cancer (NSCLC), accounting for the remaining 85% [4]. Therapeutic strategies commonly used for lung cancer include surgery, radiotherapy, and chemotherapy, which can be used either alone or in combination with each other or other therapies [5]. However, these treatment types may cause different side effects such as diarrhea, anemia and neutropenia [6, 7]. Notably, targeted therapy is a novel method for cancer treatment. Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKI) such as erlotinib and gefitinib has produced significant clinical responses in NSCLC patients with EGFR mutation [8, 9]. However, patients with different EGFR mutations respond differently to EGFR-TKI treatment. Like other treatments, targeted drug can cause side effects such as rash, vomiting and mouth sores and it can be very expensive [10, 11]. Therefore, finding a novel compound with low toxicity and high selectivity for killing cancer cells is necessary.

Reactive oxygen species (ROS) contain oxygen ions including, hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot-}$), and hydroxyl radical ($\cdot OH$) [12]. Numerous studies have indicated that ROS functions as effector molecules to modulate various signaling pathways such as STAT3, PI3K/Akt and MAPK/ERK, which in turn induce apoptotic cell death [12-15]. Many chemotherapeutic agents including cisplatin and carboplatin, platinum-base drugs, have shown to inhibit cancer cell growth by inducing apoptotic cell death through ROS generation [16, 17].

Cepharanthine (CEP) is a biscochlorine alkaloid isolated from the roots of *Stephania cepharanthine* Hayata. It is also found in *Stephania venosa* Spreng and *Stephania erecta* Craib, which is widely distributed under the local name “Kra thom luead” and “Sabu leuad or blood-soap” in Thailand [18]. CEP is approved by the Japanese Ministry of Health for the treatment of many diseases including, radiation-induced leukopenia [19-22], alopecia areata [23], bronchial asthma [24] and venomous snakebites [25]. Although CEP is widely used, its side effects are very rare [24, 26]. It should also be noted that, CEP has shown to possess diverse pharmacological activities such as anti-inflammatory, anti-angiogenic, anti-oxidant, and anti-allergic effects [24, 27]. Additionally, several studies have demonstrated that CEP exhibits potent anticancer in diverse types of cancer cells such as ovarian cancer [28], nasopharyngeal carcinoma [29], oral squamous cell carcinoma [30] and cholangiocarcinoma [31] both in *in vitro* and *in vivo*. There were evidenced that CEP inhibits tumor growth via the modulation of STAT3, PI3K/Akt and MAPK/ERK signaling pathways [32-34]. For example, Chen et al. reported that CEP effectively inhibited cancer cell growth *in vitro* and *in vivo* by inhibiting STAT3 signaling pathway, leading to downregulation of the STAT3 target genes, including Bcl-X_L, c-Myc and cyclin D1 in oral squamous carcinoma [32]. Furthermore, CEP-induced apoptosis was shown to be associated with suppression of Akt and induction of ROS production in hepatocellular carcinoma [33]. In leukemia, apoptosis-inducing effect of CEP was mediated through activation of caspase-3 and cleavage of PARP and activation of p38 in both Jurkat and K562 cells and activation of ERK in K562 cells [34]. Additionally, CEP was found to induce apoptosis by modulating the expression of Bcl-2 family proteins in ovarian cancer [28] and NSCLC [35]. Remarkably, many previous studies found that ROS was partly responsible for apoptosis-inducing effect of CEP in several cancer cells, including NSCLC [35], choroidal melanoma [36] myeloma [37] and colorectal cancer [38]. Despite cytotoxicity of CEP has been extensively studied, there is still limited information regarding to anticancer activity of CEP against non-small cell lung cancer. The present

study aimed to study cytotoxic effects and the underlying mechanisms of CEP in non-small cell lung cancer cells.

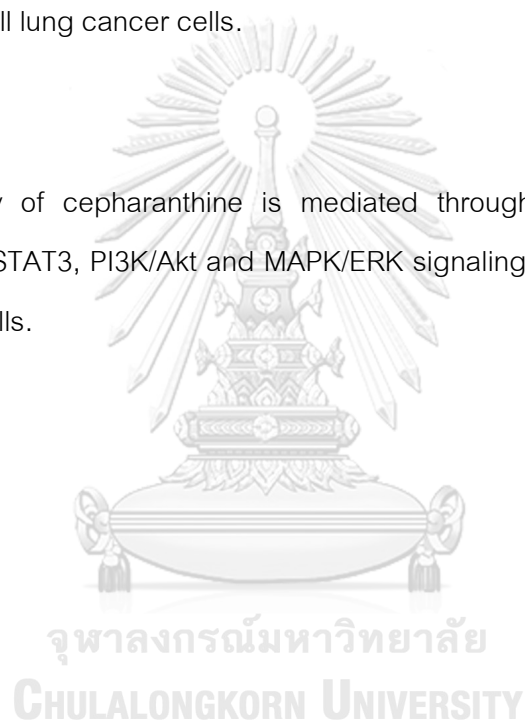
1.2 Objectives

1.2.1 To study the cytotoxic effect of cepharanthine in non-small cell lung cancer cells.

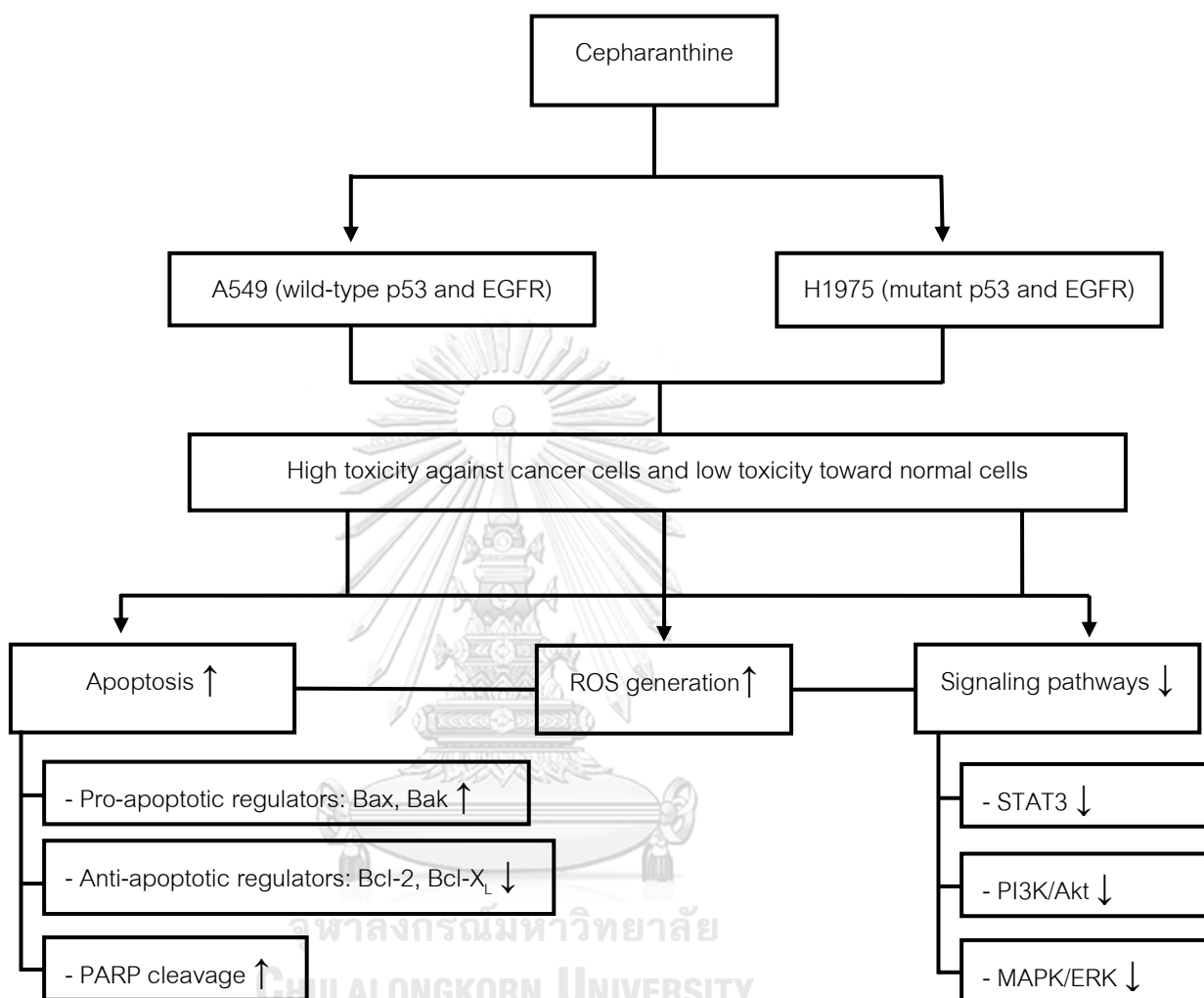
1.2.2 To determine the mechanisms underlying the cytotoxicity of cepharanthine in non-small cell lung cancer cells.

1.3 Hypothesis

Cytotoxicity of cepharanthine is mediated through generation of ROS and modulation of STAT3, PI3K/Akt and MAPK/ERK signaling pathways in non-small cell lung cancer cells.



1.4 Conceptual framework



CHAPTER II

LITERATURE REVIEWS

2.1 Lung cancer

Lung cancer is the most lethal cancer worldwide. Base on GLOBOCAN 2018 database compiled and disseminated by the International Agency for Research on Cancer (IARC), lung cancer accounts for 2.09 million new cases (11.6% of total cancer cases) and 1.76 million deaths (18.4% of total cancer deaths) (Figure 1) [1]. In Thailand, lung cancer remains the leading form of new cancer cases diagnosed in cancer patients in both men and women. Moreover, it has been the second mortality rate after liver cancer for many years [39].

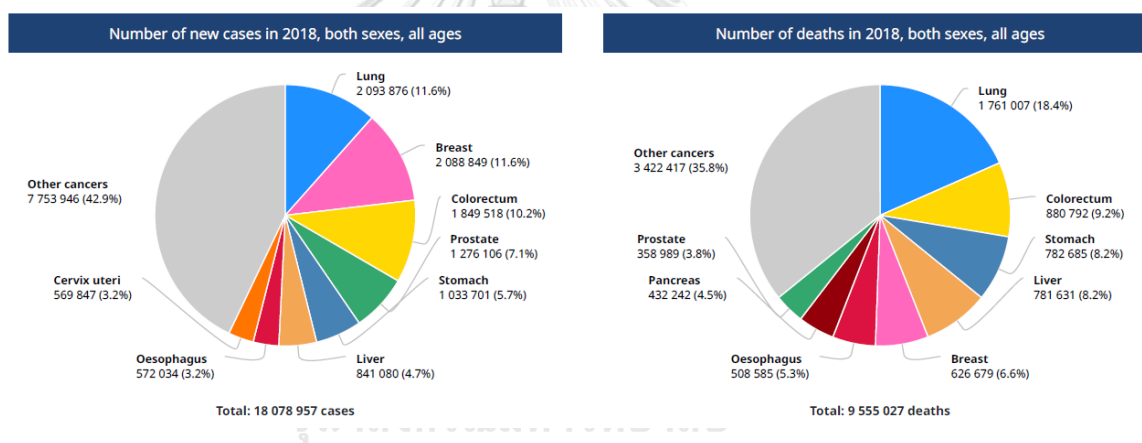


Figure 1 The number of new case patients and deaths of lung cancer

[40].

2.1.1 Types of lung cancer

Lung cancer is usually classified into two main types, called small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), depending on their morphology [4, 41-43].

1. Small-cell lung cancer (SCLC) represents about 15% to 20% of lung cancers and it is frequently found in the large airways of the lungs near the center. SCLC can grow

rapidly and spreads faster than NSCLC. It is strongly associated with a history of tobacco smoking.

2. Non-small cell lung cancer (NSCLC) comprises about 80% to 85% of lung cancers. This type of lung cancer occurs mainly in current or former smokers. It is more common in women than men. The main subtypes of NSCLC are adenocarcinoma, squamous cell carcinoma, and large cell carcinoma.

2.1) Adenocarcinoma, accounting for approximately 40% of all lung cancers, is the most general types of NSCLC and tends to grow slower than SCLC. It commonly occurs in women and people who have never smoked.

2.2) Squamous cell carcinoma, accounting for about 25%-30% of all lung cancers, occurs in epithelium of the bronchi, which are flat cells that line the inside of the airways in the lungs. It is more common in men who have history of tobacco smoking than women.

2.3) Large cell carcinoma are the least common type of NSCLC which accounts for about 10%- 15 of all lung cancers. It grows faster and spread more rapidly than other types of NSCLC, making treatment more difficult.

2.4) Other types of lung cancer such as adenosquamous carcinoma and sarcomatoid carcinoma, are much less common.

2.1.2 Risk factors for lung cancer

1. Smoking: about 80% to 90% of lung cancer deaths are caused by smoking, including the secondhand smoke.

2. Occupational exposure: exposure to some substances such as radon, silica and asbestos increase the risk of developing lung cancer among people who works in industry and construction.

3. Family history: someone who has a family member with lung cancer are more likely to be diagnosed with lung cancer than someone without a family history of lung cancer.

4. Some diseases: patients with chronic obstructive pulmonary or pulmonary tuberculosis disease are at increased risk of lung cancer [43, 44].

2.1.3 Treatments of lung cancer

The choice of treatment for lung cancer patients depends on the specific cell type and stage of cancer. The current therapeutic approaches for lung cancer comprise with surgery, radiation therapy, chemotherapy and targeted therapy [45-47].

1. Surgery is to completely remove the lung cancer and the nearby lymph nodes for early-stage NSCLC. It provides the best opportunity to treat the disease.

2. Radiation therapy is the use of high-energy radiation to kill cancer cells, especially if the cancer cannot be removed because of its size or location that cancer spread to other areas such as the brain or bone.

3. Chemotherapy is the use of drugs to kill cancer cells that are rapidly dividing and growing. SCLC are sensitive to chemotherapy more than non-small cell lung carcinoma. Most advanced NSCLC patients are treated with platinum-based chemotherapeutics. Cisplatin (CDDP) is an alkylating agent that interacts with nucleophilic N7-sites of purine bases in DNA to form inter- and intra-strand crosslinks, resulting in DNA damage and apoptosis. However, CDDP has several side effects including hair loss, mouth sores, nausea and ototoxicity [6, 7].

4. Targeted therapy is a treatment that targets specific genes that contributes to cancer growth and survival. Gefitinib and erlotinib are epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs), that are commonly used to treat NSCLC patients with common activating mutations of the EGFR gene. EGFR-TKIs act by interacting with ATP binding pocket of EGFR, blocking the signal transduction. Although targeted drugs are less harmful to normal cells, they still have side effects such as rash, diarrhea and vomiting. Moreover, they are extremely expensive [5, 8].

2.2 Apoptosis

Apoptosis is the induction of programmed cell death. It is generally characterized by cell shrinking, membrane blebbing, chromatin condensation, DNA fragmentation and formation of apoptotic bodies [48]. Many currently available chemotherapeutic drugs destroy tumor cells by inducing apoptosis [49]. Apoptosis can be divided into two major pathways, including the extrinsic and intrinsic pathways [50].

1. The extrinsic pathway

The extrinsic pathway is activated through the binding of death ligands to death receptors, resulting in the formation of the so-called death-inducing signaling complex (DISC) [48]. The recruitment of procaspase-8 to DISC results in activation of caspase, which can further directly activate caspase-3, a principle effector caspase of apoptosis [49].

2. The intrinsic pathway

The intrinsic apoptotic pathway is mediated by intracellular signals in mitochondrial. It can be initiated by DNA damage, reactive oxygen species (ROS) and chemotherapeutic agents [49, 51], which leads to activation of pro-apoptosis members of the Bcl-2 family such as Bax and Bak and inactivation of anti-apoptosis members of Bcl-2 family including Bcl-2 and Bcl-X_L, resulting in permeabilization of the outer mitochondrial membrane and the dissipation of inner membrane potential. The release of cytochrome c from the mitochondria into the cytosol promote oligomerization of a cytochrome c, Apaf-1 and procaspase-9 to form a complex, called the apoptosome. Thereafter, activated caspase-9 initiates the activation of effector caspases such as caspase-3, leading to apoptosis [52-54].

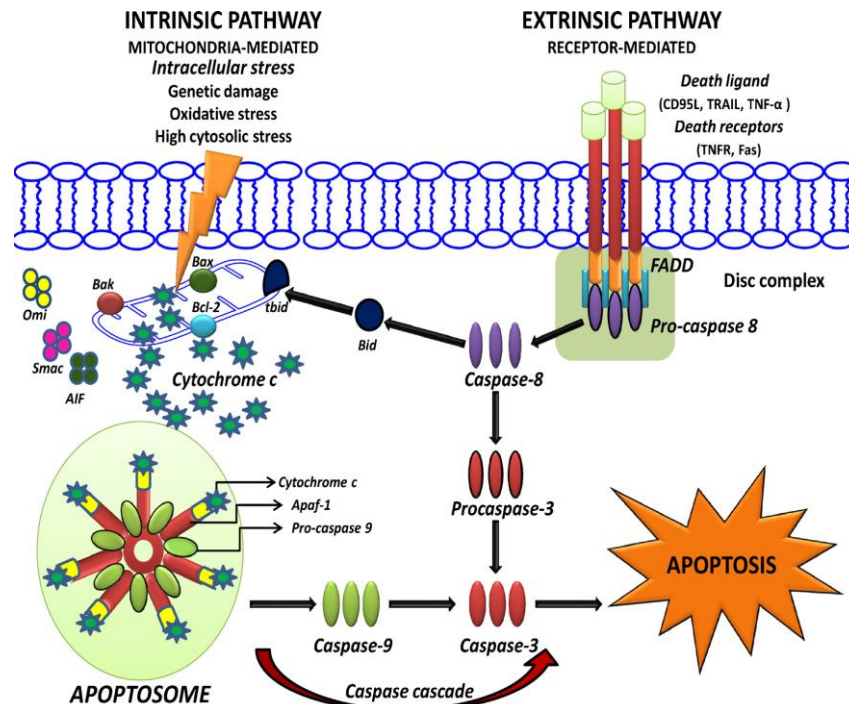


Figure 2 Extrinsic and intrinsic apoptosis pathways

[52]

The intrinsic apoptotic pathway is regulated by Bcl-2 family of proteins which are divided into 3 subgroups, based on the presence of BH domains and function [55-57].

1. Anti-apoptosis proteins, including Bcl-2, Bcl-X_L and MCL-1, contain all four BH domains (BH1-4). Their function is to preserve outer mitochondrial membrane integrity by directly binding and inhibiting the pro-apoptotic Bcl-2 proteins.

2. Pro-apoptosis effector proteins, including Bak and Bax, contain three BH domain (BH1- 4). In response to an apoptotic stimulus, these proteins undergo conformational changes that lead to Bax/Bak oligomerization in the outer mitochondrial membrane, promoting mitochondrial outer membrane permeabilization and subsequent apoptosis.

3. BH3- only proteins, including Bad, Bid, Bim and Noxa, contains only a conserved BH3 domain that can suppress anti-apoptotic proteins and induce apoptosis after receiving lethal stress.

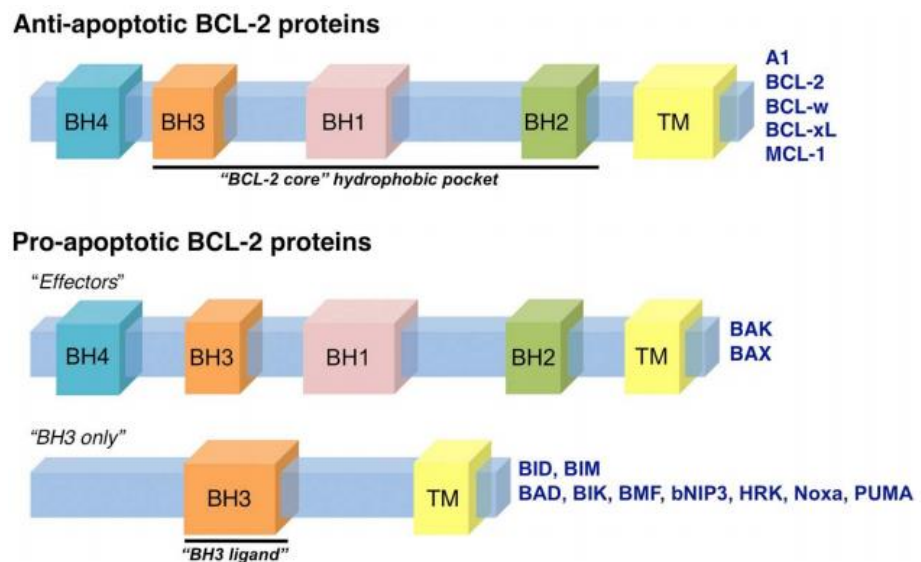


Figure 3 The Bcl-2 family of proteins

[57]

2.3 Epidermal growth factor receptor cell proliferation signaling pathways

Upon binding of a specific ligand such as epidermal growth factor (EGF) or transforming growth factor- α (TGF- α), EGFR, a transmembrane receptor tyrosine kinase protein, undergoes conformational change, causing receptor dimerization. The transphosphorylation of the cytoplasmic tyrosine kinase domain of EGFR creates binding sites for various effector molecules, resulting in activation of several downstream signaling pathways such as signal transducer and activator of transcription3 (STAT3), phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt) and mitogen-activated protein kinase (MAPK) / extracellular signal-regulated kinase (ERK) [58]. These signaling pathways play important roles in activation and regulation of cellular proliferation,

especially development and progression of NSCLC [59, 60]. In fact, overexpression or mutation of EGFR have been detected in many NSCLC patients [61, 62]. The most common EGFR mutation reported are in-frame deletion within exon19 and the L858R point mutation within exon 21 [63]. It is well known that EGFR mutations are associated with adenocarcinoma histology, women, Asians and never smoker [59]. Notably, the incidence of EGFR mutation predominantly in lung adenocarcinoma occurs in Asians, which was reported at a much higher mutation rate in Japanese, Taiwanese and Thai patients with approximately 50%, 90% and 60% respectively, whereas approximately 10% was found in Americans [64-68].

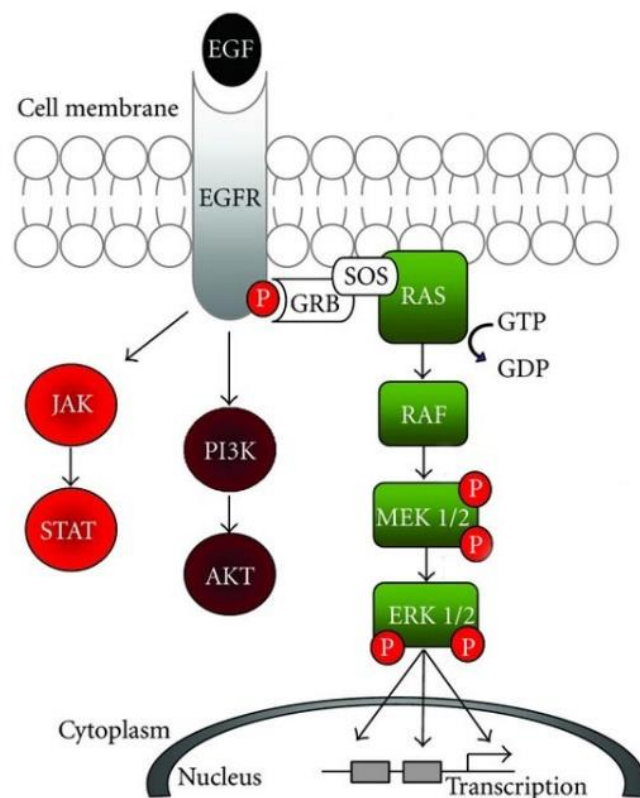


Figure 4 The EGFR signaling cascade

[69]

2.4 STAT3 signaling pathway

Signal transducer and activator of transcription 3 (STAT3) is a member of the STAT family of cytoplasmic transcription factors that promotes cell survival, proliferation, inflammation and motility [70-72]. STAT3 signaling pathway is initiated with the binding of cytokines, such as IL-6 or growth factors, such as EGF to its specific cell-surface receptors, leading to activation of receptor-associated kinases, including Janus kinases (JAK) and Src kinases [73, 74]. Phosphorylation of the tyrosine kinase receptors allow the recruitment of STAT3 to its docking sites, resulting in activation and dimerization of STAT3. These STAT3 heterodimers or homodimers can then translocate to the nucleus, eventually stimulating transcription of target genes which regulate cell differentiation, proliferation, survival and apoptosis [71, 75]. It was reported that activation and phosphorylation of STAT3 is involved in activation of cell proliferation, angiogenesis and metastasis in NSCLC [70, 76, 77]. It was also shown that STAT3 is persistently activated by approximately 22% - 65% of NSCLC and high STAT3 expression is associated with poor prognosis in patients with NSCLC [73, 78, 79].

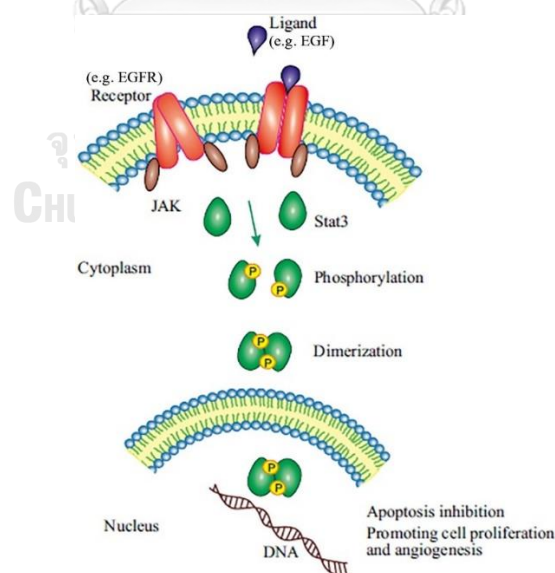


Figure 5 STAT3 signaling pathway

[71]

2.5 PI3K/Akt signaling pathway

Phosphatidylinositol-3-kinase/protein kinase B (PI3K/Akt) is one of the intracellular pathways that promotes cell survival, proliferation, migration and invasion [80]. The pathway is initially activated via binding of receptor tyrosine kinases (RTK) such as EGFR to its specific ligand, EGF. Activated RTK subsequently activate PI3K to catalyzes the phosphorylation of phosphatidylinositol bisphosphate (PIP2), forming phosphatidylinositol triphosphate (PIP3), at the plasma membrane. PIP3 serves as a docking site for pleckstrin homology domains (PH domains)-containing proteins, including phosphoinositide-dependent protein kinase-1 (PDK1) and Akt. After binding, Akt is activated by PDK1 and mammalian target of rapamycin (mTOR) at T308 and S473, respectively. Activated Akt could then translocated from the plasma membrane to the cytosol to phosphorylate several proteins, regulating cell growth and survival [81-83]. Additionally, activated Akt can directly phosphorylate a pro-apoptotic protein, Bad, resulting in the release of Bcl-X_L, anti-apoptotic protein, that is then free to inhibit apoptosis [83-85]. Several evidences suggested that PI3K/Akt signaling pathway plays important roles in promoting cell survival, cell proliferation and tumorigenesis of NSCLC. Activated Akt have been observed in approximately 50%-70% of patients with NSCLC and it was reported to be associated with poor prognosis [86].

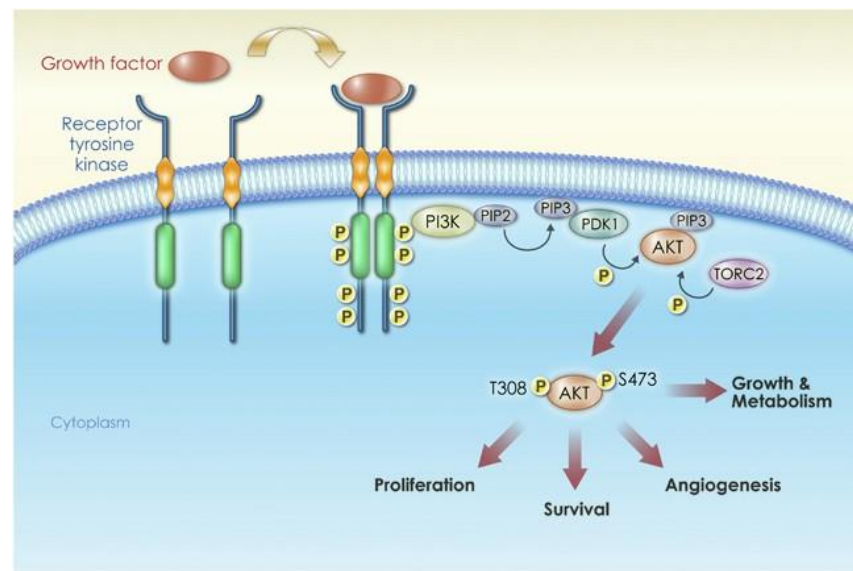


Figure 6 PI3K/Akt signaling pathway

[87]

2.6 MAPK/ERK signaling pathway

Mitogen-activated protein kinases (MAPK), serine/threonine-specific protein kinases, are involved in cell survival, differentiation, proliferation and apoptosis [88, 89]. The three most important subfamilies of MAPK pathway include the c-Jun N-terminal kinase (JNK), the p38 MAPK and the extracellular-signal-regulated kinases (ERK) [90]. These signaling processes are initiated by binding of external stimuli, for example, oxidative stress, cytokines and growth factors, such as epidermal growth factor (EGF), insulin-like growth factor and transforming growth factor, to transmembrane receptors, recruiting Son of sevenless (SOS) [91]. Then, SOS activates Ras through a conversion from the inactive GDP-bound form to the active GTP bound form. Ras subsequently recruits and activates Raf, which further activates MEK1/2, subsequently leading to activation of ERK1/2. Activated ERK1/2 can phosphorylate several substrates and regulate different transcription factors, leading to alteration of gene expression [92, 93]. The activation of ERK was found to play a role in cell survival by upregulation of anti-apoptosis Bcl-2 family proteins, including Bcl-2 and Mcl-1 and downregulation of a pro-

apoptotic Bcl-2 family member protein, Bim [93]. The MAPK/ERK pathway has been shown to be involved in progression of NSCLC [94].

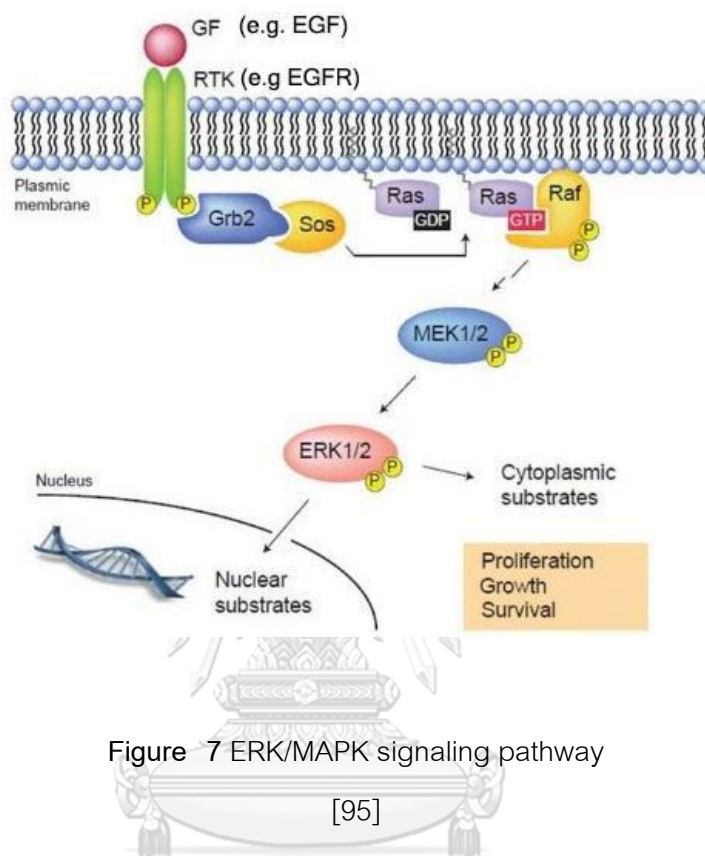


Figure 7 ERK/MAPK signaling pathway

[95]

2.7 Reactive oxygen species

Reactive oxygen species (ROS) are chemically reactive molecules containing unpaired electron which can be described as O_2 derived free radicals such as superoxide anion ($O_2^{\cdot-}$), hydroxyl radical (OH^{\cdot}), hydrogen peroxide (H_2O_2) and nitric oxide (NO^{\cdot}). They are normally found in metabolism process [96]. The Intracellular ROS levels are controlled by antioxidant enzymes including catalase (CAT), glutathione peroxidase (GPX) and superoxide dismutase (SOD) [97]. ROS are essential for regulation of normal physiological functions such as cell proliferation, differentiation, migration and cell death [98].

Roles of ROS are dependent on the concentrations of ROS. At low to moderate levels, ROS act as importance signaling molecules in essential survival pathways whereas, at higher levels, ROS can induce damage to biomolecules such as lipid, protein

and DNA, lead to cell death processes including apoptosis [98, 99]. It is known that ROS can activate various cellular signaling pathways, including STAT3, PI3K/ Akt and MAPK/ERK [96, 99]. Many chemotherapeutic agents such as CDDP, carboplatin and doxorubicin triggered apoptosis through ROS generation [100, 101]. Furthermore, a previous study has demonstrated that tetrandrine, a bisbenzylisoquinoline alkaloid could induce apoptosis through ROS-mediated PI3K/ Akt pathway in human hepatocellular carcinoma [102]. In addition, sanguinarine, a quaternary benzophenanthridine alkaloid extracted from *Sanguinaria canadensis*, induced apoptosis by inhibiting ROS-mediated ERK activation [103]. Recently, He et al. have found that apoptosis-inducing effect of chelerythrine, a natural benzophenanthridine alkaloid, was mediated via ROS-mediated STAT3 inactivation in human renal cell carcinoma [104].

2.8 Cepharanthine

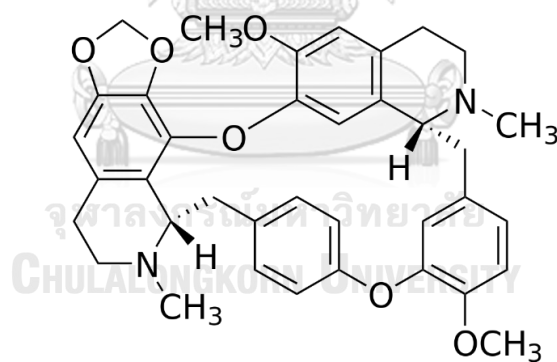


Figure 8 The chemical structure of cepharanthine

[24]

Cepharanthine (CEP) is a natural alkaloid extracted from the roots *Stephania cepharantha* Hayata that has been found in Japan, Taiwan, Laos, Thailand and a few other Southeast Asian countries [24, 105]. In Thailand, it can also be isolated from *Stephania venosa* Spreng and *Stephania erecta* Craib, which is widely distributed under

the local name “Kra thom luead” and “Sabu leuad or blood-soap” [18]. CEP is approved by the Japanese Ministry of Health for the treatment of many diseases including, radiation-induced leukopenia [19-22], alopecia areata [23], bronchial asthma [24] and venomous snakebites [25]. It also possesses a wide range of pharmacological activities, such as anti-cancer [33, 35, 106], anti-inflammation [107, 108], anti-oxidant [109], anti-malarial [110] and anti-HIV [111]. Additionally, CEP exhibits potent cytotoxicity in diverse types of cancer cells such as, leukemia [34], hepatocellular carcinoma [33], oral squamous cell carcinoma [30], cholangiocarcinoma [31] and nasopharyngeal carcinoma [29]. Previous clinical studies in patients with head and neck cancer [88], lung cancer [86] and breast cancer [87] demonstrated that CEP could effectively prevent radiation-induced leukopenia. It was safe and side effects were very rare.

Anticancer

Previous *in vitro* studies reported that CEP exhibited potent cytotoxicity against several cancer cells, including colorectal cancer (HT29, IC_{50} 5.18 μ M and COLO-205, IC_{50} 20.74 μ M) ovarian cancer (CaOV-3, IC_{50} 10.93 μ M and OVCAR-3, IC_{50} 31.20 μ M) and osteosarcoma cells (SaOS2 IC_{50} 3.18 μ M). Moreover, in nude mice xenografts with HT29 and SaOS2 cells, intraperitoneal administration of CEP inhibited the tumor volume and tumor weight while the body weight of the mice was no significantly changed [32, 112]. It was reported that CEP induced apoptosis by reducing the expression of Bcl- X_L and Bcl-2 proteins as well as inducing the expression of Bax protein in ovarian cancer cells [28]. In NSCLC, CEP could trigger apoptosis through downregulation of Bcl-2 protein and upregulation of Bax and c-PARP proteins [35]. Additionally, treatment of leukemia cells with CEP induced apoptosis by inducing of Bid and PARP cleavage and activation of p38 in both Jurkat and K562 and MAPK/ERK in K562 cells [34]. Moreover, CEP treatment was shown to inhibit Akt pathway, leading to apoptosis in hepatocellular carcinoma cells [33]. In 2012, Chen et al. demonstrated that CEP inhibited phosphorylation of STAT3, resulting

in downregulation of the STAT3 target genes, including Bcl-X_L, c-Myc and cyclin D1 [32]. Moreover, many studies illustrated that CEP induced apoptosis through ROS production in several cancer cells including NSCLC [35], choroidal melanoma [36] and myeloma [37].



CHAPTER III

MATERIALS AND METHODS

3.1 Equipment

- Analytical balance 0.001 g (Mettler Toledo, Switzerland)
- Analytical balance 0.00001 g (Sartorius, Germany)
- Autoclave (Sanyo, Japan)
- Autopipette (Brand, Germany)
- Biohazard laminar flow hood (Labconco, USA)
- Centrifuge (Hettich, Germany)
- CO₂ incubator (Thermo, USA)
- Controller pipette (Gilson, USA)
- Electrophoresis system (Bio-Rad, USA)
- Fluorescence microplate reader (Thermo, Finland)
- Fluorescence flow cytometer (BD Bioscience, USA)
- Light microscope (Nikon, Japan)
- Microplate reader (Thermo, Finland)
- Temperature control centrifuge (Eppendorf, Germany)
- Vortex mixer (Scientific Industries, USA)

3.2 Materials

- 6 well plate (Corning Inc., USA)
- 15 mL conical tube (Corning Inc., USA)
- 25 and 75 cm² rectangular cell culture flask (Corning Inc., USA)
- 96 well black polystyrene plate (Corning Inc., USA)
- 96 well plate (Corning Inc., USA)
- Polyvinylidene difluoride (PVDF) membrane (Merck, Germany)

3.3 Reagents

- Ammonium persulfate (Sigma, USA)

- Annexin V, fluorescein conjugate (FITC) (Invitrogen, USA)
- Anti-IgG, horseradish peroxidase (HRP)-linked antibody (Cell Signaling Technology, USA)
- Bromophenol blue (Sigma, USA)
- β -mercaptoethanol (Sigma, USA)
- *Cis*-diammineplatinum (II) dichloride (Sigma, USA)
- Dimethyl sulfoxide (DMSO) analytical grade (Merck, Thailand)
- Dimethyl sulfoxide (DMSO) molecular grade (Sigma, USA)
- 2,7-dichloro-dihydro-fluorescein diacetate (Sigma, USA)
- Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA)
- Fetal bovine serum (Gibco, USA)
- Glycerol (Sigma, USA)
- Glycine (Sigma, USA)
- Hank's buffered salt solution (HBSS) (Sigma, USA)
- Western horseradish peroxidase substrate (Merck, Germany)
- *N,N,N,N*-tetramethyl ethylenediamine (TEMED) (Sigma, USA)
- *N*-acetylcysteine (Sigma, USA)
- Penicillin-streptomycin (Gibco, USA)
- Primary antibodies (Cell Signaling Technology, USA)
 - AKT (pan) rabbit monoclonal antibody
 - BAK rabbit monoclonal antibody
 - BAX rabbit monoclonal antibody
 - Bcl-2 rabbit monoclonal antibody
 - Bcl-X_L rabbit monoclonal antibody
 - GAPDH rabbit monoclonal antibody
 - p44/42 MAPK (Erk1/2) rabbit monoclonal antibody
 - PARP rabbit monoclonal antibody
 - Phospho-AKT rabbit monoclonal antibody
 - Phospho-STAT3 rabbit monoclonal antibody

- Phospho-p44/42 MAPK (Erk1/2) rabbit monoclonal antibody
- STAT3 rabbit monoclonal antibody
- Propidium iodide (BD Pharmingen, USA)
- Protease inhibitors (Sigma, USA)
- Protein assay kit (Bio-Rad, USA)
- Protogel (National Diagnostic, USA)
- RIPA lysis buffer (Thermo scientific, USA)
- Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, USA)
- Secondary antibody (Cell Signaling, USA)
 - Anti-IgG, horseradish peroxidase (HRP)-linked antibody
- Sodium dodecyl sulfate polyacrylamide gel (SDS) (EM science, USA)
- Thiazolyl blue tetrazolium bromide (MTT) (Sigma, USA)
- Tris base (Vivantis, USA)
- Triton X-100 (Sigma, USA)
- Trypsin/EDTA (Gibco, USA)
- Tween 20 (Sigma, USA)
- 0.4% Trypan blue dye (Sigma, USA)

3.4 Methods

3.4.1 Preparation of cepharanthine and cisplatin

Stock solutions at 50 mM of cepharanthine (CEP) and cisplatin (CDDP) were prepared in dimethyl sulfoxide (DMSO). In the experiments, the stock solution was diluted in culture medium to give appropriate final concentrations. The 0.2% DMSO was used as a vehicle control.

3.4.2 Cell culture

Human NSCLC adenocarcinoma cell lines, A549 and H1975 as well as the human normal lung myofibroblast cells (MRC-5) were from the American Type Culture Collection (ATCC Manassas, VA, USA). A549 cells were maintained in Dulbecco's modified Eagle's

medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 μ /mL streptomycin. H1975 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 μ /mL streptomycin. MRC-5 cells were maintained in Minimum Essential Media (MEM) with supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 μ /mL streptomycin. Cells were maintained at 37°C in a humidified 5% CO₂ atmosphere.

3.4.3 Cytotoxicity assay

The cytotoxic effects of CEP were determined using methyl thiazolyl tetrazolium (MTT) assay. Briefly, A549, H1975 or MRC5 cells were seeded at a density of 3×10^4 cells/mL in 96-well plates and incubated at 37°C and 5% CO₂ overnight. Then, the cells were treated with CEP or CDDP (positive control) at concentrations of 0.1, 1, 10 and 100 μ M or 0.2% DMSO in complete medium for 48 h. After that 15 μ l of MTT (5 mg/mL) was added into each well. After 4 h incubation, the medium was discarded and 150 μ l of DMSO was added into each well. The optical density was measured at a wavelength of 570 nm using a microplate reader (Thermo, Finland). The percentage of cell viability was calculated using the following equation:

$$\% \text{ cell viability} = (\text{Absorbance}_{\text{Sample}} / \text{Absorbance}_{\text{Control}}) \times 100$$

The values of half inhibitory concentration (IC₅₀) were obtained using GraphPad Prism 7 (GraphPad Software, USA).

The selective index (SI) was calculated according to the following equation:

$$\text{SI} = \text{IC}_{50} \text{ for normal cells} / \text{IC}_{50} \text{ for cancer cells}$$

3.4.4 Apoptosis analysis using Annexin V-FITC/PI staining

A549 or H1975 cells were seeded at a density of 3×10^4 cells/mL in 6-well plates and incubated at 37°C and 5% CO₂ overnight. A549 cells were treated with CEP at concentrations of 2.5, 5 and 10 μ M or CDDP at 10 μ M for 24 h whereas H1975 cells were

treated with CEP at concentrations of 5, 10 and 20 μM or CDDP at 20 μM for 24 h. After treatment, the cells were collected by trypsinization and centrifugation at 1,500 rpm for 5 min. Subsequently, the cells were washed with ice-cold PBS twice and re-suspended in Annexin V binding buffer. The cells were stained with 3 μl of Annexin V FIT-C (Invitrogen, USA) and 1 μl of PI (Santa Cruz Biotechnology, USA) for 15 min in the dark. The stained cells were analyzed using FACS Calibur. (BD, Franklin Lakes, NJ)

3.4.5 Intracellular reactive oxygen species (ROS) generation using H₂DCF-DA assay

A549 or H1975 cells were seeded at a density of 3×10^4 cells/mL in 6-well plates and incubated at 37°C and 5% CO₂. After overnight incubation, the cells were incubated with H₂DCF-DA (Sigma, USA) in Hank's buffered salt solution (Sigma, USA) at 37°C for 30 min in dark. Then, the cells were washed with PBS. A549 cells were treated with CEP at concentrations of 2.5, 5 and 10 μM or 10 μM of CDDP whereas H1975 cells were treated with CEP at concentrations of 5, 10 and 20 μM , 20 μM of CDDP or 500 μM of H₂O₂ (positive control) for 1 h. After incubation, the cells were collected by trypsinization and centrifugation at 1,500 rpm for 5 min. The cells pellets were re-suspended with 300 μl of PBS. The fluorescence intensity was analyzed by FACS Calibur. (BD, Franklin Lakes, NJ)

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3.4.6 Evaluation of protein expression using western blotting

A549 and H1975 cells were seeded at a density of 3×10^4 cells/mL in 6-well plates and incubated at 37°C and 5% CO₂ overnight. A549 cells were treated with CEP at concentrations of 2.5, 5 and 10 μM or 10 μM of CDDP whereas H1975 cells were treated with CEP at concentrations of 5, 10 and 20 μM or 20 μM of CDDP for 24 h. After incubation, the cells were lysed with RIPA buffer (Thermo scientific, USA) containing protease inhibitors (Sigma, USA) and incubated on ice for 1 h. The cellular lysates were centrifuged at 14,000 rpm (4 °C) for 20 min. Then, the supernatants were collected and the protein contents were determined by Bio-Rad DC Protein assay reagents (Bio-Rad, USA) using

bovine serum albumin as a standard. Total protein (20 µg) were separated by 8% SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked with 3% non-fat dry milk (NFDM) for 1 h and then incubated with appropriate primary antibodies, including BAK, BAX, Bcl-2, Bcl-X_L, cleaved PARP, AKT, phospho-AKT, MAPK (Erk1/2), phospho-ERK1/2, STAT3, phospho-STAT3 or GAPDH (1:1000 dilution, Cell signaling, USA) at 4°C overnight. After incubation, the membrane was washed with TBST buffer and incubated with HRP-conjugated secondary antibody (1:2000 dilution, Cell signaling, USA) at room temperature for 1 h. Finally, the target proteins were detected using Luminata Crescendo Western HRP substrate (Merck, Germany) and analyzed using Image Studio software (LI-COR, Lincoln, NE, USA). GAPDH was used as an internal control for protein normalization.

3.4.7 Statistical analysis

All data are expressed as mean ± standard error of mean (SEM) from three independent experiments performed in triplicate. Statistical analysis of data was performed by one-way analysis of variance (ANOVA) followed by LSD using SPSS statistics 21 software (IBM Corporation, USA). Differences were considered significant if *p* value was < 0.05.

CHAPTER IV

RESULTS

4.1 Cytotoxicity of cephranthine (CEP) and cisplatin (CDDP) on non-small cell lung cancer cell lines and normal cells

The anticancer effects of CEP and CDDP on A549 cells expressing wild-type p53 and EGFR and H1975 cells expressing mutant p53 and EGFR were examined by MTT assays. As shown in Figures 9A and B, the incubation of A549 and H1975 cells with 0.1, 1, 10 and 100 μM of CEP or CDDP for 48 h decreased cell viability in a concentration-dependent manner. CEP and CDDP at 10 and 100 μM significantly inhibited the growth of A549 and H1975 cells ($P < 0.001$). Notably, 10 μM of CEP decreased viability of A549 and H1975 cells by approximately 50% and 20%, respectively, suggesting that CEP was more toxic to A549 cells than H1975 cells.

In addition to NSCLC cell lines, the cytotoxic effects of CEP and CDDP were tested on MRC5 human lung fibroblast cells. The results demonstrated that CEP and CDDP significantly inhibited the growth of MRC5 cells in a concentration-dependent manner (Figures 10A and B, $P < 0.05$).

The half maximal inhibitory concentration (IC_{50}) of CEP and CDDP on NSCLC and normal cells were listed in Table 1. The selectivity index (SI) values, the ratio of IC_{50} for normal cells to that for NSCLC cells, for CEP and CDDP on A549 cells were 1.29 and 0.89, respectively, while they were 0.47 and 0.59 for H1975 cells. Taken together, the obtained results suggest that CEP is as safe as CDDP, a commercially available chemotherapeutic drug used for NSCLC.

Table 1 IC_{50} and selectivity index values of CEP and CDDP on A549, H1975 and MRC5 cells.

Drugs	IC_{50} values (μM)			SI INDEX	
	A549	H1975	MRC5	A549	H1975
CEP	4.31 \pm 0.52	11.81 \pm 2.07	5.54 \pm 0.22	1.29	0.47
CDDP	13.37 \pm 1.46	20.32 \pm 1.51	11.89 \pm 0.52	0.89	0.59

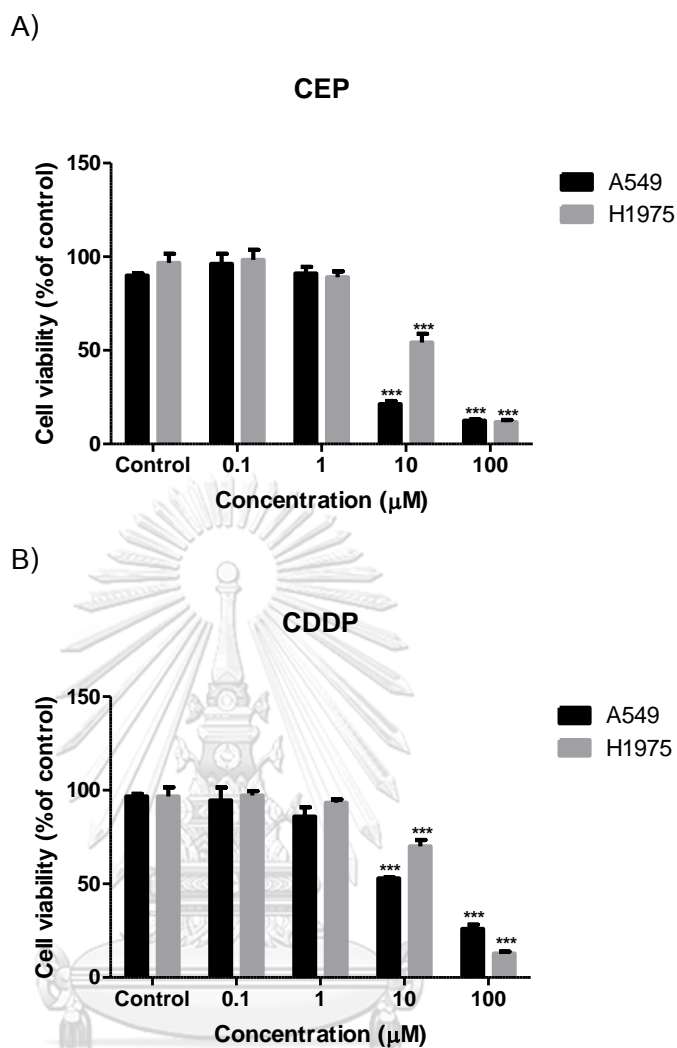


Figure 9 Effects of CEP and CDDP on viability of A549 and H1975 cells.

The cells were treated with CEP or CDDP at 0.1, 1, 10 and 100 µM for 48 h. Cell viability was determined using MTT assays. Data are the mean \pm SEM (n=3). ***P<0.001 compared with vehicle control (0.2% DMSO).

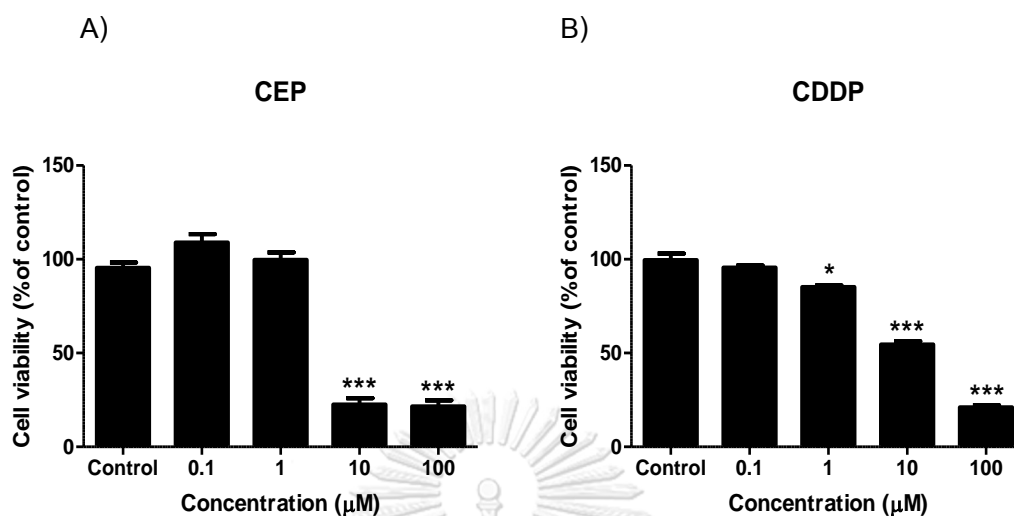


Figure 10 Effects of CEP and CDDP on viability of MRC5 cells.

The cells were treated with CEP or CDDP at 0.1, 1, 10 or 100 μM for 48 h. Cell viability was determined using MTT assays. Data are the mean \pm SEM ($n=3$). * $P<0.05$, *** $P<0.001$ compared with vehicle control (0.2% DMSO).

4.2 Effect of CEP on ROS generation in A549 cells

Elevated ROS levels can induce cell death. It is known that many chemotherapeutic drugs used in cancer therapy induce apoptosis via ROS generation [6]. Previous studies demonstrated that CEP could increase ROS level in many cancer cells, such as choroidal melanoma [36] and colorectal cancer [38]. Therefore, the effects of CEP on ROS generation in A549 cells were investigated in this study. The cells were treated with CEP at 2.5, 5 and 10 μM , CDDP at 10 μM or H_2O_2 at 500 μM for 1 h. The levels of ROS were determined by flow cytometry using $\text{H}_2\text{DCF-DA}$ as a fluorescent probe. As shown in Figure 11A, CEP at 10 μM and H_2O_2 at 500 μM significantly increased ROS levels by 3.3 and 2.4-fold, respectively when compared with the vehicle control ($P<0.05$).

We then determined whether cytotoxicity of CEP is mediated through ROS generation in A549 cells. The cells were pretreated with 2.5 mM of *N*-acetyl-L-cysteine (NAC; a ROS scavenger) for 2 h before incubation with 2.5, 5 and 10 μM of CEP or 10 μM

of CDDP for 24 h. The results obtained using MTT assay indicated that the cytotoxicity of CEP and CDDP on A549 cells was significantly decreased by the presence of NAC (Figure 11B, $P < 0.001$). These results suggest that cytotoxicity of CEP was partly mediated through ROS production in A549 cells.

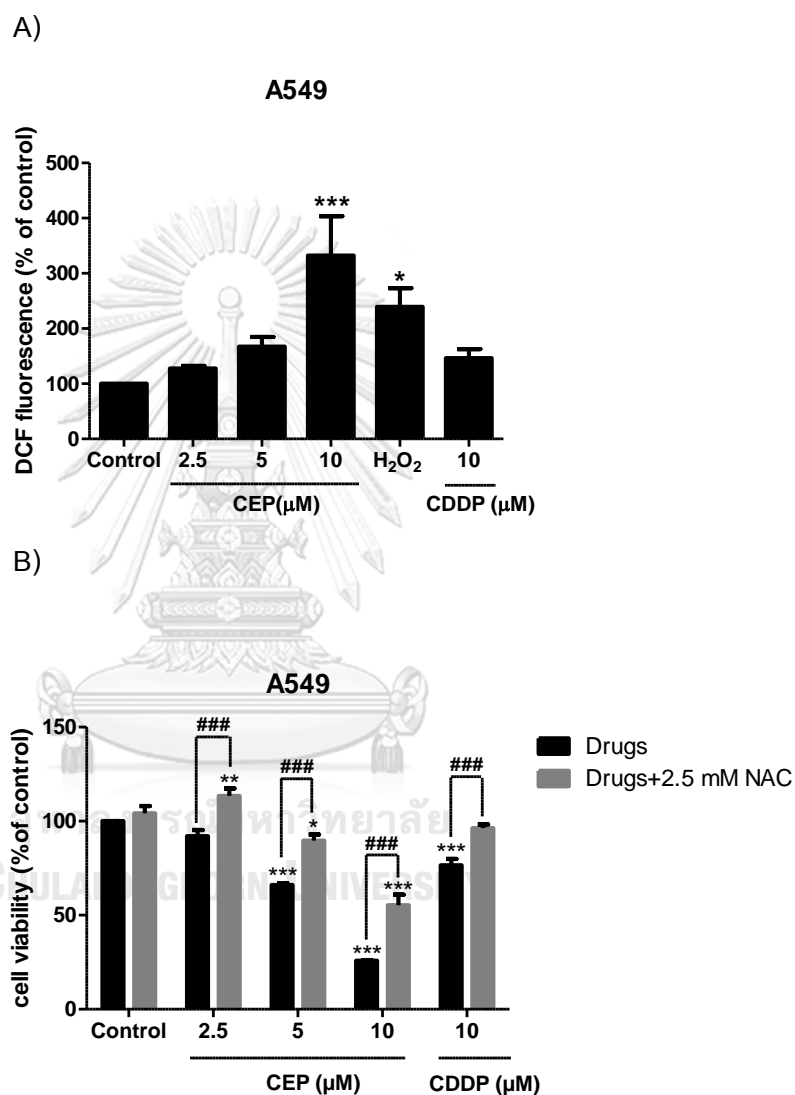


Figure 11 Effect of CEP on ROS generation in A549 cells.

A) The cells were treated with CEP at 2.5, 5 and 10, CDDP at 10 μM or H₂O₂ at 500 μM for 1 h. The levels of ROS were stained with H₂DCF-DA and analyzed with flow cytometer. B) The cells were treated with CEP at 2.5, 5 and 10 or CDDP at 10 μM for 24 h in the absence or presence of 2.5 mM NAC. The cell viability was determined using MTT assays. Data

are the mean \pm SEM (n=3). *P<0.05, **P<0.01, ***P<0.001 compared with vehicle control (0.2% DMSO). ####P<0.001 compared with drugs alone.

4.3 Effect of CEP on apoptosis induction in A549 cells

Most anticancer drugs currently used in clinic can induce cell death by apoptosis [49]. Thus, apoptosis-inducing effect of CEP was determined in A549 cells. After incubation with 2.5, 5 and 10 μ M of CEP or 10 μ M of CDDP for 24 h, apoptotic cells were determined by staining cells with annexin V and propidium iodide (PI) followed by flow cytometry analysis. As shown in Figure 12, no significant increase in apoptotic cells was observed after treatment with CDDP at 10 μ M. Conversely, CEP at 10 μ M significantly induced apoptosis by approximately 4-fold compared to the vehicle control (P<0.001). To further confirm apoptosis-inducing effect of CEP, the cleavage of poly (ADP-ribose) polymerase (PARP), an established and reliable apoptosis indicator downstream of caspase activation [113], were evaluated by monitoring the expression of cleaved PARP using western blot analysis. As shown in Figure 13, CEP and CDDP at 10 μ M significantly induced PARP cleavage. The expression of cleaved PARP proteins were approximately 25 and 20-fold above the vehicle control, respectively (P<0.05), highlighting that induction of apoptosis is partly associated with anticancer effect of CEP in A549 cells.

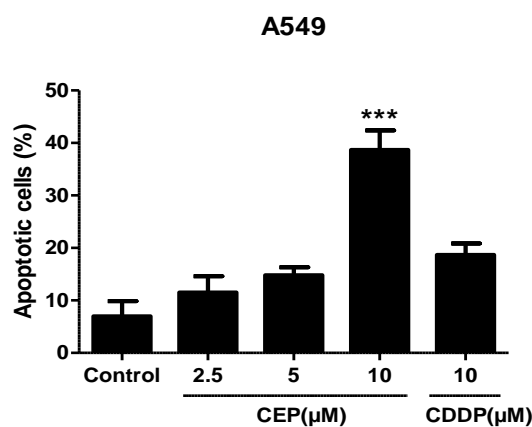


Figure 12 Apoptosis-inducing effect of CEP on A549 cells.

The cells were treated with CEP at 2.5, 5 and 10 μM or CDDP at 10 μM for 24 h and apoptotic cells were determined using annexin V-FITC/PI staining followed by flow cytometry analysis. Data are the mean \pm SEM (n=3). ***P<0.001 compared with vehicle control (0.2% DMSO).

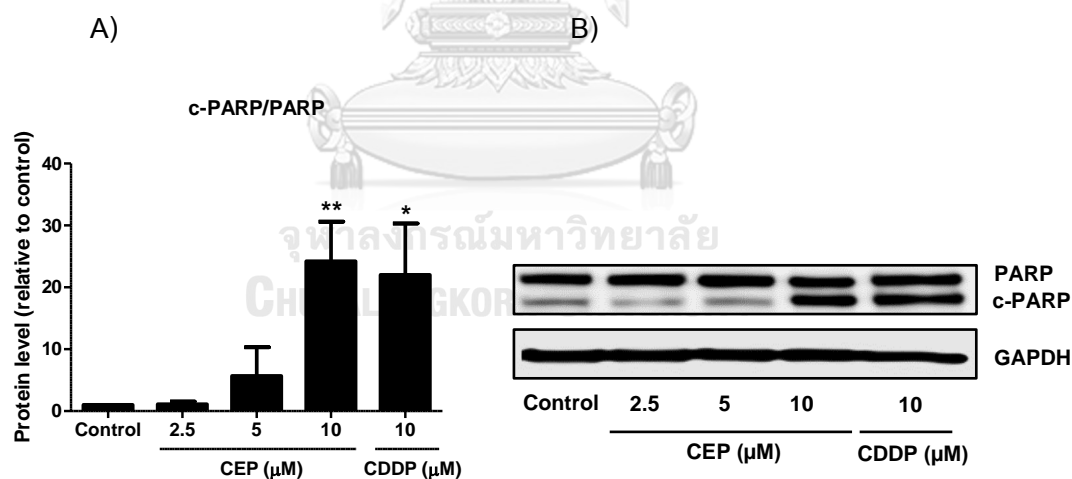


Figure 13 Effect of CEP on PARP cleavage in A549 cells.

The cells were treated with CEP at 2.5, 5 and 10 μM or CDDP at 10 μM for 24 h. A) The expression of PARP and cleaved PARP were determined by western blotting. B) Representative immunoblots corresponding to cleaved PARP. Data are the mean \pm SEM (n=3). *P<0.05, **P<0.01 compared with vehicle control (0.2% DMSO).

4.4 Effect of CEP on the expression of apoptotic proteins in A549 cells

Intrinsic apoptosis is known to be regulated by the balance of anti- and pro-apoptotic proteins in the Bcl-2 family [56]; therefore, the present study evaluated the expression of pro-apoptotic proteins, including Bax and Bak and pro-apoptosis proteins, including Bcl-2 and Bcl-X_L in CEP-treated A549 cells using western blot analysis. As shown in Figures 14A-C, CEP significantly upregulated the expression of pro-apoptotic proteins. Treatment of A549 cells with CEP increased the protein levels of Bax and Bak by approximately 2 and 6-fold above the vehicle control group, respectively ($P < 0.05$). Notably, CDDP at 10 μM significantly upregulated the expression of Bax protein ($P < 0.01$) but did not alter the expression of Bak protein in A549 cells. Along with pro-apoptotic proteins, CEP at 10 μM significantly downregulated the expression of anti-apoptotic proteins, Bcl-2 and Bcl-X_L to approximately 3 times less than the vehicle control (Figures 15A-C). In contrast, CDDP at 10 μM has no effect on the expression of Bcl-2 and Bcl-X_L proteins in A549 cells. Taken together, these results suggest that CEP triggered apoptosis by modulating the expression of Bcl-2 family proteins in A549 cells.

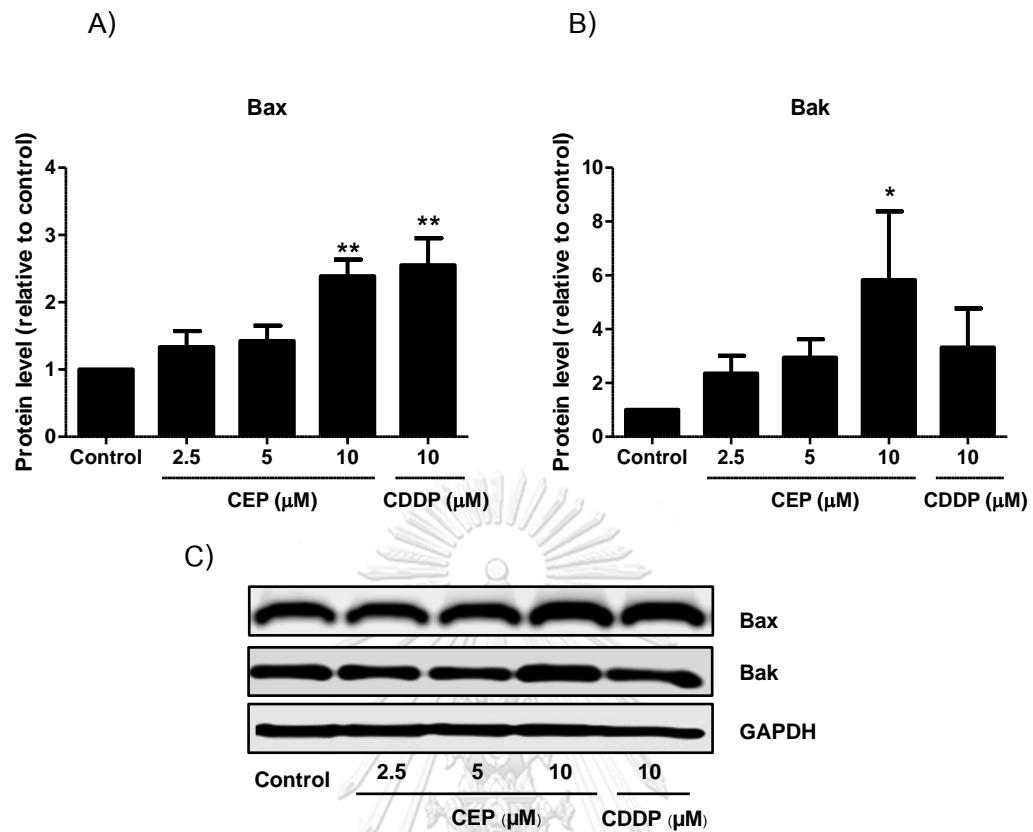


Figure 14 Effects of CEP on the expression of pro-apoptotic proteins in A549 cells.

The cells were treated with CEP at 2.5, 5 and 10 μ M or CDDP at 10 μ M for 24 h. The levels of pro-apoptotic proteins, including, A) Bax and B) Bak were determined by western blot analysis. C) Representative immunoblots to Bax and Bak. Data are the mean \pm SEM (n=3). *P<0.05, **P<0.01 compared with vehicle control (0.2% DMSO).

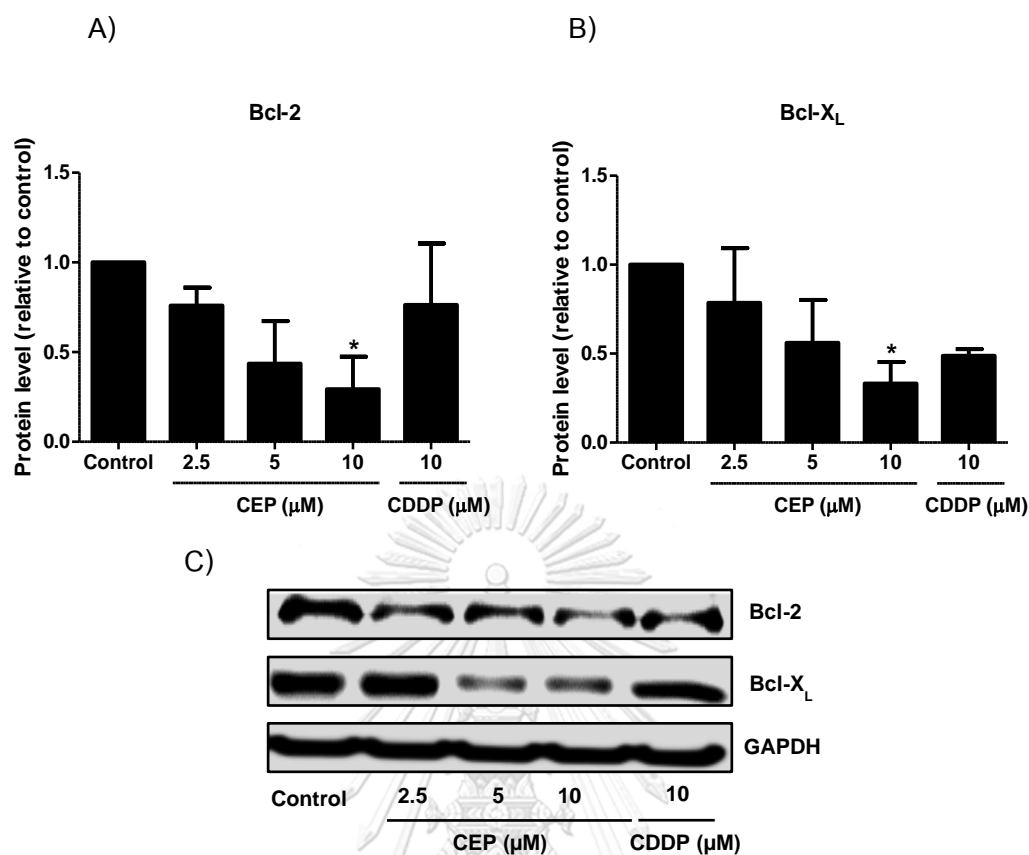


Figure 15 Effects of CEP on the expression of anti-apoptotic proteins in A549 cells. The cells were treated with CEP at 2.5, 5 and 10 μM or CDDP at 10 μM for 24 h. The levels of anti-apoptotic proteins, including A) Bcl-2 and B) Bcl-X_L were determined by western blot analysis. C) Representative immunoblots corresponding to Bcl-2 and Bcl-X_L. Data are the mean \pm SEM (n=3). *P<0.05 compared with vehicle control (0.2% DMSO).

4.5 Role of ROS in apoptosis-inducing effect of CEP in A549 cells

Previous studies reported that CEP induced apoptosis through ROS generation in myeloma [37] and non-small cell lung cancer [35]. Therefore, this study investigated whether ROS generation is involved in CEP-induced apoptosis in A549 cells. The cells were pretreated with 2.5 mM NAC for 2 h before incubation with 2.5, 5 and 10 μM of CEP or 10 μM of CDDP for 24 h. Then, apoptotic cells were investigated by staining with annexin V/PI followed by flow cytometry analysis. As shown in Figure 16, the results illustrated that NAC prevented CEP-induced apoptosis. Pretreatment with NAC

significantly decreased the percentages of apoptotic cells by approximately 2 and 2.5-fold when compared with treatment with CEP at 5 and 10 μM , respectively ($P < 0.001$). Conversely, pretreatment with NAC did not affect CDDP-induced apoptosis. These findings suggest that CEP-induced apoptosis is partly mediated through ROS generation in A549 cells.

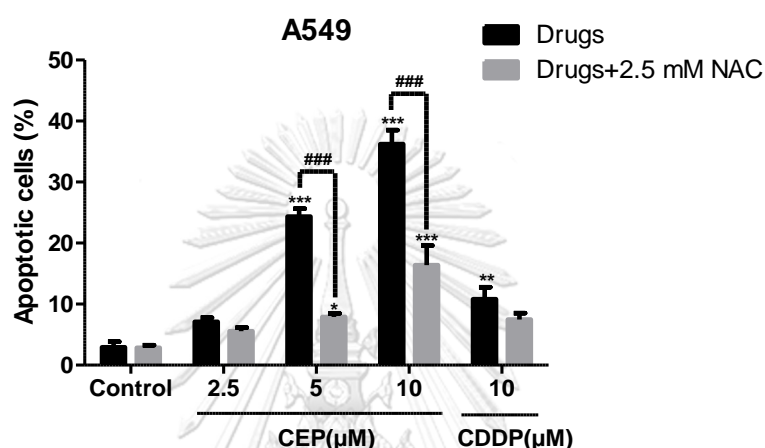


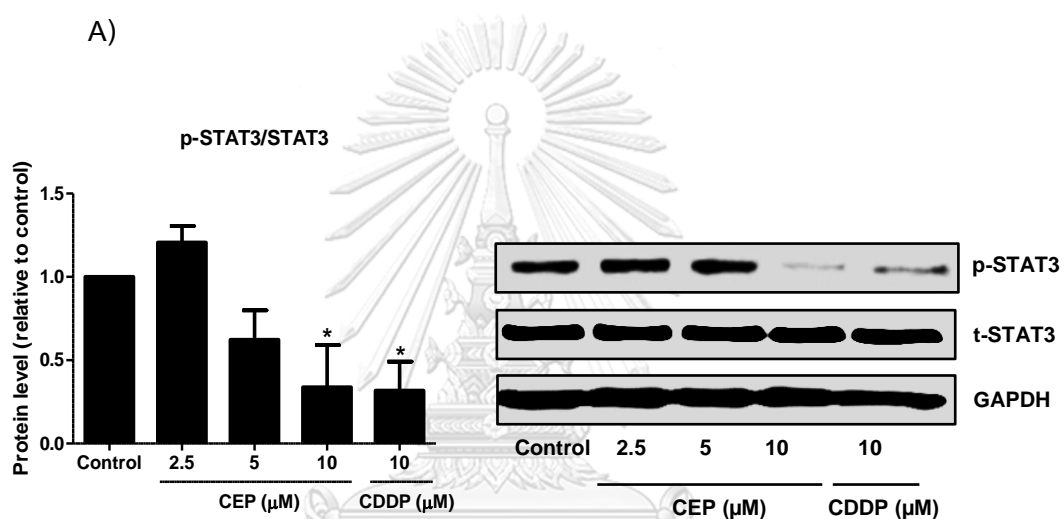
Figure 16 Role of ROS in apoptosis-inducing effect of CEP in A549 cells.

The cells were treated with CEP at 2.5, 5 and 10 μM or CDDP at 10 μM for 24 h in the absence or presence of 2.5 mM NAC. The apoptotic cells were examined by flow cytometry analysis after annexin V-FITC/PI staining. Data are the mean \pm SEM ($n=3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with vehicle control (0.2% DMSO). #### $P < 0.001$ compared with drugs alone.

4.6 Effect of CEP on STAT3, PI3K/Akt and MAPK/ERK signaling pathways in A549 cells

STAT3, PI3K/Akt and MAPK/ERK signaling pathways have been associated with tumor growth by induction of cell proliferation and inhibition of apoptosis [77, 114, 115]. Recent studies have reported that CEP induced apoptosis by modulating STAT3, PI3K/Akt and MAPK/ERK signaling pathways in osteosarcoma cells [32], human hepatocellular carcinoma cells [33] and human leukemia cells [34], respectively. To investigate the effect of CEP on STAT3, PI3K/Akt and MAPK/ERK signaling pathways in A549 cells, the

expression of p-STAT-3, p-Akt, p-ERK proteins in the cells treated with 2.5, 5 and 10 μM of CEP or 10 μM of CDDP for 24 h were evaluated using western blot analysis. Figures 17A-C exhibited that CEP at 10 μM significantly inhibited the phosphorylation of STAT3, Akt and ERK whereas CDDP at 10 μM significantly inhibited the phosphorylation of STAT3 and Akt ($P < 0.05$). Taken together, it is likely that cytotoxicity of CEP is mediated through inhibition of STAT3, PI3K/Akt and MAPK/ERK pathways in A549 cells.



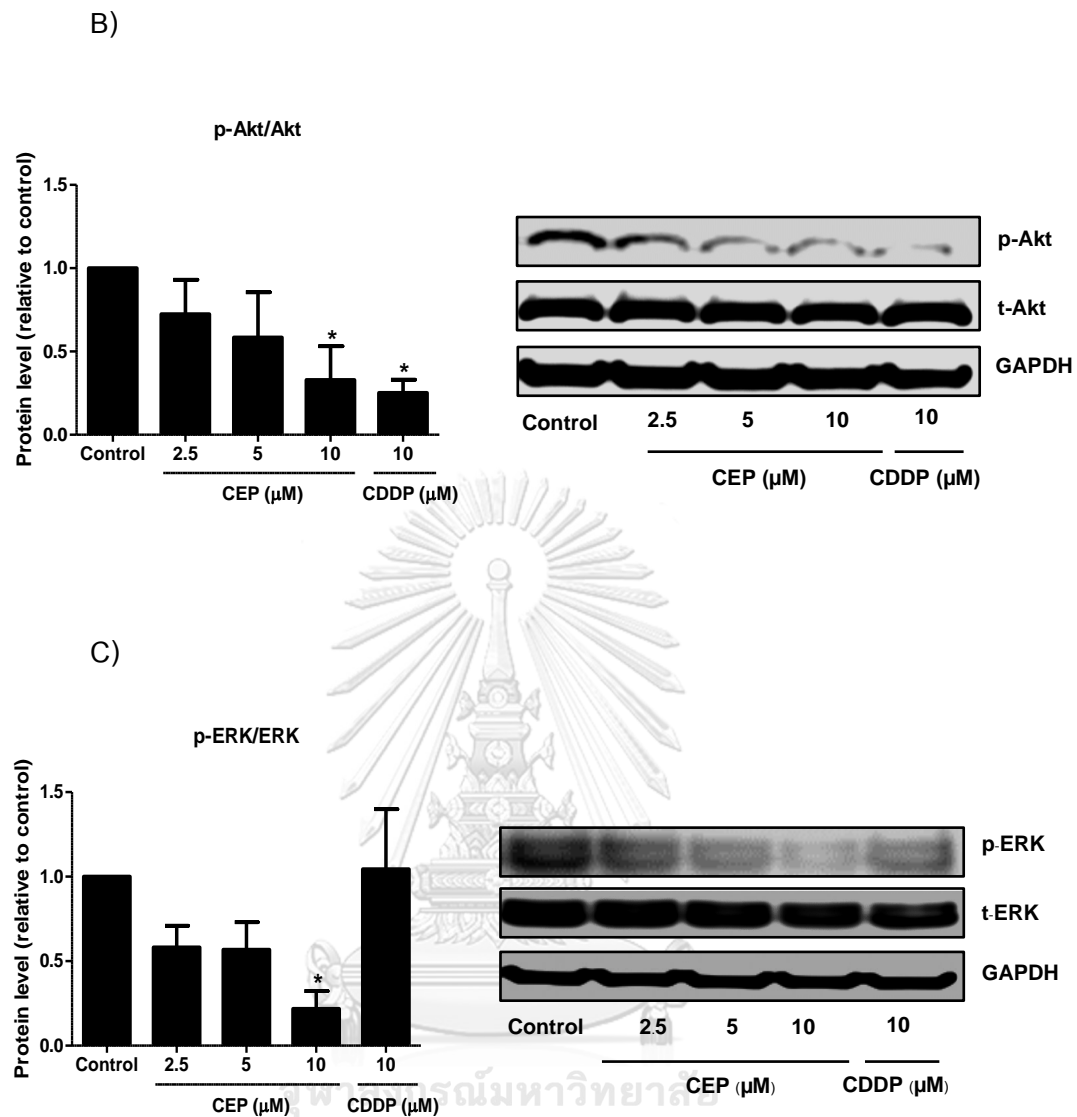


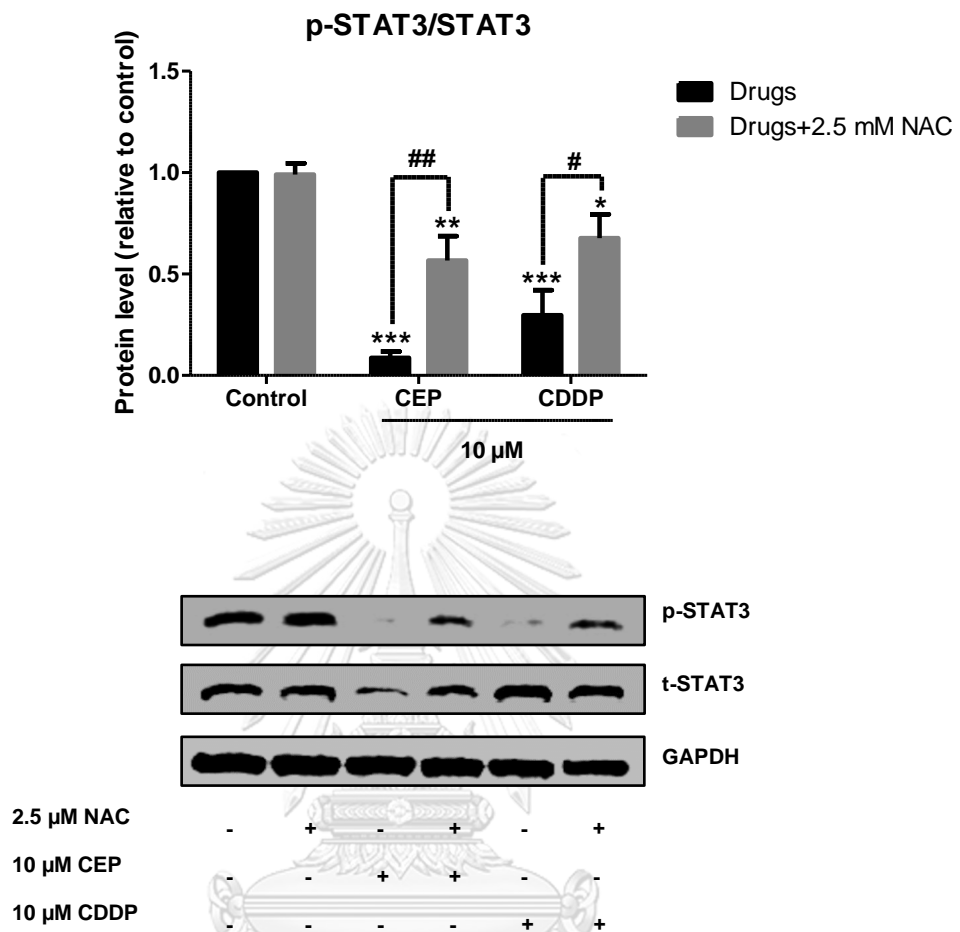
Figure 17 Effects of CEP on STAT3, PI3K/Akt and MAPK/ERK signaling pathways in A549 cells.

The cells were treated with CEP at 2.5, 5 and 10 μM or CDDP at 10 μM for 24 h. The protein expression of A) p-STAT3 and t-STAT3 B) p-Akt and t-Akt C) p-ERK and t-ERK were determined by western blotting. Representative immunoblots corresponding to p-STAT3, t-STAT3, p-Akt, t-Akt, p-ERK and t-ERK. Data are the mean \pm SEM (n=3). *P<0.05 compared with vehicle control (0.2% DMSO).

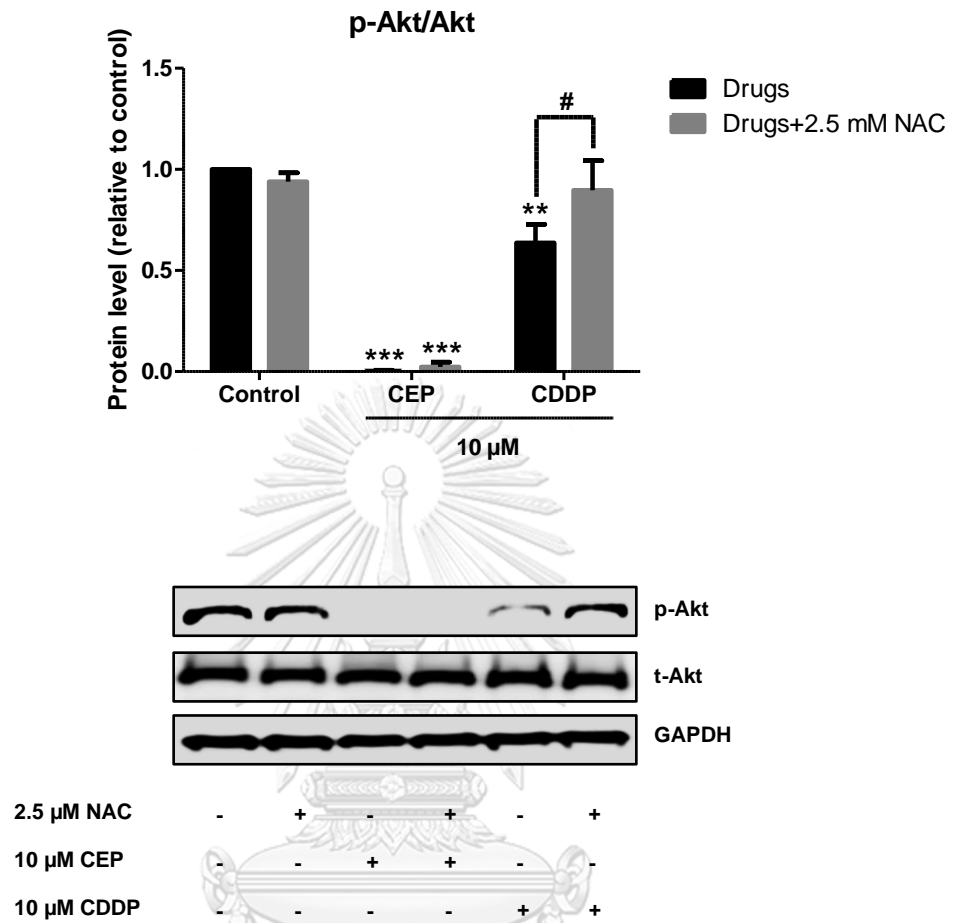
4.7 Role of ROS in CEP-mediated inhibition of STAT3, PI3K/Akt and MAPK/ERK signaling pathways in A549 cells

Previous studies showed that alkaloids modulated STAT3, PI3K/Akt and MAPK/ERK signaling pathways via ROS generation [102-104]. To investigate the roles of ROS in CEP-mediated STAT3, PI3K/Akt and MAPK/ERK signaling pathways inhibition in A549 cells, the cells were pretreated with 2.5 mM NAC for 2 h before treatment with 2.5, 5 and 10 μ M of CEP or 10 μ M of CDDP for 24 h. Then, the protein expression levels were evaluated using western blot analysis. As shown in Figures 18A-C, pretreatment with NAC significantly prevented the CEP-mediated STAT3 inhibition ($P < 0.01$). However, pretreatment with NAC did not affect CEP-mediated inhibition of Akt and ERK. Notably, NAC significantly prevented CDDP-mediated inhibition of STAT3 and Akt but did not affect CDDP-mediated ERK inhibition ($P < 0.05$). These results suggest that ROS is likely involved in the inhibitory effects of CEP on STAT3 signaling pathway in A549 cells.

A)



B)



C)

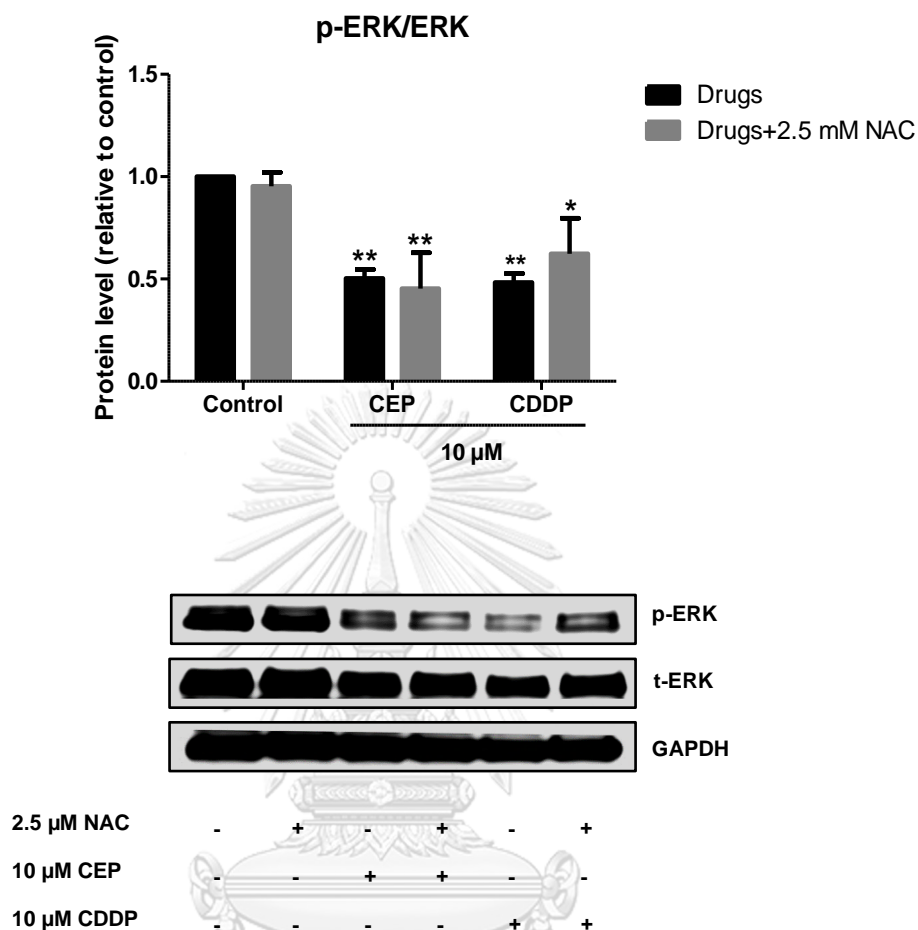


Figure 18 Role of ROS in CEP-mediated inhibition of STAT3, PI3K/Akt and MAPK/ERK signaling pathways.

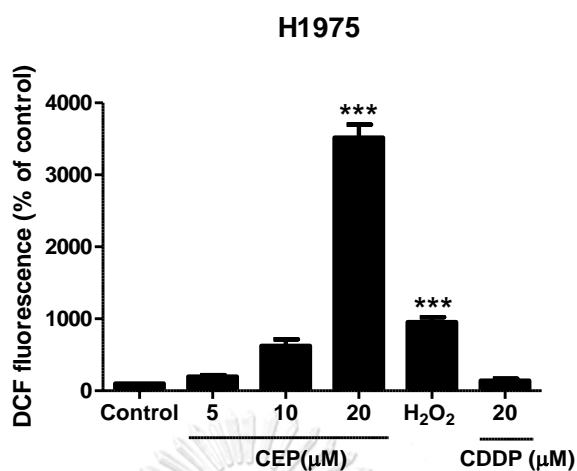
The cells were treated with CEP at 2.5, 5 and 10 μ M or CDDP at 10 μ M for 24 h in the absence or presence of 2.5 mM NAC. The protein expression of A) p-STAT3 and t-STAT3 B) p-Akt and t-Akt C) p-ERK and t-ERK were determined by western blotting. Representative immunoblots corresponding to p-STAT3, t-STAT3, p-Akt, t-Akt, p-ERK and t-ERK. Data are the mean \pm SEM (n=3). *P<0.05, **P<0.01, ***P<0.001 compared with vehicle control (0.2% DMSO). #P<0.05, ##P<0.01 compared with drugs with NAC.

4.8 Effect of CEP on ROS generation in H1975 cells

To investigate the effect of CEP on ROS generation in H1975 cells, the cells were treated with CEP at 5, 10 and 20 μM , CDDP at 10 μM or H_2O_2 at 500 μM for 1 h and the levels of ROS were determined using $\text{H}_2\text{DCF-DA}$ as a probe. As shown in Figure 19A, CEP at 20 μM and H_2O_2 at 500 μM significantly increased ROS levels by approximately 350 and 95-fold above the vehicle control, respectively ($p < 0.001$).

To further determine whether cytotoxicity of CEP is mediated through ROS generation in H1975 cells. The cells were treated with NAC for 1 h before incubation with CEP. After 24 h, the cell viability was determined by MTT assays. The results illustrated that the viability of the cells pretreated with NAC significantly higher than that of the cells treated with CEP at 5, 10 and 20 μM as well as CDDP at 20 μM alone, (Figure 19B, $P < 0.001$), suggesting that cytotoxicity of CEP and CDDP were mediated through ROS production in H1975 cells.

A)



B)

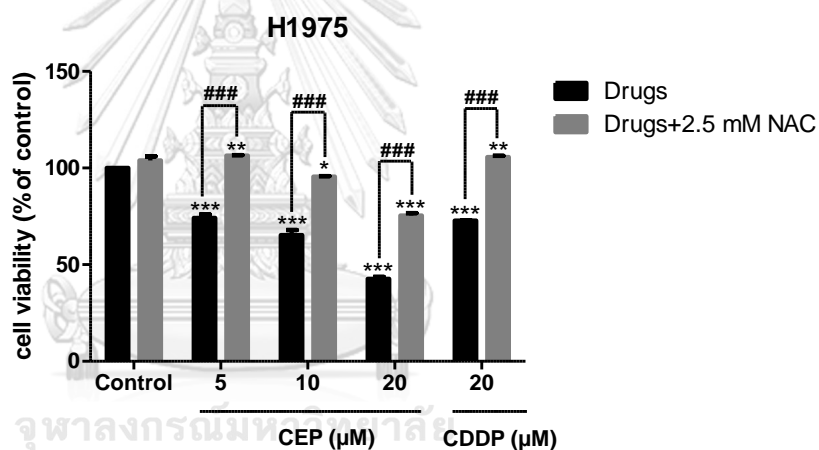


Figure 19 Effect of CEP on ROS generation in H1975 cells.

A) The cells were treated with CEP at 5, 10 and 20, CDDP at 20 μM or H₂O₂ at 500 μM for 1 h. The levels of ROS were stained with H₂DCF-DA and analyzed using flow cytometer.

B) The cells were treated with CEP at 2.5, 5 and 10, CDDP at 10 μM for 24 h in the absence or presence of 2.5 mM NAC. The cell viability was determined using MTT assays. Data are the mean ± SEM (n=3). *P<0.05, **P<0.01, ***P<0.001 compared with vehicle control (0.2% DMSO). ####P<0.001 compared with drugs alone.

4.9 Effect of CEP on apoptosis induction in H1975 cells

To investigate the apoptosis-inducing effect of CEP in H1975 cells, the cells were treated with 5, 10 and 20 μM of CEP or 20 μM of CDDP for 24 h. Apoptotic cells were evaluated by flow cytometry analysis after annexin V-FITC/PI staining. As shown in Figure 20, treatment with CEP and CDDP at 20 μM significantly increased apoptotic cells by approximately 3 and 4-fold above the vehicle control, respectively ($P < 0.01$). To further confirm the apoptosis-inducing effect of CEP in H1975 cells, the effect of CEP on PARP cleavage was evaluated. As shown in Figure 21, the results indicated that CEP and CDDP at 20 μM significantly induced the expression of cleaved PARP, which were approximately 4 and 5-fold above the vehicle control, respectively ($P < 0.05$).

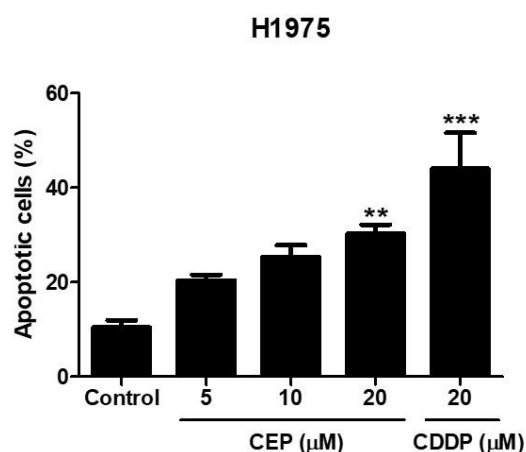


Figure 20 Apoptosis-inducing effect of CEP on H1975 cells.

The cells were treated with CEP at 5, 10 and 20 μM or CDDP at 20 μM for 24 h. Apoptotic cells were determined using annexin V-FITC/PI staining followed by flow cytometry analysis. Data are the mean \pm SEM ($n=3$). ** $P < 0.01$, *** $P < 0.001$ compared with the vehicle control (0.2% DMSO).

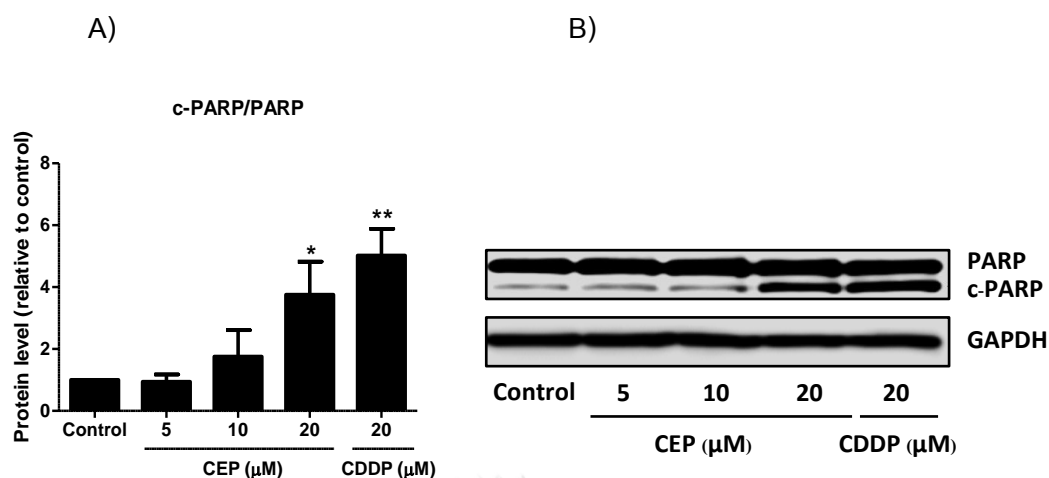


Figure 21 Effect of CEP on PARP cleavage in H1975 cells.

The cells were treated with CEP at 5, 10 and 20 μM or CDDP at 20 μM for 24 h. A) The protein expression of PARP and cleaved PARP were determined by western blotting. B) Representative immunoblots corresponding to PARP cleavage. Data are the mean ± SEM (n=3). *P<0.05, **P<0.01 compared with vehicle control (0.2% DMSO).

4.10 Effect of CEP on the expression of apoptotic proteins in H1975 cells

The effects of CEP on the expression of Bcl-2 family proteins in H1975 cells were investigated. After the cells were treated with 5, 10 and 20 μM of CEP or 20 μM of CDDP for 24 h, western blot analysis revealed that CEP or CDDP did not alter the expression of Bax and Bak proteins (Figures 22A and 22B). It however should be noted that CEP at 20 μM significantly downregulated the expression of Bcl-2 protein (Figures 23A, P<0.05) but did not affect the expression of Bcl-X_L protein. Furthermore, treatment with CDDP at 20 μM significantly down-regulated the expression of both anti-apoptotic proteins, Bcl-2 and Bcl-X_L. (Figures 23A and 23B, P<0.05). These findings suggest that CEP induces apoptosis via downregulation of Bcl-2 protein in H1975 cells.

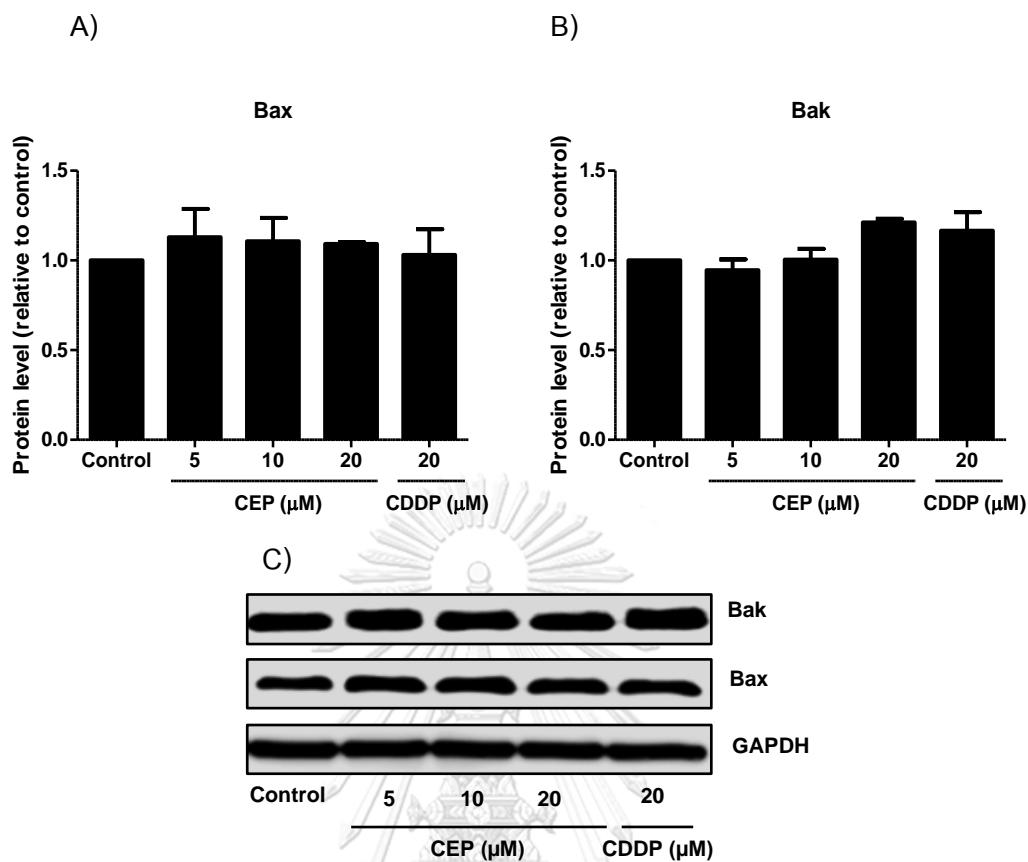


Figure 22 Effects of CEP on the expression of pro-apoptotic proteins in H1975 cells. The cells were treated with CEP at 5, 10 and 20 μM or CDDP at 20 μM for 24 h. The levels of pro-apoptotic proteins, including, A) Bax and B) Bak, were determined by western blot analysis. C) Representative immunoblots corresponding to Bax and Bak. Data are the mean ± SEM (n=3).

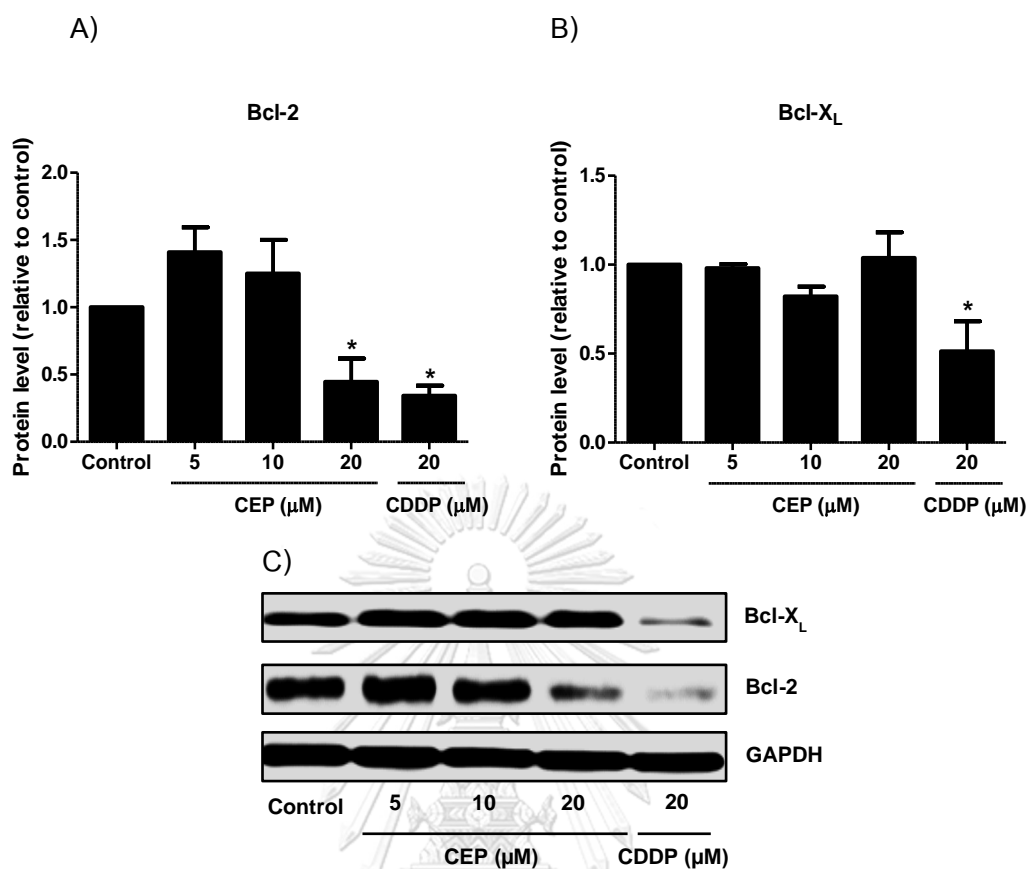


Figure 23 Effects of CEP on the expression of anti-apoptotic proteins in H1975 cells.

The cells were treated with CEP at 5, 10 and 20 μM or CDDP at 20 μM for 24 h. The levels of anti-apoptotic proteins, including, A) Bcl-2 and B) Bcl-X_L, were determined by western blot analysis. C) Representative immunoblots corresponding to Bcl-2 and Bcl-X_L. Data are the mean \pm SEM (n=3). *P<0.05 compared with vehicle control (0.2% DMSO).

4.11 Role of ROS in apoptosis-inducing effect of CEP in H1975 cells

To investigate whether the apoptosis-inducing effect of CEP is mediated through ROS generation in H1975 cells, the cells were pretreated with 2.5 mM NAC for 2 h prior to CEP treatment. After 24 h incubation, apoptotic cells were analyzed by flow cytometry analysis after annexin V/PI staining. As shown in Figure 24, pretreatment with NAC significantly decreased apoptotic cells when compared with CEP or CDDP alone (P<0.001), suggesting that CEP-induced apoptosis is mediated through ROS generation in H1975 cells.

A)

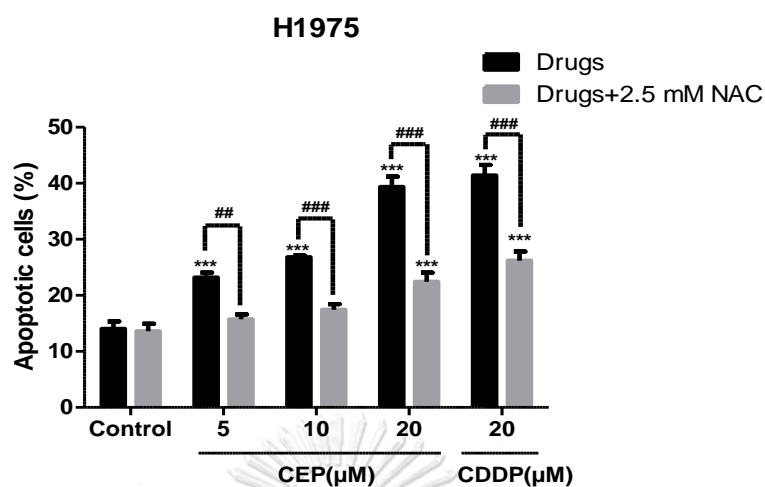


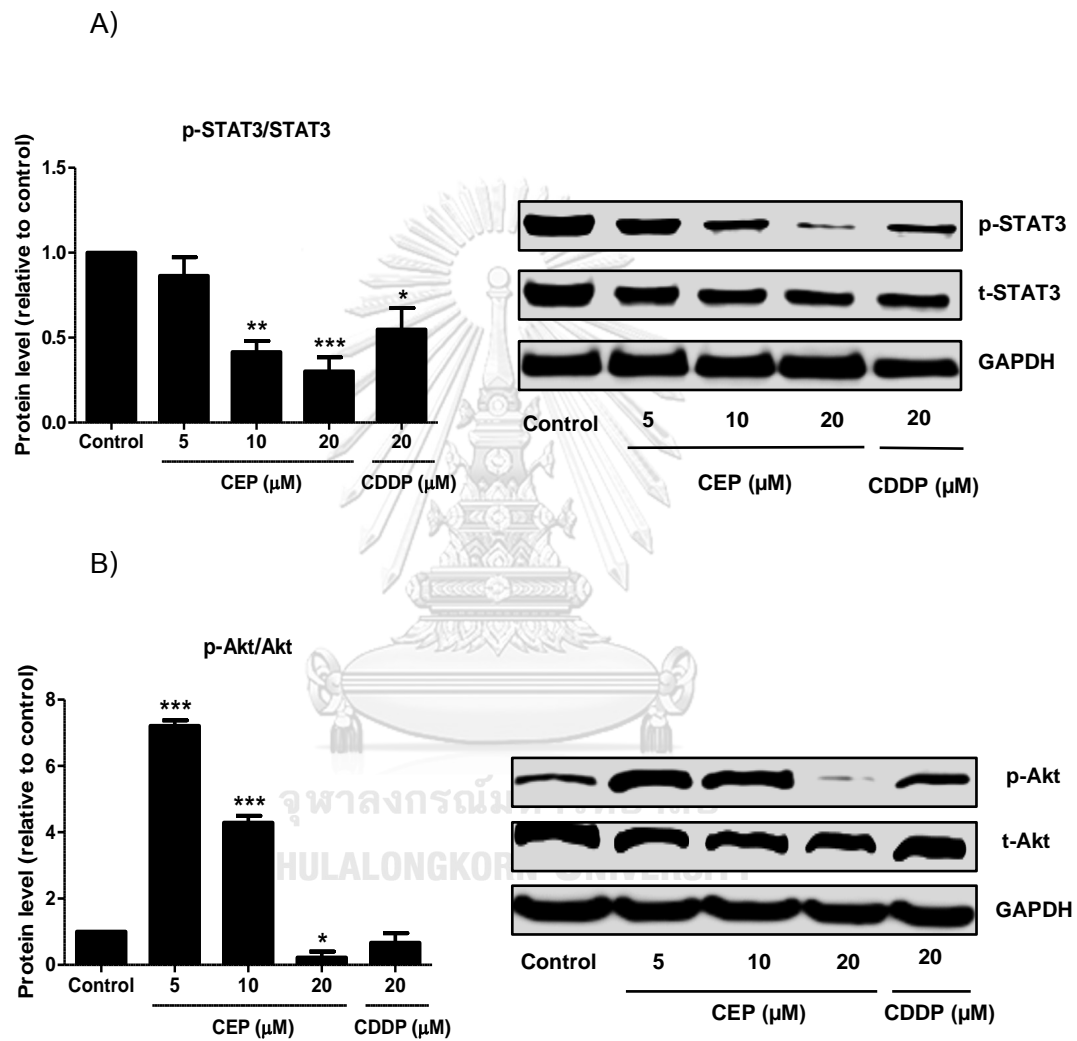
Figure 24 Role of ROS in apoptosis-inducing effect of CEP in H1975 cells.

The cells were treated with CEP at 5, 10 and 20 μM or CDDP at 20 μM for 24 h in the absence or presence of 2.5 mM NAC. The apoptotic cells were examined by flow cytometry analysis after annexin V-FITC/PI staining. Data are the mean \pm SEM ($n=3$). * * * $P<0.001$ compared with vehicle control (0.2% DMSO). ## $P<0.001$, ### $P<0.001$ compared with drugs alone.

4.12 Effect of CEP on STAT3, PI3K/Akt and MAPK/ERK signaling pathways in H1975 cells

To investigate the effect of CEP on STAT3, PI3K/Akt and MAPK/ERK signaling pathways in H1975 cells, the cells were treated with 5, 10 and 20 μM of CEP or 20 μM of CDDP for 24 h and then the protein levels of p-STAT3, STAT-3, p-Akt, Akt, p-ERK and ERK were evaluated using western blot analysis. As shown in Figure 25A, CEP and CDDP significantly inhibited the phosphorylation of STAT3. The expression of phosphorylated STAT3 were downregulated to 40% and 30% of the vehicle control after treatment with CEP at 10 and 20 μM , respectively ($P<0.01$). Of note, Akt signaling pathway was significantly induced by 5 and 10 μM of CEP but it was significantly inhibited by 20 μM of CEP (Figure 25B, $P<0.05$). Additionally, CEP and CDDP at 20 μM significantly induced

the phosphorylation of ERK (Figure 25C, $P < 0.01$). Taken together, it is possible that modulation of STAT3, PI3K/Akt and ERK pathways are associated with cytotoxicity of CEP in H1975 cells.



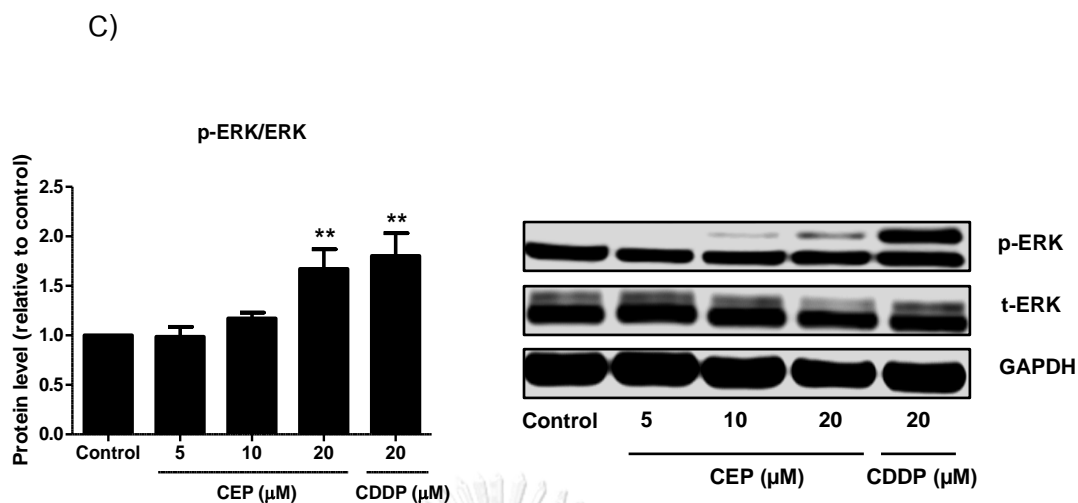


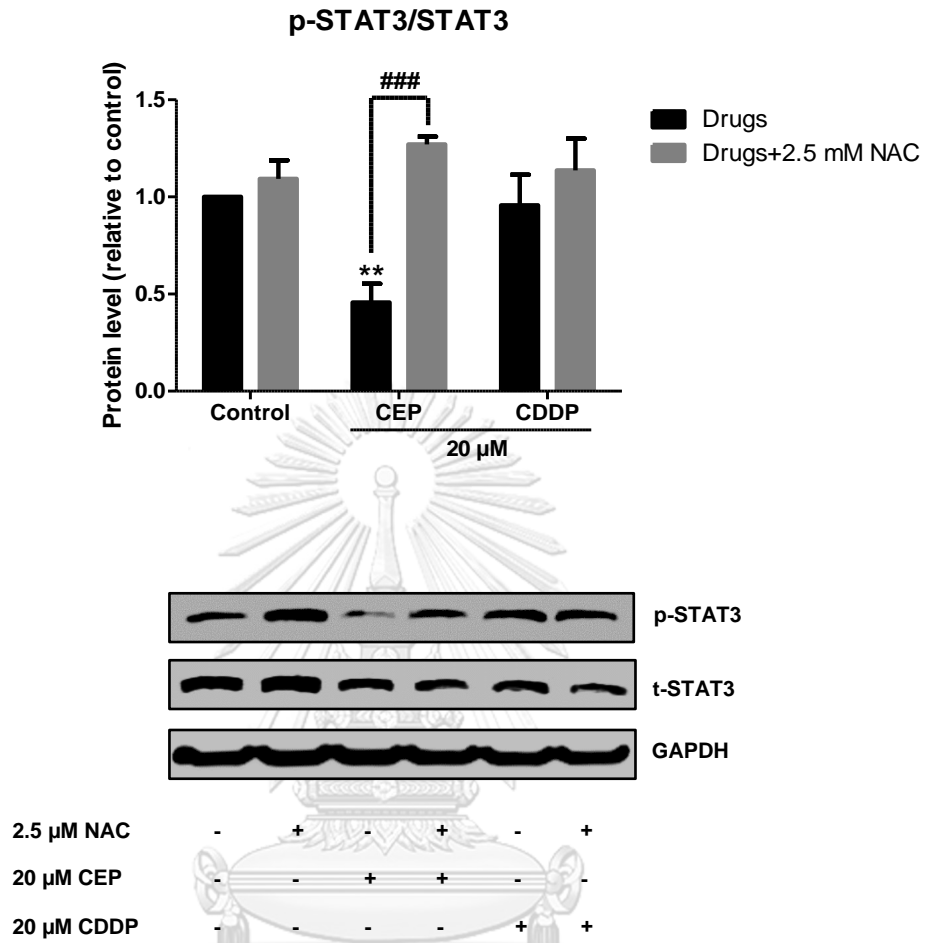
Figure 25 Effects of CEP on STAT3, PI3K/Akt and MAPK/ERK signaling pathways in H1975 cells.

The cells were treated with CEP at 5, 10 and 20 μM or CDDP at 20 μM for 24 h. The protein expression of A) p-STAT3 and t-STAT3 B) p-Akt and t-Akt C) p-ERK and t-ERK were determined by western blotting. Representative immunoblots corresponding to p-STAT3, t-STAT3, p-Akt, t-Akt, p-ERK and t-ERK. Data are the mean ± SEM (n=3). *P<0.05, **P<0.01, ***P<0.001 compared with vehicle control (0.2% DMSO).

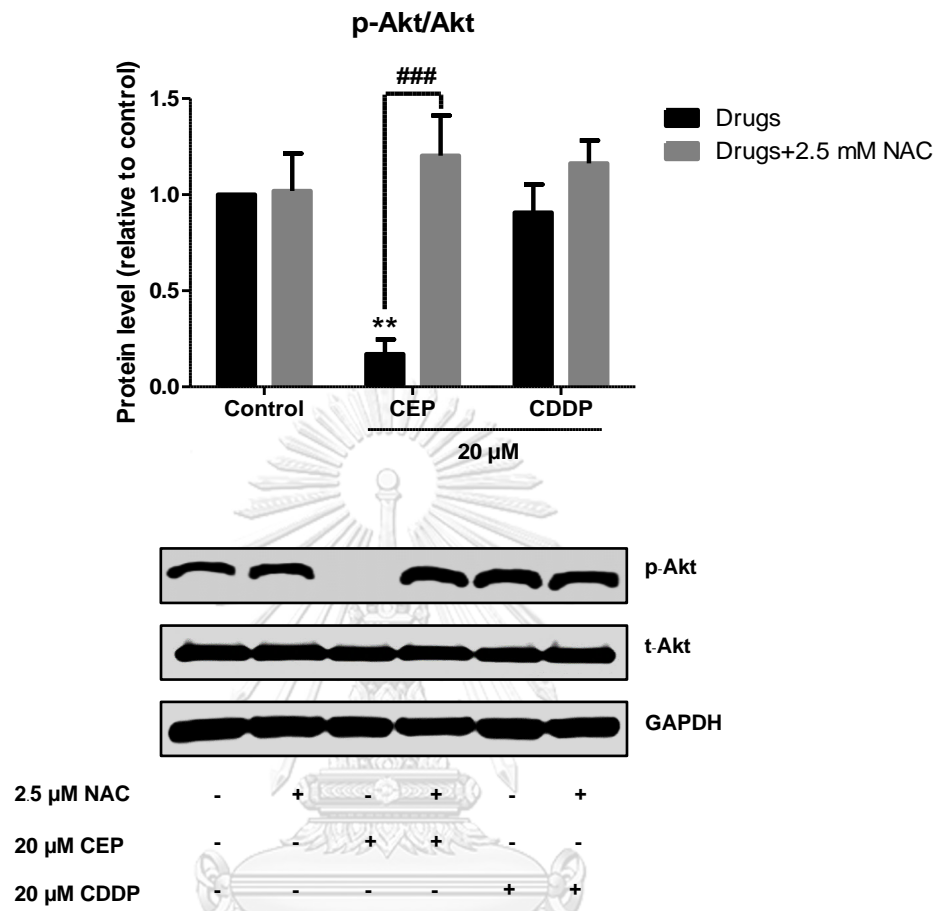
4.13 Roles of ROS on CEP-mediated modulation of STAT3, PI3K/Akt and MAPK/ERK signaling pathways in H1975 cells

To investigate whether ROS is involved in the effect of CEP on STAT3, PI3K/Akt and MAPK/ERK signaling pathways in H1975 cells, the cells were incubated with 2.5 mM NAC for 2 h before CEP treatment. Then, the levels of proteins involved in STAT3, PI3K/Akt and MAPK/ERK signaling pathways were evaluated using western blot analysis. As shown in Figures 26A and 26B, pretreatment with NAC significantly prevented the inhibition of STAT3 and Akt phosphorylation by CEP (P<0.05). Of note, NAC did not alter CEP-induced ERK activation (Figure 26C) but it could significantly prevent CDDP-induced ERK activation (P<0.01). These results suggest that ROS is partly responsible for CEP-mediated inhibition of STAT3 and PI3K/Akt signaling pathways in H1975 cells.

A)



B)



C)

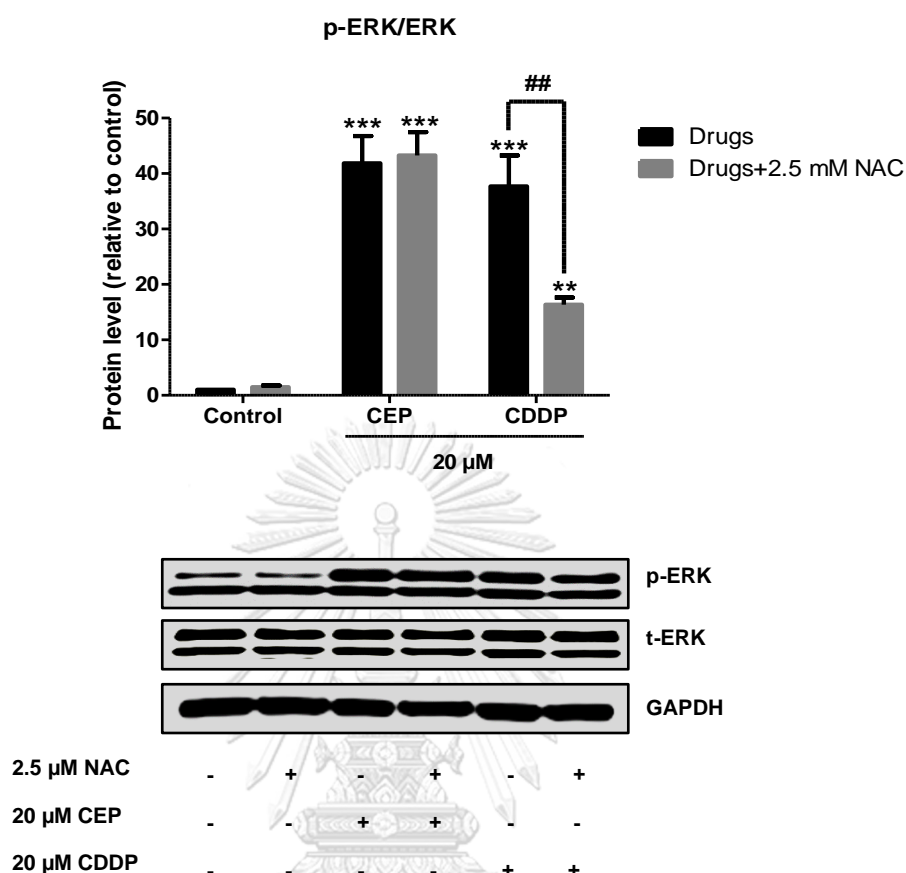


Figure 26 Roles of ROS in CEP-mediated modulation of STAT3, PI3K/Akt and MAPK/ERK signaling pathways in H1975 cells.

The cells were treated with CEP at 5, 10 and 20 μ M or CDDP at 20 μ M for 24 h in the absence or presence of 2.5 mM NAC. The protein expression of A) p-STAT3 and t-STAT3 B) p-Akt and t-Akt C) p-ERK and t-ERK were determined by western blotting. Representative immunoblots corresponding to p-STAT3, t-STAT3, p-Akt, t-Akt, p-ERK and t-ERK. Data are the mean \pm SEM (n=3). *P<0.05, **P<0.01, ***P<0.001 compared with vehicle control (0.2% DMSO). #P<0.05, ##P<0.01, ###P<0.001 compared with drugs with NAC.

CHAPTER V

DISCUSSION AND CONCLUSION

Lung cancer is the leading cause of cancer-related deaths worldwide and 80% of lung cancers are non-small cell lung cancer (NSCLC). Despite advances in NSCLC treatment, its prognosis remains very poor [40, 116]. Chemotherapy is one of the most common types of cancer treatment for NSCLC. However, its application is still limited due to drug resistance and severe side effects [6, 117]. Therefore, novel natural compounds that have a high anticancer activity and low toxicity are extremely needed. CEP, a biscochlorine alkaloid, has exhibited a potent anticancer activity against a wide range of cancers such as cervical adenocarcinoma, leukemia, cholangiocarcinoma, hepatocellular carcinoma, osteosarcoma, breast cancer, ovarian cancer, nasopharyngeal carcinoma and colorectal cancer [28, 31-34, 38, 118, 119]. Thus, the present study determined the anticancer activity of CEP in two non-small cell lung cancer cell lines, one expressing wild-type p53 and EGFR (A549) and the other one carrying mutant p53 and EGFR (H1975). The results demonstrated that CEP possessed cytotoxic activity against A549 and H1975 cells with the IC_{50} values of 4.31 ± 0.52 and 11.81 ± 2.07 μ M, respectively, indicating that CEP was more toxic to p53 and EGFR wild-type A549 cells than p53 and EGFR-mutant H1975 cells. Similar to our findings, previous studies reported that PC9 cell line with EGFR mutation is less sensitive to pemetrexed than H1993 cell line with EGFR wild-type [120]. Likewise, Fukuda et al. demonstrated that lamellarins with 21-OH selectively inhibited the growth of EGFR wild-type cells more than EGFR mutant cells [121]. Moreover, a clinical study has recently revealed that lung cancer patients with wild-type EGFR were sensitive to docetaxel than in patients with mutant EGFR [122]. Previous study reported that EGFR mutations cause over-activation of many downstream signaling pathways, including STAT3, PI3K/Akt and MAPK/ERK leading cancer cell proliferation and acquired drug resistance [123-125]. Thus, it is likely that mutations in EGFR are involved in the differences in sensitivity of EGFR wild-type A549 cells and EGFR mutant H1975 cells to CEP. It is well known that mutations in the p53 tumor suppressor gene are often involved in anticancer drug resistance [126-130]. In addition to EGFR, H1975 cells have

been reported to harbor p53 mutation [131]. Thus, it is also possible that mutant p53 plays a significant role in less sensitivity of H1975 cells to CEP and CDDP, compared to A549 cells. In addition to high anticancer activity, the present study found the SI of CEP were similar to those of CDDP, suggesting that CEP may be as safe as CDDP and have potential to be used as an alternative agent for NSCLC therapy. However, the safety of CEP needs to be further investigated.

ROS play important roles in various cellular functions such as cell growth, survival and apoptosis [132]. Several studies have reported that anticancer drugs, including CDDP, doxorubicin, 5- fluorouracil and oxaliplatin had significant cytotoxicity against ovarian cancer, breast cancer and lung cancer through ROS generation [100, 133, 134]. Moreover, alkaloid compounds such as curcumin, sanguinarine and rohitukine were shown to induce apoptosis via ROS production [135]. Notably, many *in vitro* studies have demonstrated that CEP triggered apoptosis through ROS production in a wide range of cancers, including NSCLC [35], choroidal melanoma [36] and myeloma [37]. The present study also found that increased ROS production was partly responsible for cytotoxic and apoptosis-inducing effects of CEP in A549 and H1975 cells. However, it should be noted that CEP treatment induced ROS levels in EGFR mutant H1975 cells higher than EGFR wild- type A549 cells. Similarly, following sanguinarine treatment, elevated ROS level was higher in EGFR mutant H1975 cells than EGFR wild-type A549 cells which was associated with activation of NADPH oxidase (NOX)-mediated release of ROS [136]. Furthermore, Kalo et al. have reported that mutant p53 promoted ROS production by inhibition of nuclear factor- erythroid 2- related factor 2 (NRF2) , a transcription factor that regulated antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX) and glutathione S-transferases (GST) [137]. In addition, mutation in p53 was shown to be involved in downregulation of glutathione, resulting in intracellular ROS accumulation in pancreatic cancer [138]. Therefore, it cannot be excluded that mutant p53 may be responsible for increased ROS generation in H1975 cells.

Apoptosis is one of the major mechanisms of cell death. Previous *in vitro* studies reported that CEP has exerted cytotoxic effects via apoptosis induction in various types of cancer, including oral squamous carcinoma, hepatocellular carcinoma, cervical adenocarcinoma and cholangiocarcinoma [30, 31, 33, 118]. CEP was shown to induce apoptosis through downregulation of Bcl-X_L and Bcl-2 proteins as well as upregulation of Bax protein in ovarian cancer cells [28]. Moreover, apoptosis-inducing effect of CEP was mediated via decreased Bcl-2 protein expression and increased Bax protein expression in NSCLC cell lines, A549 and H1299 [35]. Rattanawong et al. also reported that CEP triggered apoptosis through downregulation of anti-apoptosis proteins including, Bcl-2 and Bcl-X_L in colorectal cancer cells [38]. These findings were consistent with our findings that CEP induced apoptosis by modulating Bcl-2 family proteins. The expression of Bax and Bak proteins were increased and the expression of Bcl-2 and Bcl-X_L proteins were decreased in A549 cells while a decrease in Bcl-2 protein was detected in CEP-treated H1975 cells. In a manner similar to CEP, the apoptosis-inducing effects of CDDP were detected in both A549 and H1975 cells. Western blot analysis revealed that, following CDDP treatment, the expression of Bax protein was increased in A549 cells and the expression of Bcl-X_L and Bcl-2 proteins was decreased in H1975 cells.

Apoptosis-inducing effects of CEP in osteosarcoma, hepatocellular carcinoma and human leukemia cells were found to be mediated through modulation of STAT3, PI3K/Akt and MAPK/ERK signaling pathway, respectively [32-34]. In the present study, we found that CEP significantly inhibited the phosphorylation of STAT3, Akt and ERK in A549 cells. These findings were in agreement with previous studies demonstrating that sinomenine, cryptotanshinone and krukovine induced apoptosis through inhibition of STAT3, PI3K/Akt and ERK signaling pathways, respectively, in A549 cells [139-141]. Similar to A549 cells, the results from this study revealed that CEP significantly inhibited the phosphorylation of STAT3 and Akt in H1975 cells. These findings were consistent with a previous study demonstrating that *ortho*-naphthoquinone MG3 inhibited phosphorylation of STAT3 and Akt in H1975 cells [142]. It was also found that isoliquiritigenin inhibited the phosphorylation of Akt, resulting in apoptosis in H1975 cells

[143]. Moreover, apoptosis-inducing effect of cucurbitacin B was shown to be mediated through inhibition of STAT3 signaling pathway in H1975 cells [144]. It however should be noted that, in contrast to Akt and STAT3 in H1975 cells, we found that CEP significantly activated ERK signaling pathway in H1975. Similar to our findings, Kikukawa et al. reported that *Marsdenia tenacissima*-induced activation of ERK partially contributed to cell death in H1975 cells [33]. Recently, butoxy mansonone G has shown to significantly induce ERK signaling pathway, leading to apoptosis in H1975 cells [142]. It is commonly known that upregulation of ERK is involved in carcinogenesis and progression of NSCLC [145]. Conversely, recent studies suggest that ERK activation may play an important role in apoptosis induction [93, 146]. For example, an antimitotic drug, paclitaxel, was found to activate ERK and induce apoptosis in a p53 independent manner in breast cancer cells [147]. Moreover, apoptosis-inducing effect of phenethyl isothiocyanate was shown to be mediated through ERK activation in p53-deficient PC-3 prostate cell line [148]. Similarly, CEP induced apoptosis via the activation of ERK in human leukemia K562 cells carrying mutant p53 [34]. Remarkably, Thamkachy et al. reported that the diaminothiazole 1 induced apoptosis through ERK-mediated upregulation of death receptor 5 in p53 defective colon cancer cells [149]. Taken together, it is likely that activation of ERK may partly be responsible for CEP-induced cell death in p53 mutant H1975 cells.

It is commonly known that ROS function as an upstream signal for modulation of STAT3, PI3K/Akt and MAPK/ERK signaling pathway [99, 150]. Although the present study demonstrated that modulation of MAPK/ERK signaling pathway by CEP was not associated with increased ROS generation in both A549 and H1975 cells, we found that NAC significantly prevented CEP-mediated STAT3 inhibition in both A549 and H1975 cells, indicating that inhibition of STAT3 by CEP is ROS-dependent. Numerous studies have demonstrated the relationship between ROS generation and the phosphorylation of JAK2/Src-STAT3 signaling pathway in cancer cells [151, 152]. Moreover, ROS was shown to be responsible for chelerythrine-mediated apoptosis by inhibiting STAT3 signaling pathway in renal cell carcinoma [104]. In addition to STAT3, this study found the involvement of ROS in CEP-mediated inhibition of Akt signaling pathway in H1975 cells.

These findings were consistent with the findings of Liu et al. that tetrandrine induced apoptosis through ROS-dependent inactivation of PI3K/Akt signaling pathway in hepatocellular carcinoma [102]. Furthermore, a recent study has demonstrated that apoptosis-inducing effects of chaetocin was associated with ROS-mediated inactivation of the PI3K/Akt pathway [153]. Taken together, it is very likely that ROS-mediated inhibition of STAT3 and Akt is involved in apoptosis-inducing effect of CEP in A549 and H1975 cells. In the present study, we also found that treatment of H1975 cells with a high concentration of CEP resulted in downregulation of Akt but at low concentrations of CEP led to Akt activation. Accumulating evidence has shown that high ROS levels also activate PI3K/Akt signaling by inhibiting the activity of its negative regulator PTEN [154] but excessive oxidative stress might also inhibit Akt [155]. Previous studies have revealed that ROS could directly oxidize and dephosphorylate Akt, resulting in their inactivation [156, 157]. Unfortunately, roles of ROS on CEP-mediated Akt activation have not been investigated in this study but it is possible that CEP-modulated Akt signaling pathway differently depending on the level of ROS in H1975 cells. Additional studies focusing on the interaction between Akt signaling and ROS metabolism may provide novel therapeutic approaches for treating cancer.

Conclusions

The findings of this study clearly demonstrated that CEP exhibited potent cytotoxicity against p53 and EGFR wild-type A549 non-small cell lung cancer cells and p53 and EGFR mutant H1975 non-small cell lung cancer cells. In A549 cells, CEP induced apoptosis via generation of ROS, upregulation of Bax and Bak and downregulation of Bcl-2 and Bcl-X_L. In addition, CEP could inhibit STAT3, Akt and ERK signaling pathways and the inhibitory effect of CEP on STAT3 signaling pathway was mediated through ROS. Similar to A549 cells, increased ROS formation and decreased Bcl-2 expression were found to be partly responsible for apoptosis-inducing effect of CEP in H1975 cells. Moreover, we found that CEP inhibited STAT3, and Akt signaling pathways but activated ERK signaling pathway. Notably, ROS was partly responsible for CEP-mediated STAT3 and Akt inhibition in H1975 cells. Taken together, the results from this study suggest that CEP may potentially be developed as a novel anticancer agent for NSCLC. However, a better understanding of molecular mechanisms underlying anticancer activity of CEP remains necessary.

APPENDIX A

PREPARATION OF REAGENTS

1. DMEM stock solution (1 L)

DMEM powder	10.4 g
NaHCO ₃	3.7 g
ddH ₂ O	950 mL

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

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

Store at 4°C

2. RPMI 1640 stock solution (1 L)

RPMI powder	10.4 g
NaHCO ₃	1.5 g
Glucose	4.5 g
Sodium pyruvate	0.11 g
HEPES (1M)	10 mL
ddH ₂ O	950 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

Store at 4°C

3. MEM stock solution (1 L)

MEM powder	10.4 g
NaHCO ₃	3.7 g
ddH ₂ O	950 mL

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

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

Store at 4°C

4. 1X Phosphate Buffer Saline (PBS) (1 L)

NaCl	8.065 g
KCl	0.2 g
KH ₂ PO ₄	0.2 g

Na_2HPO_4	1.15 g
ddH ₂ O	950 mL

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

Add ddH₂O to 1 L and sterilized by autoclaving

Store at room temperature

5. 1x Assay Buffer for Flow Cytometer (100 mL)

HEPES (1M)	1.0 mL
CaCl_2 (0.1M)	2.8 mL
NaCl (5M)	2.5 mL
ddH ₂ O	93.7 mL

Add ddH₂O to 100 mL

Store at 4°C

6. Hank's buffered salt solution (HBSS) (1 L)

Hank balance salt powder	9.8 g
NaHCO_3	0.35 g
ddH ₂ O	950 mL

Adjust pH to 7.24 with 1 N HCl or 1 N NaOH

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

Store at room temperature

7. Separating buffer (500 mL)

Tris base	45.43 g
ddH ₂ O	450 mL

Adjust pH to 8.8 with 1 N HCl or 1 N NaOH

Add ddH₂O to 500 mL

Store at 4°C

8. Stacking buffer (500 mL)

Tris base	15.14 g
ddH ₂ O	450 mL

Adjust pH to 6.8 with 1 N HCl or 1 N NaOH

Add ddH₂O to 500 mL

Store at 4°C

9. Sample diluting buffer (SDB) (225 mL)

Stacking buffer	31.25 mL
10% Sodium dodecyl sulfate (SDS)	50 mL
Pyronin Y (0.5% stock)	5 mL
Bromophenol blue (0.5% stock)	5 mL
Glycerol	50 mL

Add ddH₂O to 225 mL

Store at room temperature

10. 10X Running buffer (1L)

Tris base	30.25 g
Glycine	144 g
Sodium dodecyl sulfate (SDS)	10 g
ddH ₂ O	800 mL

Add ddH₂O to 1 L

Store at 4°C

11. 1X Running buffer (1L)

10X Laemli buffer	100 mL
ddH ₂ O	900 mL

Store at 4°C

12. 10X Tris-Buffered Saline (TBS) (1L)

Tris base	12.1 g
NaCl	87.5 g
ddH ₂ O	900 mL

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

Add ddH₂O to 1 L

Store at 4°C

13. 1X Tris-Buffered Saline (TBS) (1L)

10X Tris-Buffered Saline (TBS)	100 mL
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ddH ₂ O	900 mL
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Store at 4°C

14. 1X Tris-Buffered Saline (TBS)/Tween buffer (1L)

Tween 20	0.5 mL
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1X Tris-Buffered Saline (TBS)	999.5 mL
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Store at 4°C

15. 10X Transfer buffer (1L)

Tris base	30 g
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Glycine	144 g
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Sodium dodecyl sulfate (SDS)	1 g
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ddH ₂ O	800 mL
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Add ddH₂O to 1 L

Store at 4°C

16. 1X Transfer buffer (1L)

10X Transfer buffer	100 mL
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Methanol	200 mL
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ddH ₂ O	700 mL
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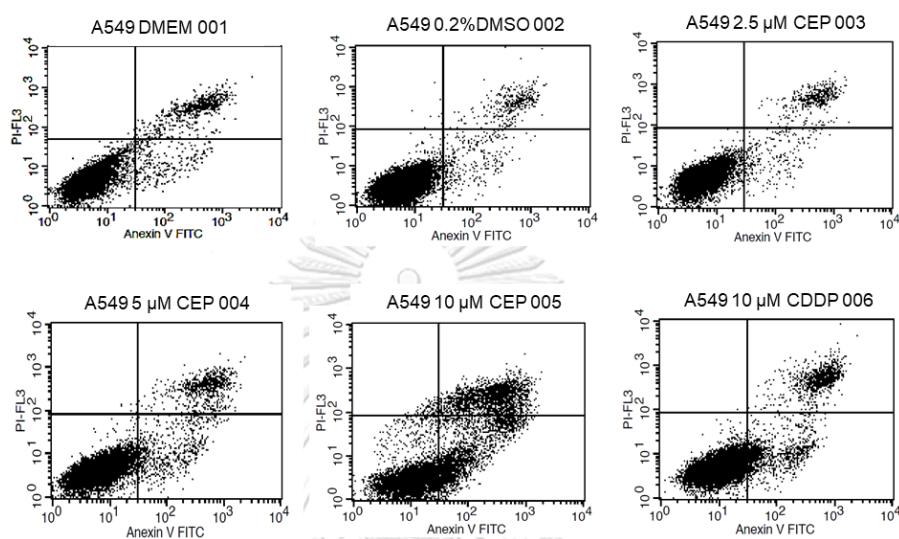
Store at 4°C



APPENDIX B

RESULTS

Appendix B-1: Representative cytograms of cell apoptosis analysis of A549 cells after treatment with 2.5, 5 and 10 μM of CEP or 10 μM of CDDP for 24 h

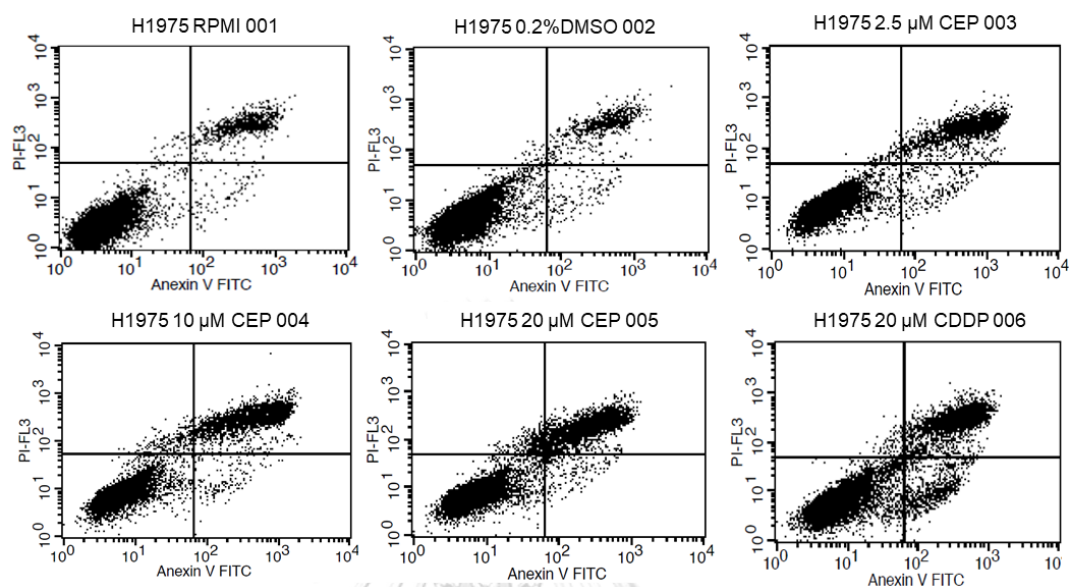


Appendix B-2: The percentage of apoptotic cells of A549 cells after treatment with 2.5, 5 and 10 μM of CEP or 10 μM of CDDP for 24 h

Treatments	Cell population (%)		
	Alive	Apoptosis	Necrosis
Untreated	82.01 \pm 7.86	17.39 \pm 7.75	0.61 \pm 0.17
0.2%DMSO	92.72 \pm 3.04	6.97 \pm 2.88	0.30 \pm 0.18
2.5 μM CEP	87.65 \pm 3.36	11.52 \pm 3.09	1.22 \pm 0.76
5 μM CEP	84.34 \pm 1.66	14.81 \pm 1.49	0.84 \pm 0.40
10 μM CEP	59.67 \pm 3.92	38.68 \pm 3.72	1.65 \pm 0.65
10 μM CDDP	80.87 \pm 2.16	18.70 \pm 2.15	0.43 \pm 0.22

Data represent mean \pm SEM from three independent experiments.

Appendix B-3: Representative cytograms of cell apoptosis analysis of H1975 cells after treatment with 5, 10 and 20 μM of CEP or 20 μM of CDDP for 24 h

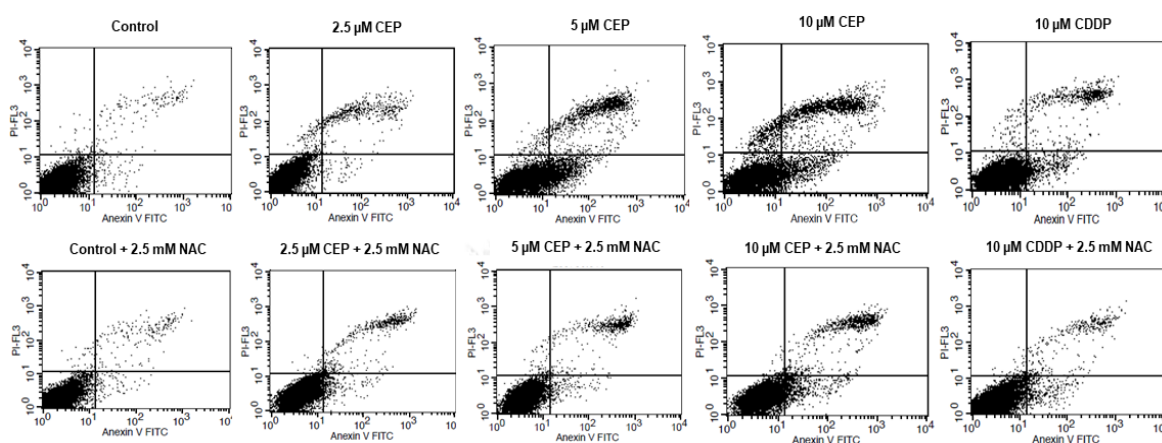


Appendix B-4: The percentage of apoptotic cells of H1975 cells after treatment with 5, 10 and 20 μM of CEP or 20 μM of CDDP for 24 h

Treatments	Cell population (%)		
	Alive	Apoptosis	Necrosis
Untreated	85.06 \pm 5.96	14.11 \pm 5.73	0.82 \pm 0.25
0.2%DMSO	86.18 \pm 4.66	13.15 \pm 4.39	0.66 \pm 0.27
5 μM CEP	78.41 \pm 1.43	20.36 \pm 1.21	1.20 \pm 0.23
10 μM CEP	73.10 \pm 2.70	25.38 \pm 2.45	1.52 \pm 0.30
20 μM CEP	68.05 \pm 2.31	30.28 \pm 1.91	1.66 \pm 0.40
20 μM CDDP	54.53 \pm 7.42	44.01 \pm 7.61	1.46 \pm 0.49

Data represent mean \pm SEM from three independent experiments.

Appendix B-5: Representative cytograms of cell apoptosis analysis of A549 cells after treatment with or without 2.5 mM of NAC for 1 h followed by treatment with 2.5, 5 and 10 μM of CEP or 10 μM of CDDP for 24 h

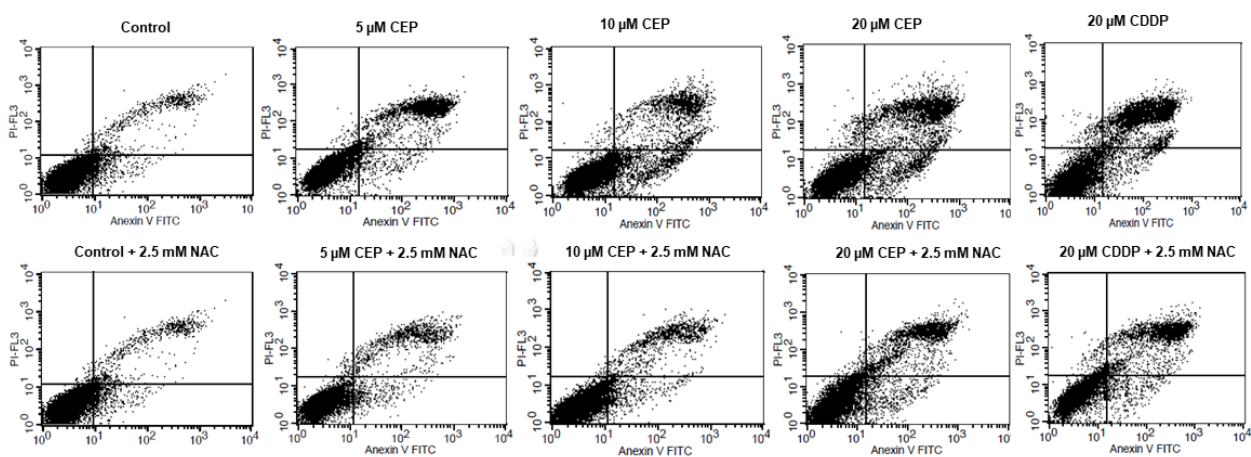


Appendix B-6: The percentage of apoptotic cells of A549 cells after treatment with or without 2.5 mM of NAC for 1 h followed by treatment with 2.5, 5 and 10 μM of CEP or 10 μM of CDDP for 24 h

Treatments	Cell population (%)	
	2.5 mM NAC	
	-	+
0.2%DMSO	2.94±0.92	2.87±0.41
2.5 μM CEP	7.10±0.67	5.58±0.58
5 μM CEP	24.35±1.30	7.91±0.55
10 μM CEP	36.24±2.30	16.39±3.22
10 μM CDDP	10.81±1.98	7.46±1.07

Data represent mean±SEM from three independent experiments.

Appendix B-7: Representative cytograms of cell apoptosis analysis of H1975 cells after treatment with or without 2.5 mM of NAC for 1 h followed by treatment with 5, 10 and 20 μM of CEP or 20 μM of CDDP for 24 h



Appendix B-8: The percentage of apoptotic cells of H1975 cells after treatment with or without 2.5 mM of NAC for 1 h followed by treatment with 5, 10 and 20 μM of CEP or 20 μM of CDDP for 24 h

Treatments	Cell population (%)	
	2.5 mM NAC	
	-	+
0.2%DMSO	14.04 \pm 1.30	13.60 \pm 1.34
5 μM CEP	23.17 \pm 0.90	15.74 \pm 0.86
10 μM CEP	26.81 \pm 0.29	17.42 \pm 1.02
20 μM CEP	39.34 \pm 1.84	22.46 \pm 1.59
20 μM CDDP	41.39 \pm 1.89	26.17 \pm 1.64

Data represent mean \pm SEM from three independent experiments.

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