ฤทธิ์ต้านมะเร็งของเซฟราแรนทีนต่อเซลล์มะเร็งรังไข่ของมนุษย์

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ANTICANCER EFFECTS OF CEPHARANTHINE ON HUMAN OVARIAN CANCER CELLS

Miss Vilawan Payon

A Thesis Submitted in Partial Fulfillment of the Requirements
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วิลาวรรณ พยนต์ : ฤทธิ์ต้านมะเร็งของเซฟราแรนทีนต่อเซลล์มะเร็งรังไข่ของมนุษย์ (ANTICANCER EFFECTS OF CEPHARANTHINE ON HUMAN OVARIAN CANCER CELLS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ดร. ปิยนุช วงศ์อนันต์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: พญ. ดร. วรรณรัศมี เกตุชาติ, 81 หน้า.

Cepharanthine (CEP) เป็นสารที่มีฤทธิ์ทางยาซึ่งพบได้ในรากของต้น *Stephania cepharantha* Hayata มีฤทธิ์ต้านมะเร็งหลายชนิด เช่น มะเร็งเม็ดเลือดขาว, มะเร็งท่อน้ำดี, มะเร็งปอด และมะเร็งตับ แต่อย่างไรก็ตามยังไม่มีการรายงานถึงฤทธิ์ต้านมะเร็งรังไข่ของ CEP ดังนั้นในการวิจัยครั้งนี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ต้านมะเร็งของ CEP รวมไปถึงกลไกการออกฤทธิ์ต่อเซลล์มะเร็งรังไข่ของมนุษย์ 2 ชนิดคือ เซลล์ CaOV-3 ซึ่งเป็นเซลล์ที่ตอบสนองต่อยาเคมีบำบัด และเซลล์ OVCAR-3 เป็นเซลล์ที่ดื้อต่อยาเคมีบำบัด cisplatin และ adriamycin ในการศึกษาครั้งนี้แสดงให้เห็นว่า CEP ยับยั้งการเจริญเติบโตของเซลล์มะเร็งรังไข่ทั้งเซลล์ CaOV-3 และเซลล์ OVCAR-3 ซึ่งแปรผันตามความเข้มข้นที่ใช้ในการทดสอบ โดยพบว่า CEP มีความเป็นพิษต่อเซลล์ CaOV-3 (IC_{50} = 10.93±0.14 ไมโครโมลาร์) มากกว่าเซลล์ OVCAR-3 (IC_{50} = 31.20±1.27 ไมโครโมลาร์) นอกจากนี้ CEP สามารถยับยั้งการเจริญเติบโตของเซลล์ CaOV-3 ผ่านทางการชักนำให้วัฏจักรเซลล์หยุดที่ระยะ G1 ในขณะที่ CEP ไม่มีผลต่อการเปลี่ยนแปลงในวัฏจักรเซลล์ของเซลล์ OVCAR-3 และเมื่อทำการศึกษาในระดับ mRNA พบว่า CEP เพิ่มระดับของ p21 อย่างชัดเจนทั้งในเซลล์ CaOV-3 และเซลล์ OVCAR-3 อีกทั้ง CEP ยังสามารถลดระดับ mRNA ของ cyclin A ในเซลล์ CaOV-3 และลดปริมาณ mRNA ของ cyclin A และ cyclin E และเพิ่ม mRNA ของ cyclin D ในเซลล์ OVCAR-3 เมื่อศึกษาผลของ CEP ต่อการตายแบบอะพอพโทซิสและกลไกการออกฤทธิ์ต่อเซลล์มะเร็งรังไข่ของมนุษย์พบว่า CEP สามารถชักนำให้เซลล์ CaOV-3 ตายแบบอะพอพโทซิสและลดระดับ mRNA ของ Bcl-xl และเพิ่มปริมาณ mRNA ของ Bax และ Bak และเช่นเดียวกัน CEP ชักนำให้เซลล์ OVCAR-3 เกิดการตายแบบอะพอพโทซิส และเพิ่มระดับ mRNA ของ Bak ดังนั้นจากผลการศึกษาครั้งนี้แสดงให้เห็นความเป็นไปได้ในการนำ CEP มาใช้ในการรักษาโรคมะเร็งรังไข่

สาขาวิชา เภสัชวิทยา ลายมือชื่อนิสิต .....

ปีการศึกษา 2559 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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KEYWORDS: CEPHARANTHINE, OVARIAN CANCER, APOPTOSIS, CELL CYCLE ARREST, CYTOTOXICITY

VILAWAN PAYON: ANTICANCER EFFECTS OF CEPHARANTHINE ON HUMAN OVARIAN CANCER CELLS. ADVISOR: PIYANUCH WONGANAN, Ph.D., CO-ADVISOR: WANNARASMI KETCHART, M.D., Ph.D, 81 pp.

Cepharanthine (CEP) is a medicinal product derived from *Stephania cepharantha* Hayata, which possesses potent anticancer activities against several types of cancers such as leukemia, cholangiocarcinoma, non-small-cell lung cancer and hepatocarcinoma. However, its anticancer activity against ovarian cancer cells has never been reported. In this study, the anticancer effect of CEP and mechanisms underlying the anticancer effect of CEP were investigated in two human ovarian cancer cell lines, CaOV-3 (chemosensitive) and OVCAR-3 (chemoresistance), which has been reported to be resistant to clinically relevant concentrations of cisplatin and adriamycin. The present study demonstrated that CEP significantly inhibited the growth of CaOV-3 and OVCAR-3 cells in a concentration-dependent manner. Interestingly, CEP was more toxic to CaOV-3 cells (IC_{50} = 10.93±0.14 μM) than OVCAR-3 cells (IC_{50} = 31.20±1.27 μM). Additionally, CEP could induce cell cycle arrest at G1 phase in CaOV-3 cells but did not alter cell cycle distribution in OVCAR-3 cells. Mechanistic studies demonstrated that CEP markedly increased mRNA level of p21, a cyclin-dependent kinase inhibitor, in both ovarian cancer cell lines. CEP also decreased mRNA level of cyclin A in CaOV-3 as well as decreased the expression of cyclin A and E genes while increased cyclin D gene expression in OVCAR-3 cells. Moreover, CEP was found to trigger apoptosis, increase Bcl-xl mRNA and decrease Bax and Bak mRNA in CaOV-3 cells. Similarly, CEP induced cancer cells to undergo apoptosis and increased Bak mRNA level in OVCAR-3 cells. Therefore, the results from this study suggest that CEP could potentially be used as a novel anticancer drug for ovarian cancer treatment.

Field of Study: Pharmacology Student's Signature .....

Academic Year: 2016 Advisor's Signature .....

Co-Advisor's Signature .....

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AML	Acute myelogenous leukemia
ANOVA	Analysis of variance
Apaf-1	Apoptotic protease-activating factor-1
ATCC	American Type Culture Collection
ATP7A	ATPase copper transporting alpha
ATP7B	ATPase copper transporting beta
Bad	BCL-2 antagonist of cell death
Bak	BCL-2 antagonist/killer-1
Bax	BCL-2 associated X protein
Bcl-xl	B-cell lymphoma-extra large
Bcl-2	B-cell lymphoma-2
Bid	BH3-interacting domain death agonist
Bim	BCL-2-like-11
Caspase	Cysteine aspartic acid specific protease
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
CEP	Cepharanthine
$CHCl_3$	Chloroform
CIP/KIP	CDK interacting protein/Kinase inhibitory protein
c-JNK	c-Jun N-terminal protein kinases
CKI	Cyclin-dependent kinase inhibitor
CO_2	Carbon dioxide
CT	Cycle threshold
DISC	Death-inducing signaling complex
DMEM	Dulbeco's Modified Eagle Medium

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTX	Docetaxel
EDTA	Ethylene diamine tetraacetic acid
ERK	Extracellular signal–regulated kinase
E2F	E2 factor
FADD	Fas-associated death domain
Fas	Fibroblast associated antigen
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FIGO	International Federation of Gynecology and Obstetrics
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehydes-3-phosphate dehydrogenase
h	Hour
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl-1-piperazineethanesulfonic acid)
IARC	International Agency for Research on Cancer
IC_{50}	Half inhibitory concentration
INK4	Inhibitor of CDK4
LSD	Least Square Difference
MAPK	Mitogen-activated protein kinase
MDR1	Multidrug resistance protein 1
mg/ml	Milligram per milliliter
MMR	DNA mismatch repair
mRNA	Messenger RNA
MRP1	Multidrug resistance-associated protein 1

MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
NCI	National Cancer Institute
NF	Nuclease free water
NF-**K**B	Nuclear factor kappa-light-chain-enhance of activated B
NICE	National Institute for Clinical Excellence
nm	Nanometer
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffer saline
PI	Propidium iodide
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PI3K	Phosphatidylinositol-3-kinase
PKB	Protein kinase B (or AKT)
PS	Phosphatidylserine
PTEN	Phosphatase and tensin homolog
PUMA	p53 upregulated modulator of apoptosis
p53	Tumor suppressor protein 53
RB	Retinoblastoma protein
RNA	Ribonucleic acid
RNase A	Ribonuclease A
RPMI	Roswell Park Memorial Institute
ROS	Reactive oxygen species
rpm	Round per minutes
RT-PCR	Reverse transcription polymerase chain reaction

SEM	Standard error of mean
STAT3	Signal transducer and activator of transcription 3
tBid	Truncated Bid
TNF-α	Tumor necrosis factor alpha
TRAIL	TNF-related apoptosis including ligand
µg/ml	Microgram per milliliter
µl	Microliter
µM	Micromolar

CHAPTER I
INTRODUCTION

1.1 Background and rationale

Ovarian cancer is the seventh most common cancer and the leading cause of cancer death in women worldwide [1]. Most patients are diagnosed with advanced stage disease (stage III and IV) because the early-stage malignancy is generally asymptomatic and very difficult to diagnose. Thus, the 5-year survival rate for these patients is less than 40% [2]. The current standard treatment for ovarian cancer patients includes cytoreductive surgery, radiotherapy, systemic chemotherapy and targeted therapy. Usually, first-line therapies for ovarian cancer are cytoreductive surgery followed by six cycle of intravenous chemotherapeutic agents [3]. The conventional cytotoxic drugs are platinum coordination complexes, including cisplatin, carboplatin and oxaliplatin as well as taxane, including paclitaxel and docetaxel. The cytotoxic drugs are generally used in combination rather than single compound [4]. In addition to platinum-based drugs and taxane, doxorubicin is approved by the Food and Drug Administration (FDA) of United States for the treatment of metastatic ovarian cancer in patients with disease refractory to both platinum-based and taxane chemotherapy. Similarly, National Institute for Clinical Excellence (NICE) reported in 2002 that, in the United Kingdom, doxorubicin is a drug of choice for many patients with advanced ovarian cancer after failure of first-line therapy [5]. Although complete clinical response in patients with ovarian cancer can be achieved in clinic but the mortality remains high due to acquired drug resistance. Besides chemoresistance, highly toxic side effects of chemotherapeutic agents have also limited their uses in cancer patients [6-9]. Therefore, it is

necessary to find novel compounds that have efficient anticancer activity with minimal side effects for ovarian cancer treatment.

Cepharanthine (CEP) is a biscoclaurine alkaloid isolated from *Stephania cepharantha* Hayata [10]. Currently, cepharanthine is approved by the Japanese Ministry of Health for treatment of many acute and chronic diseases such as radiation-induced leukopenia, alopecia areata and alopecia pityrodes [11]. Although it has been widely used, serious side effects of cepharanthine have never been reported [11, 12]. Previous studies have reported various pharmacological effects of cepharanthine such as anti-malarial [13], antioxidant [10, 14], anti-allergic [15], anti-inflammatory [16, 17], and anticancer [11]. Cepharanthine has been found to exert cytotoxicity against several types of cancers such as leukemia, cholangiocarcinoma and hepatocarcinoma [18-21]. Apoptosis-inducing effects of cepharanthine was reported to be mediated through reducing Bcl-2 expression [22], inducing Bax expression [23, 24] and triggering several signaling pathways such as STAT [25] and NF-κB [26]. Similarly, this alkaloid could induce cell cycle arrest at G1 phase by altering cell cycle regulators such as $p21^{WAF1}$ [27], $p27^{Kip1}$, and cyclin E [28]. Moreover, cepharanthine has demonstrated to potentiate the anticancer activity of numerous chemotherapeutic agents, including doxorubicin, vincristine and adriamycin by inducing p53-deficient cells and P-glycoprotein (P-gp) overexpressing cells to undergo apoptosis following treatment [29, 30].

Recently, Rattanawong et al. (2015) reported that cepharanthine could efficiently inhibit the proliferation of p53-mutant HT-29 colorectal cancer cells which are resistant to chemotherapeutic drugs, suggesting that cepharanthine may be a novel agent for treatment of cancer patients who are not respond to commonly used chemotherapeutic drugs. In addition, the

anticancer effects of cepharanthine on human ovarian cancer cells have never been investigated. Therefore, in the present study, the anticancer activity of cepharanthine and its underlying mechanism(s) were determined in both chemosensitive (CaOV-3) and chemoresistant (OVCAR-3) human ovarian cancer cell lines.

1.2 Objectives

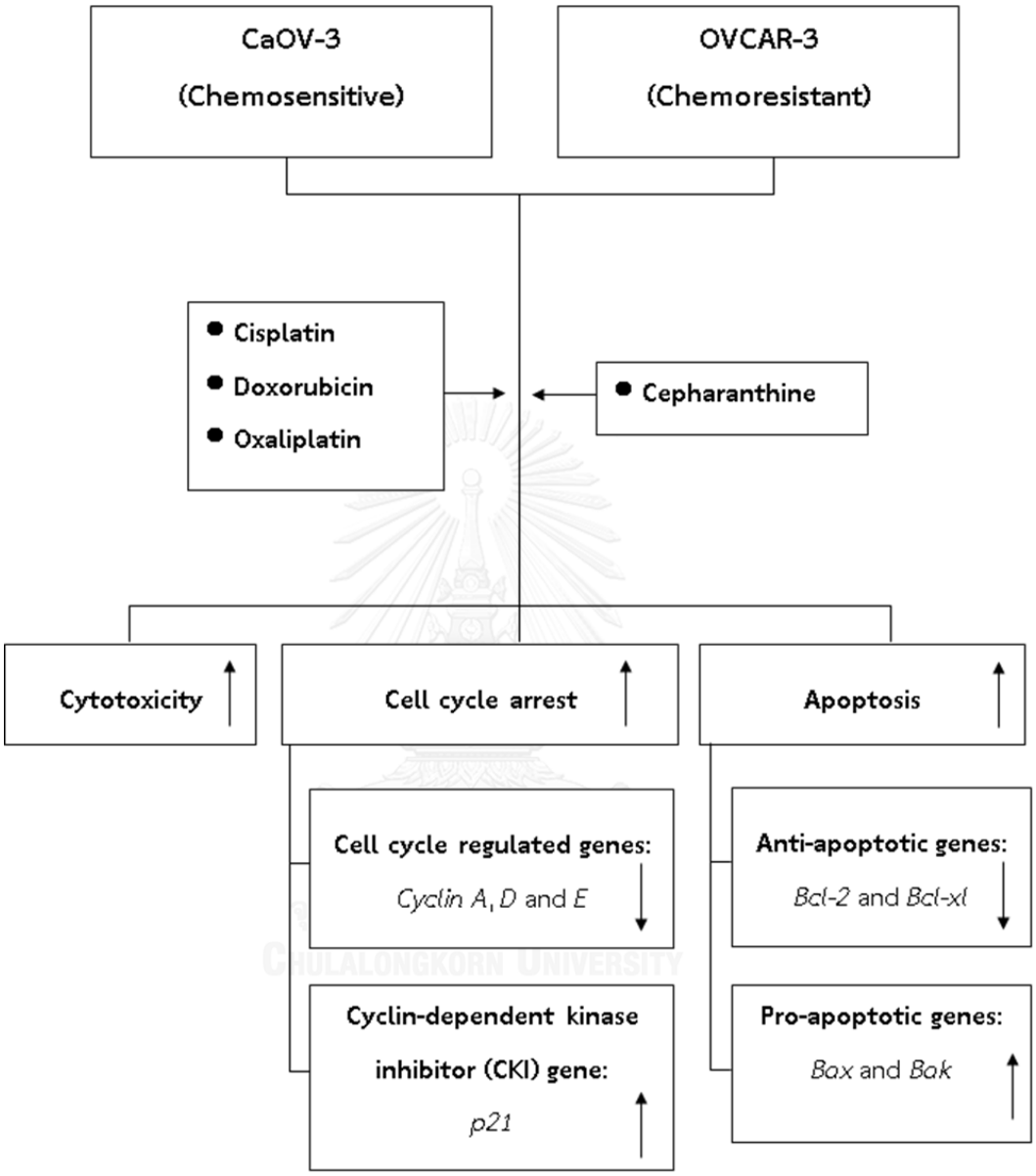
1. To investigate the anticancer effects of cepharanthine in chemosensitive (CaOV-3) and chemoresistant (OVCAR-3) human ovarian cancer cells.

2. To examine the mechanism(s) responsible for the anticancer effects of cepharanthine on both human ovarian cancer cell lines.

1.3 Hypothesis

Cepharanthine has effective anticancer activity against both chemosensitive and chemoresistant ovarian cancer cells.

1.4 Conceptual framework



CHAPTER II
LITERATURE REVIEWS

2.1 Ovarian cancer

Ovarian cancer is one of the most common gynecologic cancers and the leading cause of cancer-related death in women worldwide. More than 70% of patients are present with advanced stage disease (stage III-IV) because the early-stage is generally asymptomatic and very difficult to diagnose. Thus, the 5-year survival rate for these patients is less than 40% [2]. According to estimates from the International Agency for Research on Cancer (IARC): Globocan, in 2012, ovarian carcinoma was the seventh most common cancers among women in the world and there were 238,700 new ovarian cancer cases and 151,900 ovarian cancer deaths. The incidence of ovarian cancer was found to be the highest in Europe and Northern America. In the United State, American Cancer Society has reported that ovarian cancer is the fifth leading cause of deaths in US women which is estimated that about 22,280 women develop ovarian cancer and there are approximately 14,240 ovarian cancer deaths in 2016. The incidences in American women are slightly increased from 2015 around 5% , while the mortality rates remain approximately the same which have been about 14,000 cases since 2013 to 2016. In Asia, the International Agency for Research on Cancer (IARC) reported that there were estimated 111,887 new cases of ovarian cancer and 66,215 deaths, which Thailand is presented in the seventh of ovarian cancer incidence and deaths among Asia women in 2012. According to National Cancer Institute (NCI) demonstrated in 2013, there were estimated about 73 new cases or 3.10% of gynecological cancer occurred in Thai women.

Ovarian cancer is a term of cancer that begins in the ovary, a reproductive gland found only in females. The ovaries produce eggs for reproduction and also the main source of the female hormones, including estrogen and progesterone. The ovaries consist of three main types of cells, which each type can develop into different types of tumor, including epithelial tumors, stromal tumors and germ cell tumors (Figure 1) [31].

(i). ***epithelial tumors*** develop from the cells that cover the outer surface of the ovary.

(ii). ***stromal tumors*** develop from structural tissue cells that hold the ovary together and produce the female hormones, estrogen and progesterone.

(iii). ***germ cell tumors*** develop from the cells that produce the eggs.

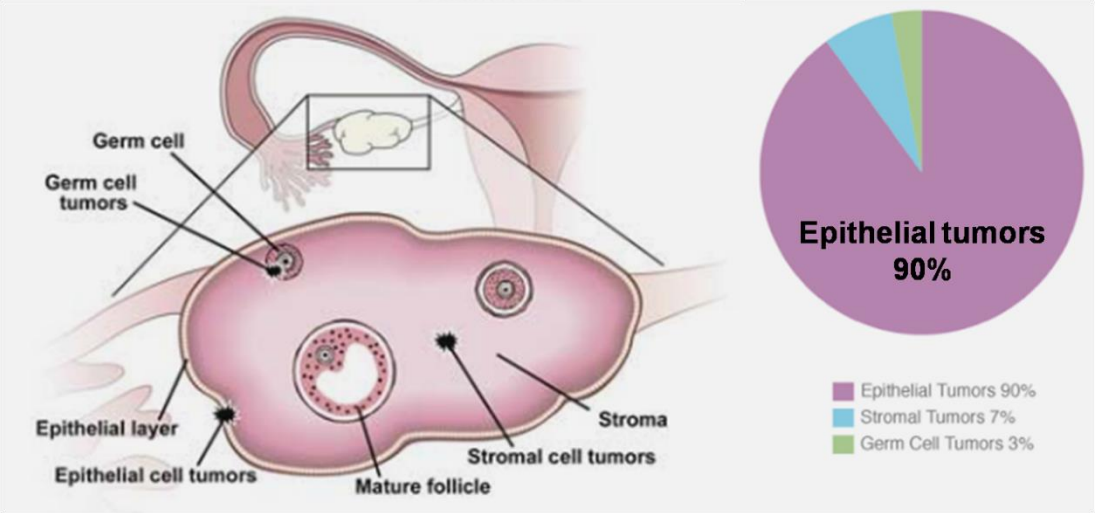


Figure 1 Types of ovarian cancer

More than 90% of ovarian cancers are epithelial tumors, which develop on the surface layer of the ovary [32]. There are various subtypes of epithelial ovarian cancer but the most common are high-grade serous cancer subtypes [33]. High-grade serous ovarian cancers frequently contain p53 mutation [34, 35]. In addition to p53 mutation, aging (more than 50 years old), obesity, hormone replacement and family history of ovarian cancer are highly associated with ovarian cancer [36].

Genetic mutations have widely been identified in ovarian cancer patients. Mutation and loss of tumor protein 53 (*TP53* or p53) function is the most common genetic abnormalities (approximately 96%) in ovarian cancer [37, 38]. Several studies reported that ovarian cancer cells with abundant p53 mutation are associated with poor prognosis of patients [39]. In addition, mutation of p53 has been involved with resistance to platinum-based therapy for ovarian cancer treatment [37, 39, 40]. Previous studies revealed that the mutation of p53 gene causes the development of cisplatin-resistance in ovarian cancer cells such as OVCAR-3 [41, 42]. Besides p53, mutation and amplification of oncogene in the PI3K/AKT family were detected in more than 40% of patients with epithelial ovarian cancer. It was reported that there are correlation between mutations of PI3KCA and AKT and progression of ovarian cancer [38]. Several studies have found increase in copy number of PIK3CA in ovarian cancers, leading to higher PI3K activity [43-45]. Over-activation of AKT was also found to be related to ovarian cancer progression [45, 46]. Furthermore, activation of the PI3K signaling pathway through mutation of PIK3CA or AKT has been found to be involved in chemoresistance of ovarian cancer cells [35, 47, 48].

2.1.1 Staging of ovarian cancer

Cancer staging, classified based on the severity of disease, is used to plan treatment and predict the clinical outcome. According to the International Federation of Gynecology and Obstetrics (FIGO), the stage of ovarian cancer can be classified into four stages as follows: [33]

Stage I: Tumor is confined to one or both ovaries and has not yet spread to other areas. At this stage, up to 90% of patients can be cured using currently available therapy.

Stage II: Tumor can be found outside of one or both ovaries but has spread into the pelvic region (uterus, bladder, lower intestine).

Stage III: Tumor involves one or both ovaries and has spread beyond the pelvis into the abdominal cavity (but not the liver) and/or metastasis to near lymph nodes.

Stage IV: Tumor has spread to other parts of the body such as the liver, lung and brain. At this stage, the cure rate and survival rate decrease substantially.

2.1.2 Treatment of ovarian cancer

The current standard treatments for ovarian cancer patients include cytoreductive surgery, radiotherapy, systemic chemotherapy and targeted therapy depending on the stage of the disease. Surgery is the main option for early-stage disease. However, the majority of ovarian cancer patients are diagnosed in advanced-stage disease. Thus, these patients are usually treated by a combination of cytoreductive surgery and standard chemotherapy [31].

The standard chemotherapeutic agents for ovarian cancer are platinum coordination complexes, including cisplatin, carboplatin, and oxaliplatin as well as taxane, including paclitaxel, and docetaxel, which are generally used in combination rather than single compound [49]. Although, the platinum- based chemotherapy has had a major impact for clinical treatment of ovarian cancers [50], more than 70% of patients with advanced ovarian cancer will experience disease recurrence within 6 months after primary chemotherapy. Thus, the second- line chemotherapy, such as doxorubicin, becomes candidate for treatment with relapsed ovarian cancer [31, 51, 52].

2.1.2.1 Platinum coordination complexes

The platinum coordination complexes have board anticancer activity against several types of cancer such as head and neck, bladder, lung, colon and ovarian. The members of platinum compounds consist of cisplatin, carboplatin and oxaliplatin [53]. It has been shown that the combination between cisplatin or carboplatin with paclitaxel induces complete response in the majority of patients with ovarian cancer [50, 53]. In addition, oxaliplatin, a third- generation platinum derivative, has been used mainly in recurrent ovarian cancer as a single or in combination with other platinum and non-platinum compounds. Some studies reported that oxaliplatin/ cisplatin combination treatment has also shown higher activity comparable to high-dose of cisplatin or high-dose of carboplatin in patients with recurrent ovarian cancer [54, 55].

Mechanism of action

Once inside the cell, the platinum compound reacts with N-7 of guanine site on DNA, which results in intra-strand and inter-strand DNA crosslinks, and DNA single- or double- strand breaks, inhibiting transcription and DNA replication (Figure 2) [49, 56].

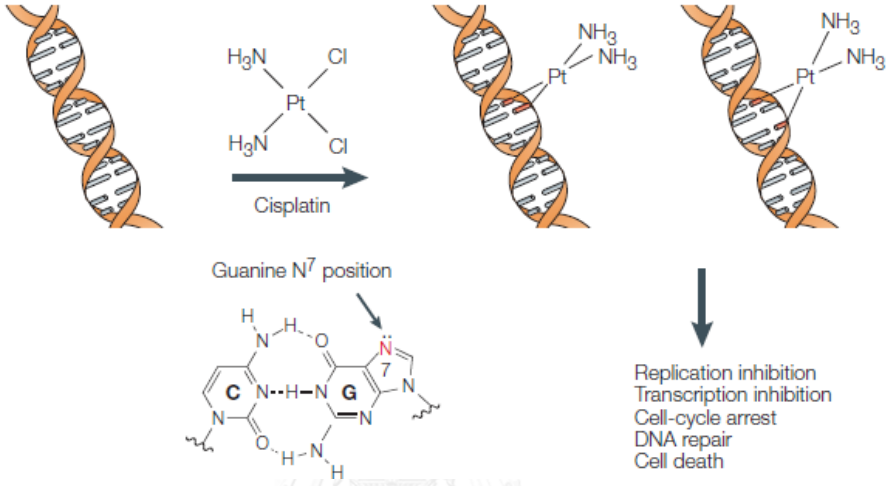


Figure 2 Mechanism of action of cisplatin [53]

Clinical toxicity

Similar to other chemotherapeutic agents, common side effects of the platinum drugs are bone marrow suppression, nausea and vomiting. Moreover, specific side effects of platinum-based antineoplastic drugs have also been reported. Of all the platinum compounds, nephrotoxicity and ototoxicity have been often found in cisplatin treatment [57]. Additionally, the treatment of cisplatin at high or multiple doses causes neurotoxicity by approximately 50% of patients receiving cisplatin [58, 59]. It was also reported that cisplatin could induce anaphylactic-like reaction [56]. Moreover, cisplatin has been associated with the development of acute myelogenous leukemia (AML) after treatment approximately over 4 years [59]. Compared to cisplatin, carboplatin, a second generation of anticancer drug, is relatively well

tolerated. It causes less nausea, nephrotoxicity, ototoxicity, and neurotoxicity than cisplatin [60, 58]. However, studies have shown that approximately 4-6% of patients who receive carboplatin develop peripheral neuropathy [59, 60]. In contrast to carboplatin, oxaliplatin, the third generation platinum analog, produces significant neurological dysfunction. Peripheral neuropathy is the most common dose-limiting toxicity of oxaliplatin [6]. Oxaliplatin also induces acute allergic response with urticaria and bronchoconstriction. Furthermore, pulmonary fibrosis has been detected within months to years after treatment with oxaliplatin. This drug, however, reduces renal toxicity as compared to the cisplatin. Additionally, oxaliplatin- associated ototoxicity is very uncommon [55, 59].

2.1.2.2 Anthracycline antibiotics

Anthracycline drugs are derived from the fungus *Streptomyces peucetius var. caesius*. Doxorubicin is one of the members of anthracycline drugs, which display board activity against human solid tumors such as breast cancer, lung cancer, gastric cancer, thyroid cancer, non-Hodgkin's & Hodgkin's lymphoma, multiple myeloma, sarcoma and also ovarian cancer [61]. Doxorubicin has commonly been used as the first-choice non-platinum agent for recurrent ovarian cancers. It was reported that this drug is effective and well tolerated in patients with relapsed ovarian cancer [5]. A phase II clinical trial demonstrated that the response rates were 17- 20% and a median progression- free survival was approximately 5- 6 months in the recurrent ovarian cancer patients receiving doxorubicin [62, 63].

Mechanism of action

There are three proposed mechanisms by which doxorubicin act in the cancer cell [64, 65].

(i). The drug can intercalate with DNA, directly affecting transcription and replication.

(ii). The drug can form a complex with topoisomerase II and DNA. Topoisomerase II is an ATP- dependent enzyme that binds to DNA and produces double- strand break and then re- ligates the DNA strands for replication processing. Binding of the topoisomerase/ DNA complex with anthracyclines inhibits the re- ligation of the broken DNA strands, leading to DNA damage and induce apoptosis.

(iii). The drug generates free radicals, which can form semiquinone radical intermediates that can react with oxygen to produce superoxide anion radicals. The production of free radicals can lead to lipid peroxidation and DNA and membrane damage, triggering apoptotic cell death.

Clinical toxicity

The major limitation for the use of doxorubicin is cardiotoxicity. Several studies demonstrated that doxorubicin- induced cardiomyopathy is the most important long-term toxicity and may proceed to clinical congestive heart failure many months, or longer after the completion of treatment [64]. In addition, the generally toxicities of the drug are myelosuppression, thrombocytopenia, anemia and leukopenia. Previous study found that the high dose anthracyclines (doxorubicin ≥ 120 mg/m^2) cause severe neutropenia. Furthermore, mucositis, diarrhea and alopecia have been found to be common side effects of doxorubicin, but these symptoms are reversible [61].

2.2 The cell cycle

The cell cycle, a cycle of cell duplication and division, can be divided into four successive phases: G1 (gap 1), S (DNA synthesis), G2 (gap 2) and M (mitosis). During G1 phase, the cell grows and synthesizes mRNA and proteins, which are required for DNA replication during the S phase. Once DNA synthesis is completed, the cell enters the next phase, G2 phase. During this phase, the cells check the completion of DNA replication and the integrity of genome before cell division starts. The M phase is composed of mitosis and cytokinesis, resulting in two identical daughter cells. However, when the cell ceases proliferate due to the absence of proper mitogenic signaling, the cell can exit the cell cycle and enter a resting state known as G0 (G zero) [66].

The cell cycle progression is tightly controlled by two protein families, cyclin-dependent kinase (CDKs) and the cyclins (Figure 3). CDKs are a family of serine/threonine kinase, including CDK4, CDK6, CDK2 and CDK1. The activities of these kinases are activated via forming a complex with specific cyclins, such as cyclin D, E, A and B [67]. The cyclin-CDK complexes specifically regulate each phase of cell cycle. During late G1 phase, cyclin D/CDK4 or cyclin D/CDK6 complexes phosphorylate the retinoblastoma (RB), followed by another phosphorylation caused by cyclin E/CDK2 complex. Phospho-RB can then dissociate from E2F, allowing transcription and progression of the cells into the S phase. Similar to the G1-S phase, transition of the cells from the S phase into the G2 phase and G2 phase into M phase are regulated by cyclin A/CDK2 and CDK1 and cyclin B/CDK1 complexes, respectively [68].

The activity of cyclin-CDKs complexes is inhibited by two families of cyclin-dependent kinase inhibitors (CKIs). The members of the INK4 family, including $p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$, and $p19^{INK4d}$, are direct inhibitors of CDK4 and CDK6 whereas members of the CIP/KIP family, including $p21^{CIP}$, $p27^{KIP1}$

and $p57^{KIP2}$, are specific inhibitors of CDK2/cyclin E, as well as CDK2/cyclin A, CDK1/cyclin A and CDK1/cyclin B [68, 69] (Figure 3).

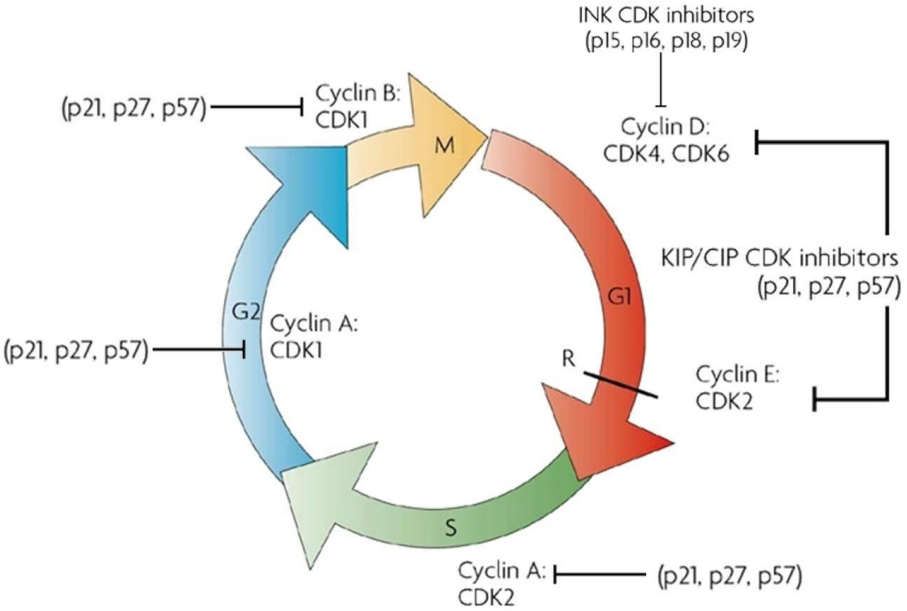


Figure 3 Cell cycle regulation [66]

2.3 Apoptosis

Apoptosis is a naturally occurring form of programmed cell death. It plays a crucial role in both physiological and pathological processes. Apoptotic cell is morphologically characterized by DNA fragmentation, cell shrinkage and membrane blebbing [70]. Most chemotherapeutic agents induce cancer cells to undergo apoptosis process [71]. There are two major apoptosis pathways, including *the extrinsic death receptor pathway* and *the intrinsic mitochondrial pathway* (Figure 4).

The extrinsic pathway can be initiated by binding of extracellular signaling proteins such as Fas ligand, tumor necrosis factor alpha (TNFα), and TNF-related apoptosis inducing ligand (TRAIL) to cell-surface death receptors, which are transmembrane proteins belonging to the tumor necrosis factor (TNF) receptor family. This leads to recruitment of intracellular adaptor proteins such as Fas-associated death domain (FADD) and initiator pro-caspases (pro-caspase-8, pro-caspase-10, or both), forming a death-inducing signaling complex (DISC). The formation of the DISC results in activation of the initiator caspase, further activating downstream executioner caspases such as caspase-3 or caspase-7, leading to apoptosis [(72)].

The intrinsic pathway is apoptosis program initiated from inside the cell, usually in response to injury, DNA damage, hypoxia, oxidative stress, tumorigenesis or anticancer drugs. The intrinsic pathway of apoptosis is tightly regulated by member of the Bcl-2 family proteins, which can be divided into pro-apoptotic and anti-apoptotic proteins. The pro-apoptotic proteins, consisting of two sub families including the BH123 proteins e.g. Bax and Bak and the BH3-only proteins e.g. Bad, Bid, Bim, PUMA and NOXA, promote apoptosis by enhancing the release of cytochrome C. Conversely, the anti-apoptotic proteins, such as Bcl-2 and Bcl-xl, inhibit apoptosis by blocking the release of cytochrome C.

Usually, the anti-apoptotic proteins, such as Bcl-2 and Bcl-xl, are mainly located on the cytosolic surface of the outer mitochondrial membrane to preserve the integrity of the mitochondrial membrane, preventing the inappropriate release of cytochrome C from mitochondrial. These proteins bind to and inhibit pro-apoptotic proteins such as Bax and Bak, resulting in inhibition of apoptosis. After receiving apoptotic stimuli, the pro-apoptotic BH3-only proteins bind and inhibit anti-apoptotic proteins,

leading to the activation of pro-apoptotic BH123 protein, Bax and Bak. Once activated, Bax and Bak promote cytochrome C release into the cytosol. The cytochrome C then bind to an apoptotic protease-activating factor 1 (Apaf1), forming an oligomeric apoptosome to recruit and activate initiator pro-caspases (pro-caspase-9). Once activated, caspase-9 can cleave and activate executioner caspase (caspase-3 or caspase-7), leading to apoptosis [73, 74].

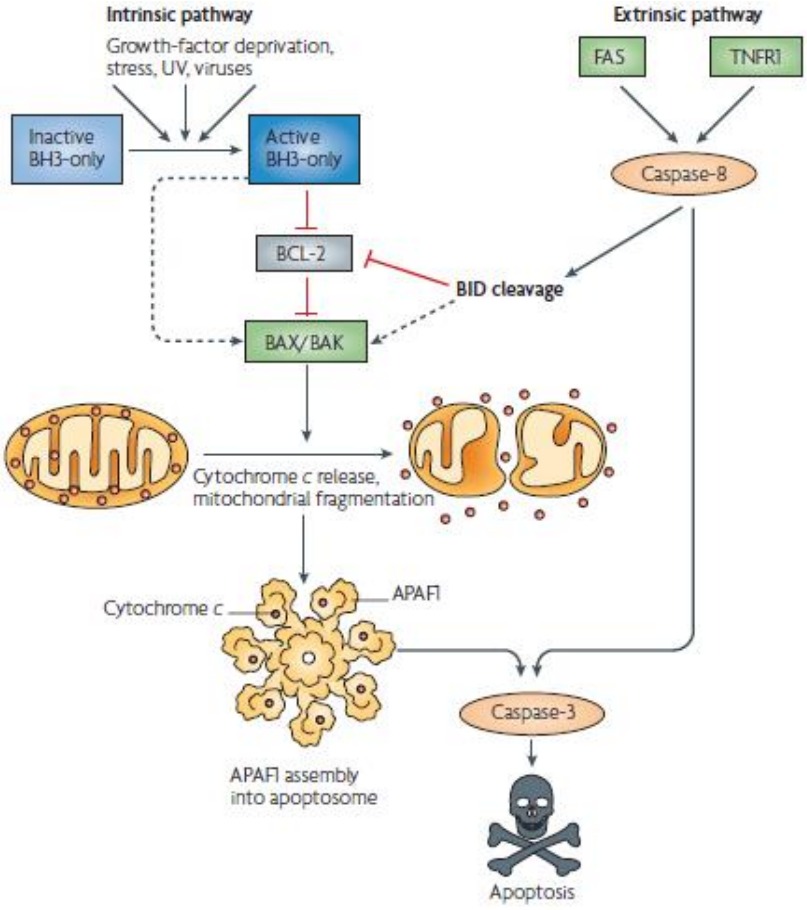


Figure 4 Apoptosis pathway [74]

2.4 Cepharanthine

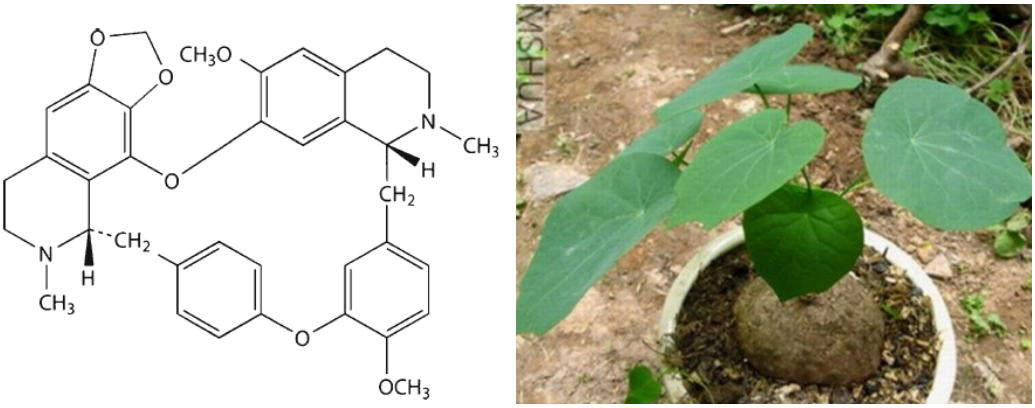


Figure 5 (A) Chemical structure of cepharanthine [11]
and (B) *Stephania venosa* (Blume) Spreng

Cepharanthine (CEP) is a biscoclaurine alkaloid, extracted from the root of *Stephania cepharantha* Hayata (Figure 5A). It can also been isolated from *Stephania venosa* (Blume) Spreng and *Stephania erecta* Craib, which are commonly found in Thailand (Figure 5B). This compound has been used in Japan for more than 40 years to treat various acute and chronic diseases. It is available in both powder and tablet forms [27]. Cepharanthine is well absorbed and mainly distributed to the liver, spleen and kidney. Pharmacokinetics studies in healthy adult males revealed the time to maximum concentration (t_{max}) following oral administration of a 10 to 60 mg dose is between 1.1 and 2.5 h as well as t_{max} for a 120 mg dose is approximately 1.2 ± 0.3 h. The 48 h cumulative urinary excretion rate of 120 mg of cepharanthine in healthy adult meals is 1.4 ± 0.3% [11]. Currently, cepharanthine is approved by the Japanese Ministry of Health for treatment of radiation-induced leukopenia, alopecia areata and alopecia pityrodes [11]. Various pharmacological effects of cepharanthine have been reported such as anti-malarial [13], antioxidant [10, 14], anti-allergic [15], anti-inflammatory [16, 17],

anti-platelet aggregation [75, 76], reversing multi-drug resistance [77, 78], and antitumor [18-21].

2.4.1 Antitumor activity

Several preclinical studies have demonstrated the antitumor activity of cepharanthine against several types of cancers such as adenosquamous cell carcinoma [27], oral squamous cell carcinoma [28], leukemia [18, 79], hepatocellular carcinoma [21], cholangiocarcinoma [19], myeloma [23], osteosarcoma [25], cervical adenocarcinoma [22] and non-small-cell lung cancer [10]. Previously, cepharanthine was found to induce apoptosis in cancer cells through triggering several signaling pathways such as STAT pathway [25], NF-ΚB pathway [19, 26], p38 MAPKs/ ERKs/ JNK1/2 pathway [18, 21] and Akt pathway [21, 79]. Moreover, Hua et al. was reported that apoptosis induction by cepharanthine is mediated through induction of Bax and reduction of Bcl-2 in non-small-cell lung cancer [10]. Additionally, cepharanthine was found to decrease the expression of Bcl-xl gene, resulting in apoptotic cell death [25]. Previous studies also illustrated that 1-10 μM of cepharanthine could induce apoptosis in human leukemia T cells, Jurkat, and human chronic myelogenous leukemia cells, K526 cells, via up-regulation of Bid, activation of caspase-3 and -9 and cleavage of poly (ADP-ribose) polymerase (PARP) [79]. Similarly, activation of caspase-3, -8, -9 and cleavage of PARP were shown to be associated with antitumor effects of cepharanthine in other numerous human cancer cells, including adenosquamous cell carcinoma, non-small-cell lung cancer, myeloma and cholangiocarcinoma [10, 19, 21, 23, 25, 27, 28]. Biswas et al. and Hua et al. also found that cepharanthine could disrupt mitochondrial membrane potential, which leads to cytochrome C release and ROS generation, resulting in apoptotic cell death [10, 21, 23]. In addition, cepharanthine could increase sub-G1 accumulation, indicating apoptotic cell

death [26]. Furthermore, this alkaloid could also trigger cell cycle arrest. Kikukawa et al. revealed that cepharanthine could induce G1 arrest through increasing CDKs inhibitor, $p15^{INK4B}$, and decreasing cyclin D1 and CDK6 in myeloma [23]. In a manner similar to myeloma, cepharanthine could inhibit expression of cyclin D1 and c-Myc, resulting in cell growth arrest at G1 phase in osteosarcoma cells [25]. Moreover, Harada et al. reported that cepharanthine induced G1 arrest in oral adenosquamous cell carcinoma by up-regulating $p21^{WAF1}$ as well as $p27^{Kip1}$, leading to cyclin E down-regulation [27, 28]. Hua et al. have recently shown that cepharanthine was able to induce cell cycle arrest at S phase and G2/M phase in H1299 and A549 non-small-cell lung cancer cells, respectively [10]. Interestingly, cepharanthine has been demonstrated to induce TYS cells, adenosquamous cell carcinoma carrying p53 mutation, to undergo cell cycle arrest by up-regulating of $p21^{WAF1}$ protein and it was suggested that cepharanthine is also capable of triggering cell growth arrest in a p53-independent pathway [27]. Recently, Rattanawong et al. (2015) reported that cepharanthine could efficiently inhibit the proliferation of p53-mutant HT-29 colorectal cancer cells which are resistant to commonly used chemotherapeutic drug [80]. Moreover, cepharanthine has demonstrated to potentiate the anticancer activity of various chemotherapeutic agents including vincristine and adriamycin by inducing apoptosis in p53-deficient osteosarcoma cell lines, SaOS2 [29]. Also, Zehedi et al. found that the combination of docetaxel (DTX) and cepharanthine resulted in a synergistic inhibition of cell growth in taxane-resistant ovarian cancer cells, HeyA8-MDR. Mechanistic studies revealed that the synergistic effect was mediated through enhanced apoptosis, increased intracellular DTX accumulation and reduced DTX efflux [81].

2.4.2 Clinical toxicity

Although cepharanthine has been widely used, severe side effects of this compound have never been reported. Previously reported that about 28 clinical cases (0.79%) from a total 3556 cases receiving the compound had headaches, dizziness and stomach discomfort but none of these complaints were serious side effects [12]. Moreover, Tanimura et al. revealed that no side effects were observed in two patients who took large doses 3 g/day and 6 g/day of cepharanthine for 35 and 23 days, respectively. Intravenous injection of cepharanthine 40-60 mg/day for 2 months was also found to be safe [82].

CHAPTER III

MATERIALS AND METHODS

3.1 Reagents

- Annexin-V, Fluorescein conjugate (FITC) (Life technologies, USA)
- Cepharanthine (Abcam, UK)
- Chloroform (Lab-scan, Thailand)
- Cisplatin (Sigma, USA)
- Dimethyl sulfoxide (DMSO) [cell culture grade] (Sigma, USA)
- Dimethyl sulfoxide (DMSO) [analytical grade] (Merck, Thailand)
- Doxorubicin (Sigma, USA)
- Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA)
- Ethanol (Merck, Germany)
- Express SYBER Green qPCR supermix universal (Life technologies, USA)
- Fetal bovine serum (Gibco, USA)
- ImProm-II™ Reverse Transcription system (Promega, USA)
- Nuclease free water (QIAGEN, USA)
- Oxaliplatin (Sigma, USA)
- Penicillin-streptomycin (Gibco, USA)
- Propidium iodide (Santa Cruz Biotechnology, USA)
- RNase (Thermo Scientific, EU)
- Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, USA)
- 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, USA)
- TRIzol Reagent (Life technologies, USA)

- 2-Propanol (Merck, Germany)
- 0.4% trypan blue dye (Sigma, USA)
- 0.25% trypsin-EDTA (Gibco, USA)

3.2 Materials and equipments

3.2.1 Materials

- 100 mm^2 cell culture dish (Corning Inc., USA)
- 15 ml conical tube (Corning Inc., USA)
- 0.1 ml low profile polypropylene thin wall PCR tube strips (Corning Life Sciences, USA)
- 6-well plate (Corning Inc., USA)
- 96-well plate (Corning Inc., USA)
- 75 cm^2 rectangular cell culture flask (Corning Inc., USA)
- 25 cm^2 rectangular cell culture flask (Corning Inc., USA)
- 5 ml round bottom polystyrene test tube (Falcon, USA)

3.2.2 Equipments

- Analytical balance 0.001 g (Mettler Toledo, Switzerland)
- Analytical balance 0.00001 g (Sartorius, Germany)
- Autopipette (Brand, Germany)
- Autoclave (Sanyo, Japan)
- Biohazard laminar flow hood (Labconco, USA)
- Controller pipette (Gilson, USA)
- CO_2 incubator (Thermo, USA)

- Centrifuge (Hettich, Germany)
- Fluorescence flow cytometer (BD Bioscience, USA)
- Light microscope (Nikon, Japan)
- Microplate reader (Thermo, Finland)
- PCR thermal cycler (Eppendrof, Germany)
- pH meter (Mettler Toledo, Switzerland)
- StepOnePlus™ Real-Time PCR system (Applied Biosystems, USA)
- Temperature control centrifuge (Eppendrof, Germany)
- Vortex mixer (Scientific Industries, USA)
- Ultrasonic bath (Bandelin, Germany)
- Water bath (IKA Labortechnik, Germany)

3.3 Methods

3.3.1 Preparation of chemotherapeutic agents and cepharanthine

Stock solutions of cisplatin, doxorubicin, oxaliplatin and cepharanthine were prepared in dimethyl sulfoxide (DMSO) at concentrations of 50 mM, 0.17 mM, 0.05 mM, and 50 mM, respectively, and stored at 4ºC until use. In the experiments, the stock solutions were diluted in culture medium to give appropriate final concentrations. The final concentration of DMSO was 0.2% (v/v).

3.3.2 Cell lines and cell culture

Two human ovarian cancer cell lines, CAOV-3 and OVCAR-3, were purchased from American Type Culture Collection (ATCC, USA). Both are epithelial cell lines. CAOV-3 cells derived from ovarian adenocarcinoma of a

54-year-old patient are classified as chemosensitive cells while OVCAR-3 cells derived from ovarian adenocarcinoma of a 60-year-old patient who was not respond to clinically relevant concentrations of adriamycin (doxorubicin) and cisplatin are classified as chemoresistant cells. It should be noted that the population doubling time of CaOV-3 and OVCAR-3 cells is approximately 40 and 57 h, respectively.

CAOV-3 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 U/ml penicillin and 100 µg/ml streptomycin. OVCAR-3 cells were maintained in Roswell Park Memorial Institute (RPMI) - 1640 medium containing 20% FBS and 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were kept at 37ºC in a humidified incubator with 5% CO_2.

3.3.3 Cell viability assay

Cell viability was evaluated using MTT assay that base on the enzymatic reduction of a yellow tetrazolium salt, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) into a purple formazan crystal in viable cells.

Briefly, CAOV-3 and OVCAR-3 cells were seeded in 96-well plates at a density of 7.5×10^3 cells/well and allowed to attach overnight in 5% CO_2 at 37 ºC. After that, cells were treated with various concentrations of cepharanthine (0.1, 1, 10, 100 µM), cisplatin (0.1, 1, 10, 100 µM), doxorubicin (0.01, 0.1, 1, 10, 100 µM) and oxaliplatin (1, 10, 100, 1000 µM) or 0.2% DMSO in complete medium (vehicle control) for 24, 48 and 72 h. After incubation,

15 µl of MTT (0.5 mg/ml in PBS) was added to each well and incubated for 3 h at 37 ºC, 5% CO_2 in the dark. Then, the supernatant was removed and 150 µl of DMSO was added to dissolve the formazan crystals. Finally, the absorbance was measured at 570 nm. The results are presented as percentage of cell viability, which was calculated using the following equation:

% cell viability = (Abs. of samples/ Abs. of control) × 100

The half inhibitory concentration (IC_{50}) was obtained using GraphPad Prism 7 (GraphPad Software, USA).

3.3.4 Analysis of cell cycle progression

Measurement of cellular DNA content to identify the proportion of cells that are in one of the three interphase stages of the cell cycle: G1, S, or G2/M phase can be performed using propidium iodide (PI), the fluorescent nucleic acid dye [83].

Briefly, CaOV-3 and OVCAR-3 cells were seeded in 6-well plates at a density of 2.5×10^5 cells/well and incubated in 5% CO_2 at 37 ºC for 24 h. According to the IC_{50} of cepharanthine and the doubling time of CaOV-3 cells and OVCAR-3 cells, CaOV-3 cells were treated with 5, 10 and 20 µM of cepharanthine, 10 µM of cisplatin (positive control), or 0.2% DMSO in complete DMEM medium (vehicle control) for 24 h whereas OVCAR-3 were exposed with 10, 20 and 40 µM of cepharanthine, 30 µM of cisplatin, or 0.2% DMSO in complete RPMI medium for 48 h. After incubation, the cells were harvested by trypsinization and collected by centrifugation at 1,500 rpm for 5 min. The cell pellets were washed with 500 µl of ice-cold PBS twice and fixed with ice-cold absolute ethanol for 30 min. After washing with ice-cold

PBS twice, samples were incubated with 5 µl of a 4 mg/ml stock of RNase at room temperature for 30 min. Cell staining was performed by incubating samples with 5 µl of 0.05 µg/ml stock of propidium iodide (PI) for 15 min at room temperature in the dark. The samples were measured by flow cytometry and analyzed using FCS Express 5 Image Cytometry software. Cells with 2n and 4n DNA content are in the G1 and G2/M phase of cell cycle, respectively, while cells contain DNA content between 2n and 4n are in the S phase. Apoptotic cells that have lost some of DNA content are in the sub-G1 phase (Figure 6).

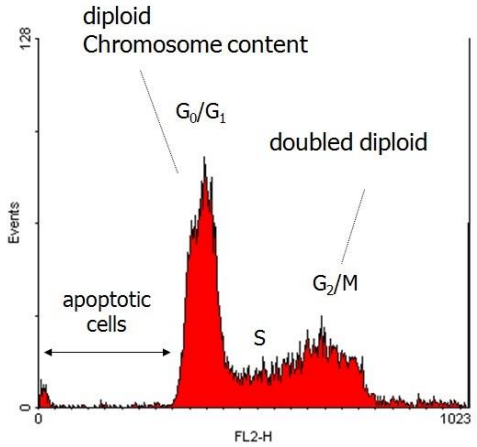


Figure 6 The cytograms of cell cycle pattern

3.3.5 Assessment of apoptosis using annexin V/PI staining

Apoptosis can be determined using annexin V-FITC conjugate and propidium iodide (PI) staining. During the early phase of apoptosis, phospholipid phosphatidylserine (PS) translocates from the inner to the outer leaflet of cellular membrane. Thus, fluorescently labeled annexin V which specifically binds with externalized PS can be used to detect early apoptotic cells. Conversely, cells undergoing necrosis lose membrane integrity, allowing entry of propidium iodide, a cell-impermeant DNA-binding dye [84].

Briefly, CaOV-3 and OVCAR-3 cells were seeded in 6-well plates at a density of 2.5×10^5 cells/well and incubated in 5% CO_2 at 37 ºC for 24 h. Then, CaOV-3 cells were treated with 5, 10 and 20 µM of cepharanthine, 10 µM of cisplatin (positive control), or 0.2% DMSO in complete DMEM medium (vehicle control) for 24 h. OVCAR-3 cells were treated with 10, 20 and 40 µM of cepharanthine, 30 µM of cisplatin, or 0.2% DMSO in complete RPMI medium for 48 h. After incubation, cells were harvested by trypsinization and collected by centrifugation at 1,500 rpm for 5 min. The cell pellets were washed with 500 µl of ice-cold PBS twice and then suspended with 100 µl of assay buffer. Thereafter, 1 µl of annexin V-FITC and 1 µl of propidium iodide (PI) were added and incubated for 20 min at room temperature in the dark. The stained cells were counted using fluorescence flow cytometer. Q1 (annexin V-/PI-), Q2 (annexin V+/PI-), Q3 (annexin V+/PI+) and Q4 (annexin V-/PI+) represent living cells, early-apoptotic cells, late-apoptotic cells and necrotic cells, respectively (Figure 7).

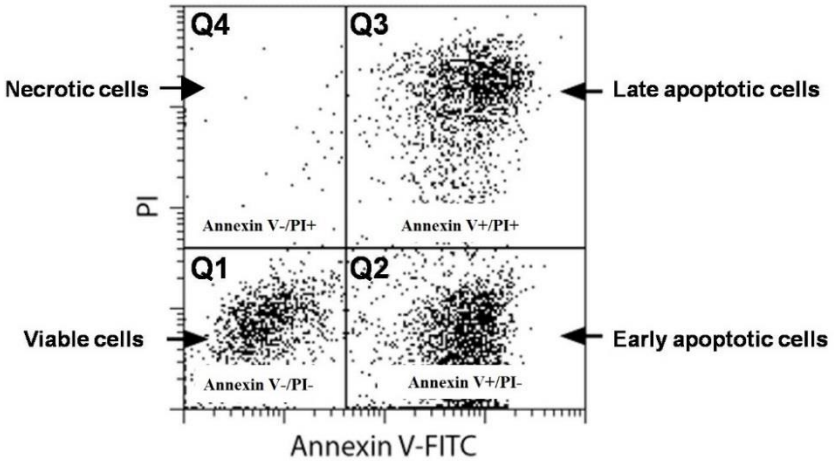


Figure 7 The cytograms of apoptotic/necrotic cell

3.3.6 Measurement of gene expression by real-time RT PCR analysis

The mRNA level of Bcl-2 family proteins, including pro-apoptotic (*Bax, Bak*) and anti-apoptotic (*Bcl-2, Bcl-xl*), and cell cycle regulators including *cyclin A*, *cyclin D*, *cyclin E* and *p21* were analyzed by real-time RT PCR using SYBR Green I as a probe.

Briefly, two human ovarian cancer cell lines, CaOV-3 and OVCAR-3, were seeded in 6-well plates at a density of 5×10^5 cells/well. After overnight incubation, CaOV-3 cells were treated with 5, 10 and 20 μM of cepharanthine, 10 μM of cisplatin (positive control) or 0.2% DMSO in complete DMEM medium (vehicle control) for 24 h whereas OVCAR-3 cells were treated with various concentrations of cepharanthine (10, 20 and 40 μM), 30 μM of cisplatin or 0.2% DMSO in complete RPMI medium for 24 h. Total RNA extraction was performed using TRIzol reagent. The concentrations of total RNA were evaluated spectrophotometrically at 260 nm. Then, the RNA (mRNA) was reverse transcribed to cDNA using Improm-II™ Reverse Transcription system according to the manufacturer's instruction (Promega, USA). Real-time RT PCR reactions were performed in a StepOne™ Real-Time PCR System using the SYBR Green I qPCR Master Mix and specific forward and reverse primers (Table 1). The reactions were carried out using the following conditions: 50ºC for 2 min, 95 ºC for 2 min, and 40 cycles of 95ºC for 30 sec, 60 ºC for 30 sec and 72ºC for 30 sec. The mRNA levels of apoptosis and cell cycle regulators normalized to housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was calculated by the $^{\Delta\Delta}$Ct method using StepOnePlus™ software.

Table 1 Sequences of primers used for real-time RT PCR

Target genes	Primer sequences
GAPDH	Forward: 5'-AAG GTC GGA GTC AAC GGA TTT GGT -3' Reverse: 5'-ATG GCA TGG ACT GTG GTC ATG AGT -3'
Bcl-2	Forward: 5'- TCA TGT GTG TGG AGA GCG TCA A -3' Reverse: 5'- CTA CTG CTT TAG TGA ACC TTT TGC -3'
Bcl-xl	Forward: 5'- TTG GAC AAT GGA CTG GTT GA -3' Reverse: 5'- GTA GAG TGG ATG GTC AGT G -3'
Bax	Forward: 5'- GAC GAA CTG GAC AGT AAC ATG -3' Reverse: 5'- AGG AAG TCC AAT GTC CAG CC -3'
Bak	Forward: 5'- ATG GTC ACC TTA CCT CTG CAA -3' Reverse: 5'- TCA TAG CGT CGG TTG ATG TCG -3'
Cyclin A	Forward: 5'- CTG CTG CTA TGC TGT TAG CC -3' Reverse: 5'- TGT TGG AGC AGC TAA GTC AAA A -3'
Cyclin D	Forward: 5'- TTG TTG AAG TTG CAA AGT CCT GG -3' Reverse: 5'- ATG GTT TCC ACT TCG CAG CA -3'
Cyclin E	Forward: 5'- TCC TGG ATG TTG ACT GCC TT -3' Reverse: 5'- CAC CAC TGA TAC CCT GAA ACC T -3'
p21	Forward: 5'- CCT GTC ACT GTC TTG TAC CCT -3' Reverse: 5'- GCG TTT GGA GTG GTA GAA ATC T -3'

3.3.7 Statistical analysis

All samples were prepared and analyzed from three independent experiments. The results are presented as means ± standard error of mean (SEM). Multiple comparisons were performed by a one-way analysis of variance (ANOVA) followed by LSD post hoc test using SPSS software. Statistical differences between two groups were evaluated by Student's *t* test using SPSS software. $P<0.05$ was considered statistically significant.

CHAPTER IV

RESULTS

4.1. Effect of chemotherapeutic agents and cepharanthine on cell viability of CaOV-3, a chemosensitive human ovarian cancer cell line.

Initially, the cytotoxic effects of chemotherapeutic agents including doxorubicin, oxaliplatin and cisplatin on a chemosensitive human ovarian cancer cell line, CaOV-3, were investigated. Cells were treated with various concentrations of the drugs: doxorubicin (0.01, 0.1, 1, 10 and 100 μM), oxaliplatin (1, 10, 100 and 1000 μM), and cisplatin (0.1, 1, 10 and 100 μM) for 24, 48 and 72 h. Then, cell viability was evaluated using MTT reduction assay. As shown in Figure 8, all chemotherapeutic agents inhibited CaOV-3 cell growth in a concentration dependent manner. Figure 8A illustrated that doxorubicin at concentrations of 0.1, 1, 10 and 100 μM significantly induced CaOV-3 cell death at 24, 48 and 72 h of incubation when compared to the vehicle control (0.2% DMSO) (P<0.01). For oxaliplatin, the drug at concentrations of 100 and 1000 μM significantly inhibited CaOV-3 cell growth following 24 h treatment and significant cell death was also detected after treatment with 10 μM of the drug for longer incubation time (48 and 72 h) (P<0.001) (Figure 8B). Similarly, at 24 h of incubation, cisplatin only at a concentration of 100 μM effectively reduced viability of CaOV-3 cells compared with the vehicle control (P<0.001) (Figure 8C). However, cytotoxicity of this compound was increased after 48 and 72 h of incubation. Cisplatin at 10 and 100 μM significantly induced CaOV-3 cell death by 56.86±1.28% and 85.35±0.13% at 48 h and 77.60±0.45% and 87.71±0.25% at 72 h, respectively.

Previously, several studies reported that cepharanthine has anticancer effect against various human cancer cells but its effect on human ovarian cancer cells has never been investigated. Therefore, the anticancer activity of cepharanthine against CaOV-3 was evaluated using MTT assay. As shown in Figure 8D, cepharanthine inhibited the growth of CaOV-3 cells in a concentration dependent manner. Treatment of cepharanthine at concentrations of 10 and 100 μM significantly decreased the viability of CaOV-3 cells, compared with the vehicle control at all-time points. The percentage of cell viability at 24, 48 and 72 h were 48.75±1.28%, 24.33±0.82% and 15.33±0.88% after treatment with 10 μM cepharanthine and 15.20±0.14%, 11.76±0.08% and 9.52±0.06% after incubation with 100 μM cepharanthine, respectively. Then, the cytotoxicity of the two-fold dilution of toxic concentrations of cepharanthine was further determined. Following 48 h treatment, cepharanthine at concentrations of 3.125, 6.25, 12.5, 25, 50 and 100 μM significantly decreased viabilities of CaOV-3 cells to 85.7, 78.2, 31.2, 9.6, 9.5 and 9.2 % of the vehicle control, respectively (Figure 8E).

The half inhibitory concentration (IC_{50}) values of chemotherapeutic agents and cepharanthine in CaOV-3 cells at 24, 48 and 72 h of incubation were illustrated in Table 2. These findings demonstrated that cepharanthine possess the potent anticancer effect on chemosensitive human ovarian cancer cells, CaOV-3. The IC_{50} values of cepharanthine in CaOV-3 cells at 24, 48 and 72 h of incubation were 2.90, 1.24 and 2.17 fold lower than those of cisplatin, one of the most commonly used drugs for ovarian cancer. To determine the mechanism(s) underlying anticancer activity of cepharanthine in CaOV-3 cells, the compound at concentrations of 5 (a half-fold IC_{50}), 10 (IC_{50}) and 20 (two-fold IC_{50}) μM were used. Cisplatin at a concentration of 10 μM (IC_{50}) was used as a positive control.

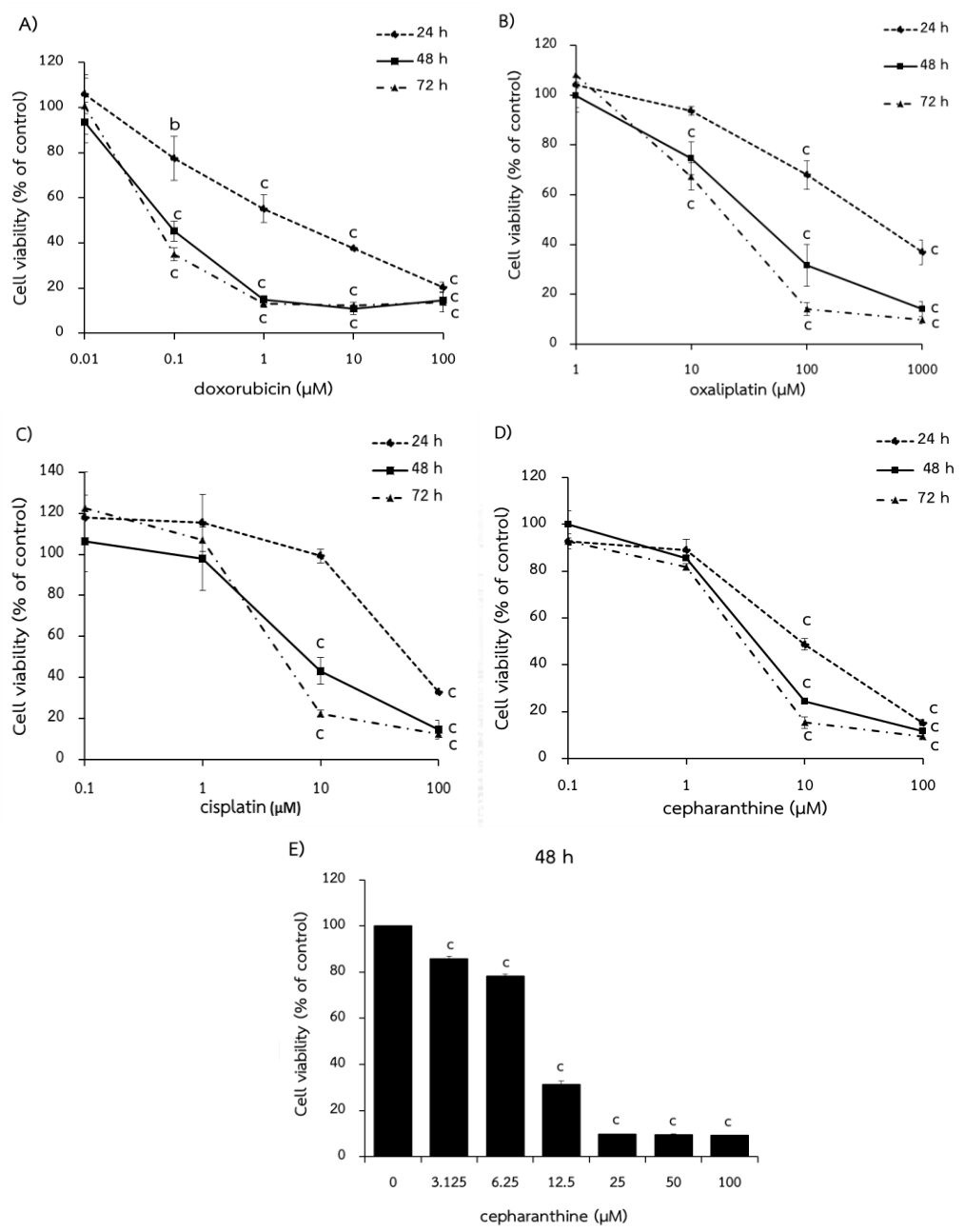


Figure 8 The effect of chemotherapeutic agents and cepharanthine on cell viability of a chemosensitive ovarian cancer cell line, CaOV-3. Cells were exposed to the various concentrations of (A) doxorubicin: 0.01, 0.1, 1, 10, 100 μM, (B) oxaliplatin: 1, 10, 100, 1000 μM, (C) cisplatin: 0.1, 1, 10, 100 μM, and (D) cepharanthine: 0.1, 1, 10, 100 μM for 24, 48 and 72 h incubation. (E) Cells were treated with 3.125, 6.25, 12.5, 25, 50 and 100 μM of cepharanthine for 48 h. Cell viability was assessed using MTT assay. The results are expressed as mean ± SEM of three independent experiments. $^{b}P<0.01$, $^{c}P<0.001$ compared to the vehicle control (0.2% DMSO).

Table 2 IC_{50} values of chemotherapeutic agents and cepharanthine on a chemosensitive human ovarian cancer cell line, CaOV-3.

Drugs	IC_{50} (μM)		
	24 h	48 h	72 h
doxorubicin	2.10 ± 0.52	0.10 ± 0.02	0.07 ± 0.02
oxaliplatin	361.13 ± 58.79	43.92 ± 12.04	21.36 ± 3.09
cisplatin	66.74 ± 4.20	13.51 ± 0.88	7.36 ± 0.13
cepharanthine	22.99 ± 7.28[b]	10.93 ± 0.14	3.39 ± 0.28[a]

- Data represent means ± SEM from three independent experiments.
- [a]$P<0.05$, [b]$P<0.01$ compared with cisplatin.

4.2. Effect of chemotherapeutic agents and cepharanthine on cell viability of OVCAR-3, a chemoresistant human ovarian cancer cells.

Resistance to chemotherapy is a major problem in cancer treatment, therefore novel compounds with potent anticancer activity against chemoresistant cancer cells are urgently need [(85)]. In the present study, the effect of chemotherapeutic agents including doxorubicin, oxaliplatin and cisplatin on a chemoresistant ovarian cancer cell line, OVCAR-3, which have been reported to be resistant to several conventional therapeutic drugs, including cisplatin and adriamycin (doxorubicin), were determined. OVCAR-3 cells were treated with various concentrations of the drugs: doxorubicin (0.01, 0.1, 1, 10 and 100 μM), oxaliplatin (1, 10, 100 and 1000 μM), and cisplatin (0.1, 1, 10 and 100 μM) for 24, 48 and 72 h and cell viability was assessed using MTT reduction assay. As shown in Figures 9A-C, all three chemotherapeutic agents reduced OVCAR-3 cell proliferation in a concentration-dependent manner. At 24 h incubation, doxorubicin at concentrations of 10 and 100 μM significantly inhibited OVCAR-3 cell growth

to 71.77±0.55% and 43.14±0.40% compared to the vehicle control, respectively (Figure 9A, $P<0.001$). Cytotoxicity of the drug was significantly increased when incubation times were extended to 48 and 72 h. Figure 9B illustrated that oxaliplatin at 100 and 1000 μM significantly induced OVCAR-3 cell death at all-time points whereas toxicity of the drug at 10 μM was also observed following 72 h incubation. Similarly, cisplatin only at a concentration of 100 μM significantly decreased cell viability at 24 h treatment ($P < 0.01$) (Figure 9C). And anticancer activity of the drug was found to be higher at longer incubation times (48 and 72 h). Cisplatin at 10 and 100 μM significantly induced OVCAR-3 cell death by 25.88±2.42% and 74.75±0.22% at 48 h and 44.72±0.81% and 77.39±0.32% at 72 h, respectively.

Recently, Rattanawong A. et al. reported that cepharanthine has anticancer effect against chemoresistant human colorectal cancer cells (HT-29). Therefore, in this study, the anticancer activity of cepharanthine on chemoresistant human ovarian cancer cells (OVCAR-3) was investigated using MTT assay. Cells were treated with 0.1, 1, 10 and 100 μM of cepharanthine for 24, 48 and 72 h and cell viability was determined using MTT reduction assay. Figure 9D showed that cepharanthine inhibited the proliferation of OVCAR-3 cells in a concentration-dependent manner. The highest concentration of cepharanthine (100 μM) significantly induced cell death at all-time points compared to the vehicle control ($P<0.001$). A significant decrease in cell viability was also noted at 10 μM of cepharanthine at 48 and 72 h of treatment. Cell viabilities were 82.16±1.76% and 72.47±1.58% after treatment at 48 and 72 h, respectively, when compared with the vehicle control. Then, the cytotoxic activity of the toxic concentrations of cepharanthine was determined using the two-fold dilution. Following 48 h treatment, cepharanthine at concentrations of 3.125, 6.25, 12.5, 25, 50 and

100 μM significantly decreased cell viabilities of OVCAR-3 cells to 99.4, 87.5, 98.2, 88.1, 56.3 and 10.3 % of the vehicle control, respectively (Figure 9E).

The half inhibitory concentration (IC_{50}) values of chemotherapeutic agents and cepharanthine in OVCAR-3 cells were illustrated in Table 3. This finding demonstrated that at 24 h treatment, of all four agents tested, cepharanthine possess the most potent anticancer activity. The IC_{50} of cisplatin at 24 h was however undetermined because the highest concentration of the compound used in this experiment inhibited OVCAR-3 cell growth approximately 56.18 ± 0.40 %. It is worth mentioning that, IC_{50} of cepharanthine at 72 h of incubation decreased by approximately 3 times that at 24 of incubation. Interestingly, at 72 h of incubation, the anticancer effect of cepharanthine was significantly greater than cisplatin in OVCAR-3 cells, indicating that cepharanthine effectively controlled the growth of ovarian cancer cells that are often resistant to commonly used chemotherapeutic agents such as cisplatin. To determine the mechanism(s) responsible for anticancer activity of cepharanthine in OVCAR-3 cells, the concentration of the compound at concentrations of 10 (a half-fold IC_{50}), 20 (IC_{50}) and 40 (two-fold IC_{50}) μM were used. Cisplatin at a concentration of 30 μM (IC_{50}) was used, as a positive control.

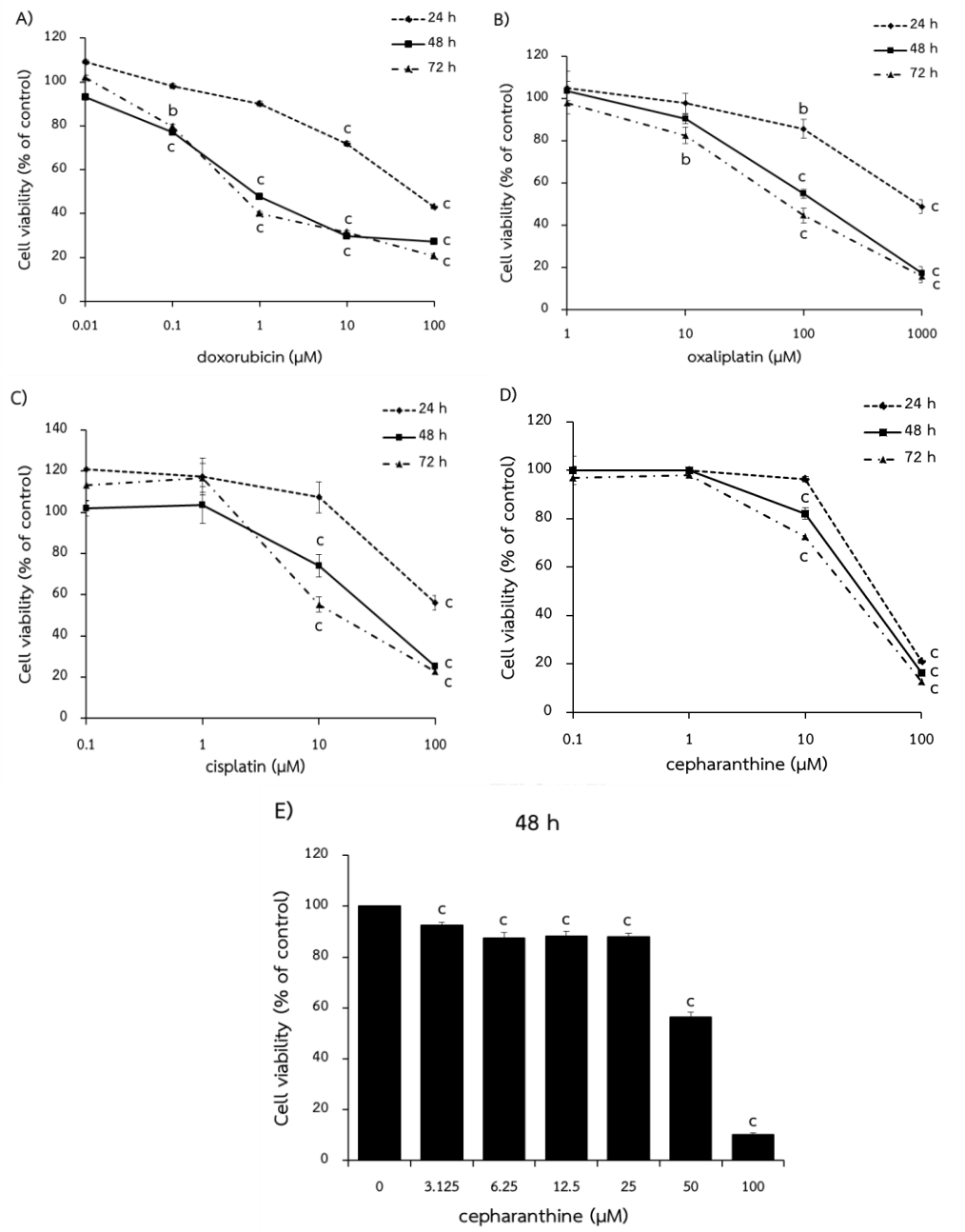


Figure 9 The effect of chemotherapeutic agents and cepharanthine on cell viability of a chemoresistant ovarian cancer cell line, OVCAR-3. Cells were exposed to the various concentrations of (A) doxorubicin: 0.01, 0.1, 1, 10, 100 μM, (B) oxaliplatin: 1, 10, 100, 1000 μM, (C) cisplatin: 0.1, 1, 10, 100 μM, and (D) cepharanthine: 0.1, 1, 10, 100 μM for 24, 48 and 72 h incubation. (E) Cells were treated with 3.125, 6.25, 12.5, 25, 50 and 100 μM of cepharanthine for 48 h. Cell viability was assessed using MTT assay. The results are expressed as mean ± SEM of three independent experiments. $^{b}P<0.01$, $^{c}P<0.001$ compared to the vehicle control (0.2% DMSO).

Table 3 IC_{50} values of chemotherapeutic agents and cepharanthine on a chemoresistant human ovarian cancer cell line, OVCAR-3.

Drugs	IC_{50} (μM)		
	24 h	48 h	72 h
doxorubicin	55.81 ± 11.69	1.06 ± 0.18	0.81 ± 0.06
oxaliplatin	899.13 ± 55.81	131.60 ± 8.29	80.49 ± 4.05
cisplatin	NA	34.26 ± 1.36	24.87 ± 0.44
cepharanthine	47.25 ± 5.74	31.20 ± 1.27	15.29 ± 1.49[a]

- Data represent means ± SEM from three independent experiments.
- NA is not applicable.
- [a]$P<0.05$ compared with cisplatin.

4.3. Effect of cepharanthine on cell cycle distribution in CaOV-3 cells.

Several anticancer agents induce cell cycle arrest at either G0/G1, S or G2/M phase, leading to apoptotic cell death [86]. Previous studies reported that cepharanthine induced cell cycle arrest at G1 phase in adenosquamous cell carcinoma [27], oral squamous cell carcinoma [28] and myeloma [23]. Therefore, induction of cell cycle arrest in CaOV-3 cells by cepharanthine was evaluated. The cells were treated with 5, 10 and 20 μM of cepharanthine, 10 μM of cisplatin or 0.2% DMSO for 24 h and cell cycle distribution was examined using propidium iodide (PI) staining followed by flow cytometry analysis. As shown in Figure 10, treatment of CaOV-3 cells with 5 μM of cepharanthine inhibited cell cycle progression at G1 phase, resulting in a significant increase in the percentage of cells at the G1 phase (from 52.65±3.44% to 64.10±0.66%, $P<0.001$) which was accompanied by reduction of the cells in the S phase (from 17.03±0.70% to 11.34±0.51%, $P<0.001$) and G2/M phase (from 24.53±3.40% to 16.53±0.74%, $P<0.017$) when compared

to the vehicle control. However, it should be noted that cepharanthine at higher concentrations, 10 and 20 μM, caused significant accumulation of CaOV-3 cells at sub-G1 phase (P<0.001), indicating apoptotic cell death. The percentage of cells in the sub-G1 phase was increased by approximately 4 and 9 times following treatment with cepharanthine at 10 and 20 μM, respectively. In contrast to cepharanthine, incubation of the cells with 10 μM cisplatin significantly induced cell cycle arrest at the S phase (Figure 10). Taken together, these findings suggest that cepharanthine has concentration-dependent dual effects by inducing G1 cell cycle arrest at a low concentration and triggering apoptosis at higher concentrations in CaOV-3 cells.

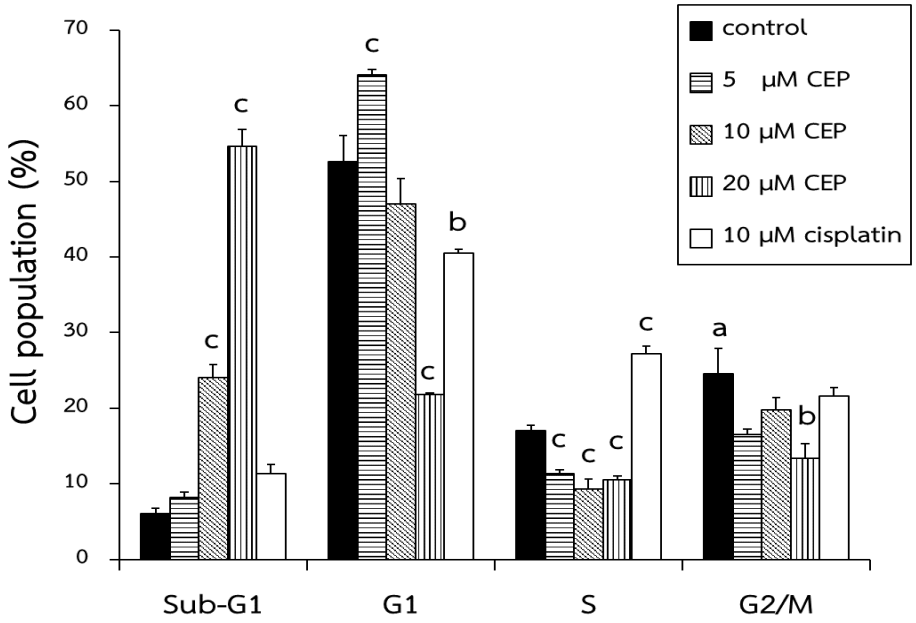


Figure 10 The effect of cepharanthine on cell cycle distribution of CaOV-3 cells. Cells were treated with 5, 10 and 20 μM of cepharanthine, 10 μM of cisplatin (positive control) or 0.2% DMSO (vehicle control) for 24 h. The percentage of CaOV-3 cells in sub-G1, G1, S and G2/M phase was determined by propidium iodide (PI) staining followed by flow cytometry analysis. Results are reported as means ± SEM of three independent experiments. Statistically significant differences compared with the vehicle control (^{a}P<0.05, ^{b}P<0.01 and ^{c}P<0.001).

4.4. Effect of cepharanthine on cell cycle distribution in OVCAR-3 cells.

The cell cycle distribution following cepharanthine treatment was evaluated using PI staining and flow cytometry analysis. OVCAR-3 cells were exposed with 10, 20 or 40 μM of cepharanthine, 30 μM of cisplatin or 0.2% DMSO for 48 h. As illustrated in Figure 11, treatment of cepharanthine did not significantly cause OVCAR-3 cells to pause at any phase of the cell cycle when compared to the vehicle control. However, cepharanthine, at the highest concentration (40 μM), induced a significant population of OVCAR-3 cells to accumulate at the sub-G1 phase, indicating apoptotic cell death. The percentage of cells in sub-G1 phase was increased by approximately 3 times that of control ($P<0.021$). In a manner similar to cepharanthine, cisplatin significantly caused accumulation of cells in sub-G1 phase approximately 4 times that of control (Figure 11, $P<0.009$). These results suggest that anticancer activity of cepharanthine is not mediated through cell cycle arrest in OVCAR-3 cells.

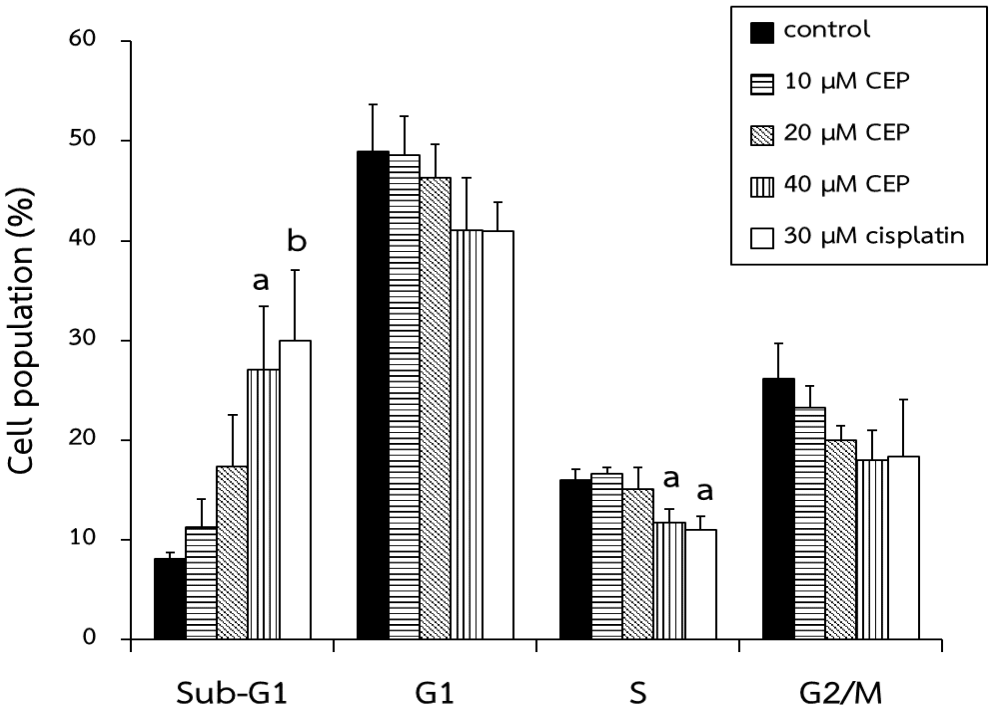


Figure 11 The effect of cepharanthine on cell cycle distribution of OVCAR-3 cells. Cells were treated with 10, 20 and 40 μM of cepharanthine, 30 μM of cisplatin (positive control) or 0.2% DMSO (vehicle control) for 48 h. The percentages of OVCAR-3 cells in each phase were measured by PI staining and flow cytometry analysis. Results are presented as means ± SEM of three independent experiments. Statistically significant differences compared to the vehicle control ($^{a}P<0.05$ and $^{b}P<0.01$).

4.5. Effect of cepharanthine on the mRNA levels of cell cycle regulators in CaOV-3 cells.

Cyclins and cyclin-dependent kinases (Cdks) are the major regulators of cell cycle progression. The activities of Cdks are tightly regulated by Cdk inhibitor (CKIs) such as p21 [87]. Previously, several studies indicated cepharanthine could down-regulate cyclin D and E in osteosarcoma cell [25] and adenosquamous cell carcinoma, respectively [27] while it could up-regulated p21 in myeloma [23]. Therefore, mRNA levels of cyclins D, E and A as well as p21, one of the most important CKIs, following cepharanthine treatment were evaluated in this study using real-time RT-PCR analysis. Figure 12A illustrated that cepharanthine significantly down-regulated expression of cyclin A gene in a concentration-dependent manner, whereas it did not alter the mRNA levels of cyclin D and cyclin E (Figures 12B-C). Interestingly, the expression of p21 gene was dramatically up-regulated in response to cepharanthine treatment. This compound at 10 and 20 μM increased p21 mRNA level by approximately 5 and 15 fold of the vehicle control, respectively, (Figure 12D, $P<0.048$). Similar to cepharanthine, treatment of CaOV-3 cells with cisplatin led to decreased cyclin A mRNA level ($P<0.005$) (Figure 12A). Expression of cyclin D, E and p21 genes however, was unaffected by cisplatin (Figures 12B-D). These results suggest that changes in mRNA levels of cyclin A and p21 might be responsible for cepharanthine-induced cell cycle arrest in CaOV-3 cells.

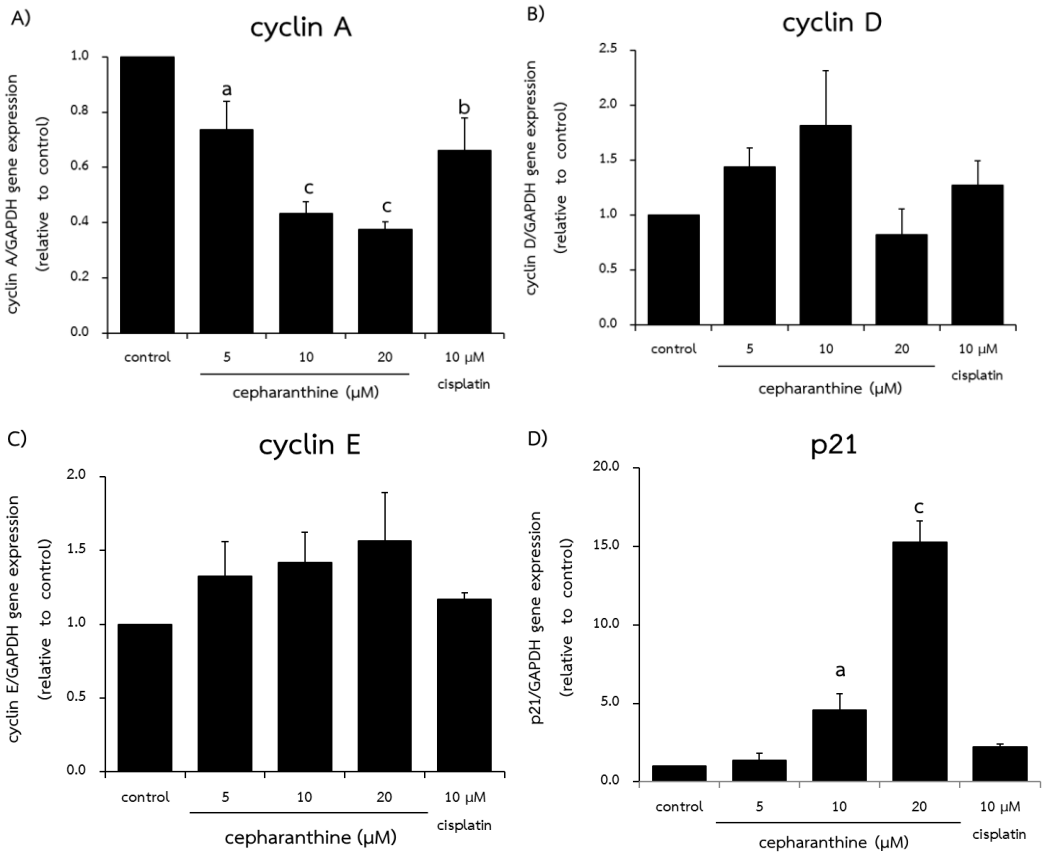


Figure 12 The effect of cepharanthine on the mRNA levels of cell cycle regulators: (A) cyclin A, (B) cyclin D, (C) cyclin E, and (D) p21 in CaOV-3 cells. Cells were treated with 5, 10 and 20 μM of cepharanthine, 10 μM of cisplatin (positive control) or 0.2% DMSO (vehicle control) for 24 h and the mRNA levels of the interested genes were monitored using real-time RT PCR analysis. The values were normalized to GAPDH and revealed as fold changes relative to the vehicle control. Data are presented as means ± SEM of three independent experiments. $^{a}P<0.05$, $^{b}P<0.01$ and $^{c}P<0.001$ compared with the vehicle control.

4.6. Effect of cepharanthine on the mRNA levels of cell cycle regulators in OVCAR-3 cells.

The mRNA levels of cell cycle regulators, including cyclins A, E, D and p21 in OVCAR-3 cells was evaluated after treatment with 10, 20 and 40 μM of cepharanthine, 30 μM of cisplatin or 0.2% DMSO. As illustrated in Figures 13A and C, cepharanthine decreased mRNA levels of cyclins A and E in a concentration-dependent manner. However, a significant reduction in mRNA levels of both cyclins was detected following treatment of the compound at 40 μM only ($P<0.024$). The mRNA levels of cyclins A and E were approximately half of those treated with the vehicle control. It should be noted that at this concentration, cepharanthine markedly up-regulated expression cyclin D gene (Figure 13B, $P<0.001$). The mRNA level of cyclin D was increased more than 6 times that of the vehicle control. In addition to cyclins, treatment with cepharanthine also changed the expression of p21, a CDK inhibitor, dramatically in OVCAR-3 cells (Figure 13D, $P<0.004$). The expression of p21 gene was increased by more than 10 fold above the vehicle control. Similar to cepharanthine, the p21 mRNA level was significantly up-regulated after treatment with cisplatin (Figure 13D, $P<0.017$). However, of all cyclins tested, only expression of cyclin D was significantly affected by cisplatin (Figure 13B, $P<0.008$). The mRNA level of cyclin D was almost doubled that of the vehicle control. Taken together, these results demonstrated that cepharanthine at high concentrations could down-regulate the gene expression of cyclin A and cyclin E and also up-regulate the gene expression of p21 and cyclin D in OVCAR-3 cells.

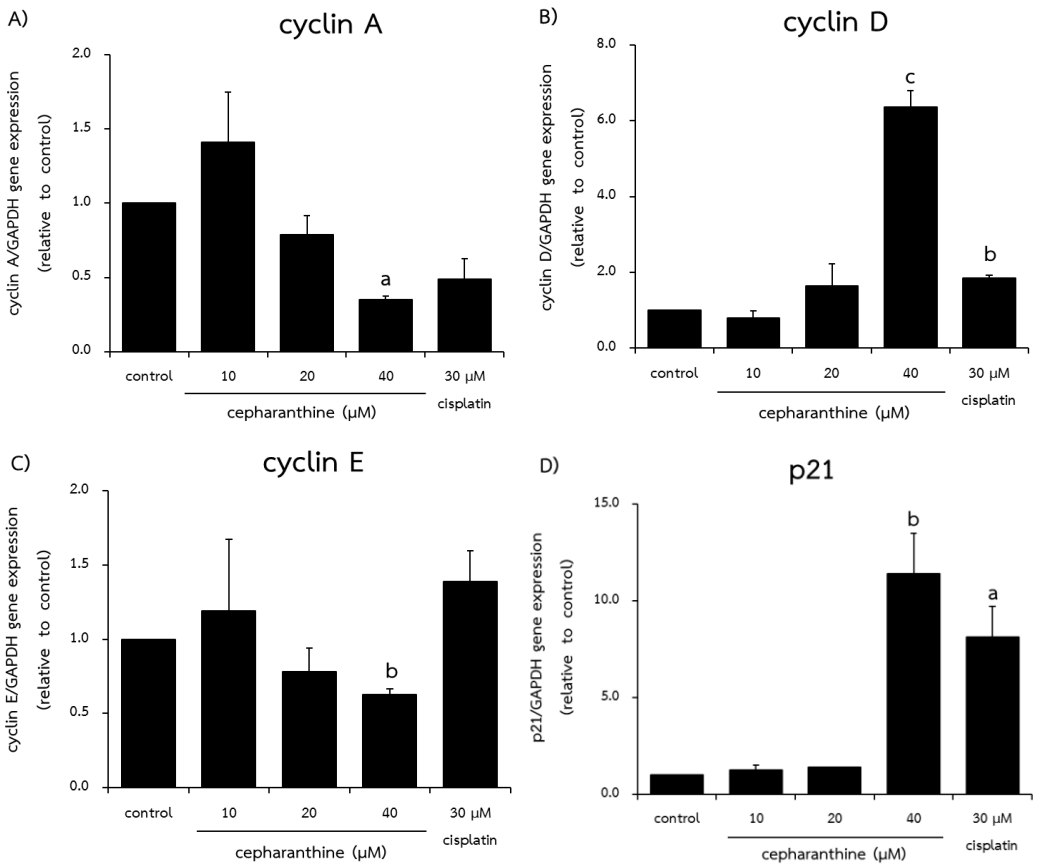


Figure 13 The effect of cepharanthine on the mRNA levels of cell cycle regulators: (A) cyclin A, (B) cyclin D, (C) cyclin E, and (D) p21 in a OVCAR-3 cells after treatment with 10, 20 and 40 μM of cepharanthine, 30 μM of cisplatin (positive control) or 0.2% DMSO (vehicle control) for 24 h. The values were normalized to GAPDH and revealed as fold changes relative to the vehicle control cells. Data are presented as means ± SEM of three independent experiments. $^{a}P<0.05$, $^{b}P<0.01$ and $^{c}P<0.001$ compared with vehicle control.

4.7. Effect of cepharanthine on apoptosis induction in CaOV-3 cells.

Apoptosis is one of the most important mechanism underlying anticancer activity of most chemotherapeutic drugs [88]. Several studies have demonstrated that cepharanthine could induce apoptotic cell death in various human cancer cells [22-25]. Thus, to determine whether anticancer activity of cepharanthine in CaOV-3 cells is mediated through apoptosis induction, the cells were exposed to 5, 10 and 20 μM of cepharanthine, 10 μM of cisplatin or 0.2% DMSO for 24 h and apoptotic and necrotic cells were monitored using annexin V/PI staining and flow cytometry analysis. As illustrated in Figure 14, 5 μM of cepharanthine significantly induced CaOV-3 cells to undergo late-apoptosis which was 2 fold higher than the control ($P<0.024$). It however should be noted that significant necrotic cell death was observed following treatment of CaOV-3 cells with cepharanthine at higher concentrations, 10 and 20 μM, ($P<0.001$), compared to the vehicle control. The percentages of cells undergoing necrosis were increased by 10 and 17 times that of the vehicle control after treatment with cepharanthine at 10 and 20 μM, respectively. On the other hand, neither significant apoptotic nor necrotic cell death was detected in CaOV-3 cells following treatment with 10 μM of cisplatin (Figure 14).

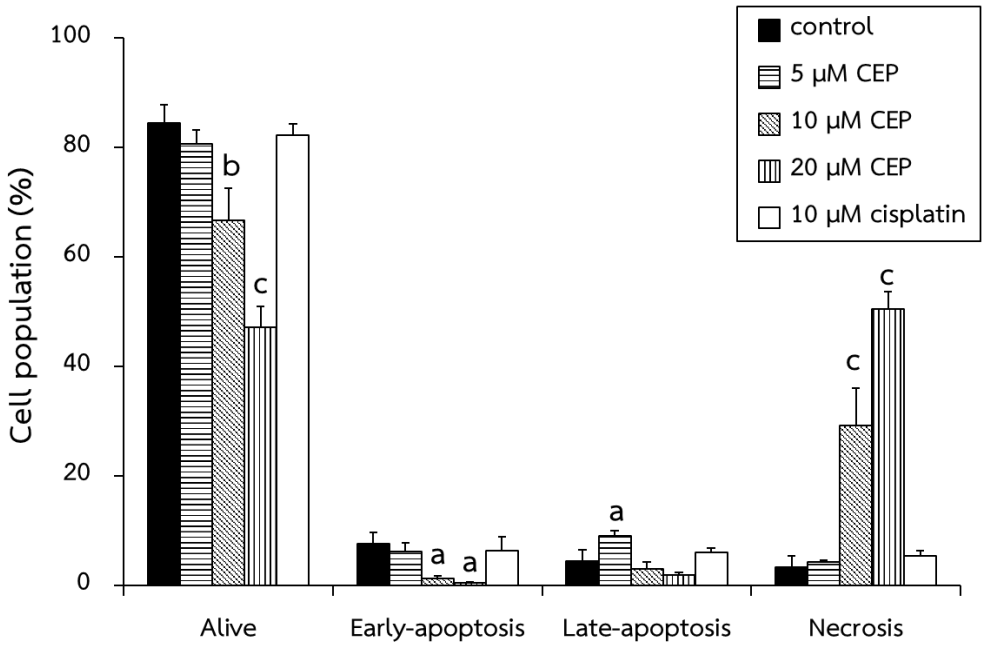


Figure 14 The effect of cepharanthine on apoptotic cell death in CaOV-3 cells. The cells were treated with 5, 10 and 20 μM of cepharanthine, 10 μM of cisplatin (positive control) or 0.2% DMSO (vehicle control) for 24 h. The percentages of apoptotic or necrotic cells were evaluated by flow cytometry after annexin V-FITC and PI staining. The results are expressed as mean ± SEM of three independent experiments. Statistically significant differences compared to the vehicle control ($^{a}P<0.05$, $^{b}P<0.01$ and $^{c}P<0.001$).

4.8. Effect of cepharanthine on apoptosis induction in OVCAR-3 cells.

To investigate whether apoptosis induction is responsible for anticancer effect of cepharanthine in OVCAR-3 cells, the cells were treated with 10, 20 and 40 μM of cepharanthine, 30 μM of cisplatin or 0.2% DMSO for 48 h and apoptotic as well as necrotic cell death were analyzed using annexin V/PI staining and flow cytometry analysis. As illustrated in Figure 15, treatment with cepharantine at 40 μM led to a marked decrease in numbers of live cells which was accompanied by significant increases in early and late apoptotic populations. Early and late apoptotic cells were significantly enhanced by 3 and 4 fold above those of the vehicle control, respectively. Flow cytometry analysis also revealed that 30 μM cisplatin significantly induced OVCAR-3 cells to undergo late apoptosis and necrosis which was in agreement with a significant decrease in living cells approximately 0.7 times that of the vehicle control (Figure 15). These results demonstrated that cepharanthine could effectively induce apoptosis in a chemoresistant ovarian cancer cell line, OVCAR-3.

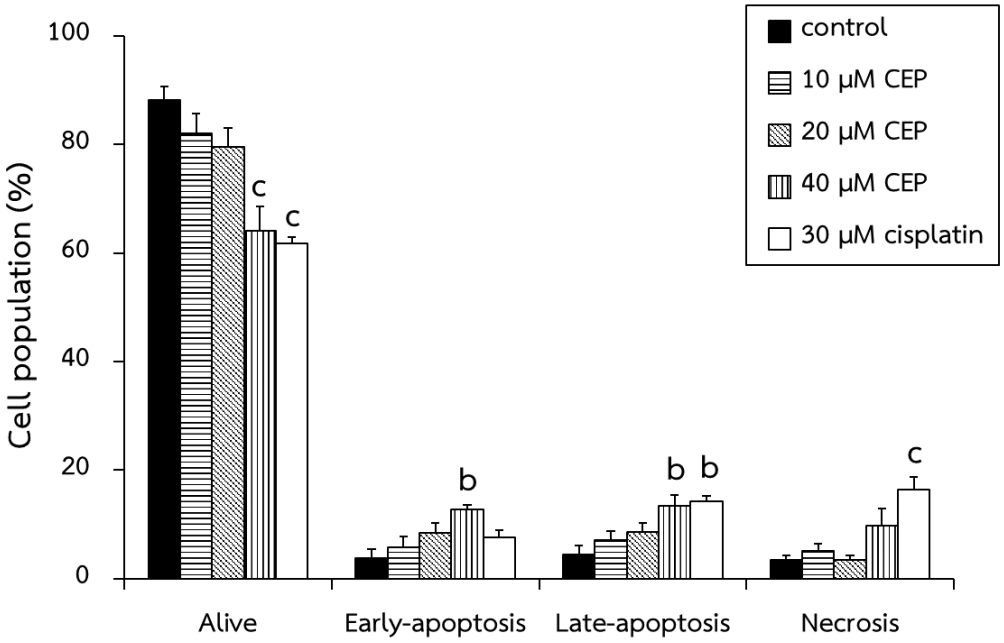


Figure 15 The effect of cepharanthine on apoptotic cell death in OVCAR-3 cells. Cells were treated with 10, 20 and 40 μM of cepharanthine, 30 μM of cisplatin (positive control) or 0.2% DMSO (vehicle control) for 48 h. The percentages of live, apoptotic and necrotic populations were evaluated by flow cytometry after staining with annexin V-FITC and PI. The results are expressed as mean ± SEM of three independent experiments. $^{b}P<0.01$ and $^{c}P<0.001$ compared to the vehicle control.

4.9. Effect of cepharanthine on the mRNA levels of Bcl-2 family members in CaOV-3 cells.

The intrinsic (mitochondrial) pathway of apoptosis is mainly responsible for apoptosis-inducing effect of several chemotherapeutic agents. This pathway is regulated by members of the Bcl-2 family proteins, including pro-apoptotic proteins such as Bax and Bak and anti-apoptotic proteins such as Bcl-2 and Bcl-xl [89, 90]. Previous studied reported that cepharanthine could increase the expression of Bax gene in non-small-cell-lung cancer [10] and myeloma [23] and decreased the mRNA level of Bcl-xl in osteosarcoma cell [25]. Therefore, the mRNA levels of Bcl-2 family including pro-apoptotic proteins, Bak and Bax and anti-apoptotic proteins, Bcl-2 and Bcl-xl were evaluated. Cells were incubated with 5, 10 and 20 μM of cepharanthine, 10 μM of cisplatin, or 0.2% DMSO for 24 h, and the mRNA levels of apoptosis-regulators were determined using real-time RT-PCR. As shown in Figures 16A-D, treatment of CaOV-3 cells with 20 μM of cepharanthine significantly up-regulated the gene expression of both tested pro-apoptotic regulators, Bak (P<0.005) and Bax (P<0.002) (Figures 16A and B) and down-regulated the gene expression of anti-apoptotic regulator, Bcl-xl (Figure 16C, P<0.004). Similarly, cisplatin significantly increased mRNA levels of Bak (P<0.005) and Bax (P<0.044). Moreover, expression of Bcl-2 and Bcl-xl genes were decreased following treatment of the cells with 10 μM of cisplatin (P<0.001). These results indicated that changes in mRNA levels of Bcl-2 family members, including pro-apoptotic (Bak and Bax) and pro-apoptotic (Bcl-xl), are involved in the cepharanthine-mediated apoptosis in CaOV-3 cells.

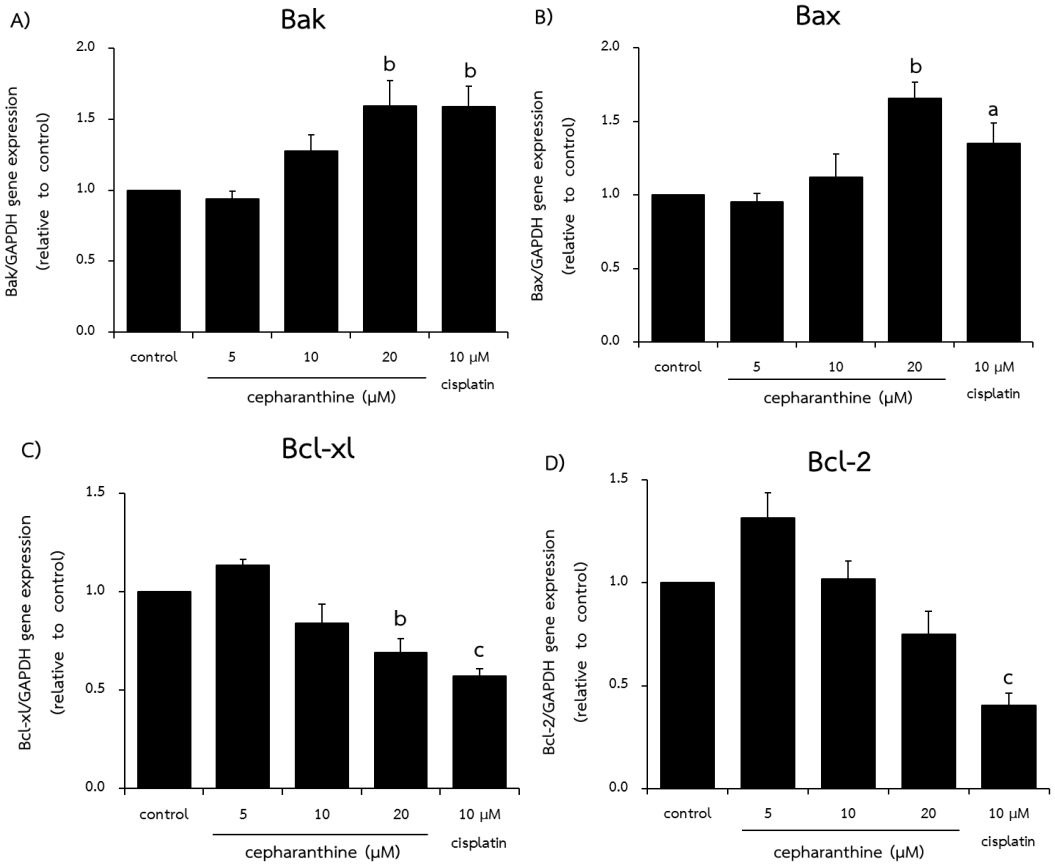


Figure 16 The effect of cepharanthine on the mRNA levels of (A) Bak, (B) Bax, (C) Bcl-xl, and (D) Bcl-2 in a chemosensitive ovarian cancer cell line, CaOV-3. Cells were treated with 5, 10 and 20 μM of cepharanthine or 10 μM of cisplatin (positive control) or 0.2% DMSO (vehicle control) for 24 h and mRNA levels of the interested Bcl-2 family members were measured using real-time RT PCR analysis. The values were normalized to GAPDH and revealed as fold changes relative to the vehicle control. Data are presented as mean ± SEM of three independent experiments. $^{a}P<0.05$, $^{b}P<0.01$ and $^{c}P<0.001$ compared with vehicle control.

4.10. Effect of cepharanthine on the mRNA levels of Bcl-2 family members in OVCAR-3 cells.

The mRNA levels of Bcl-2 family members, including pro-apoptotic (Bak and Bax) and anti-apoptotic (Bcl-2 and Bcl-xl) were analyzed using real-time RT PCR after treatment with cepharanthine (10, 20 and 40 μM), 30 μM of cisplatin or 0.2% DMSO for 24 h. The results revealed that cepharanthine at a concentration of 40 μM significantly up-regulated Bak gene expression by approximately 1.8 fold of the vehicle control (Figure 17A, $P<0.011$). The compound however did not alter the mRNA levels of Bax, Bcl-xl and Bcl-2 (Figures 17B-D). Conversely, treatment of OVCAR-3 cells with cisplatin significantly increased mRNA levels of Bak and Bax but decreased mRNA levels of Bcl-xl and Bcl-2 (Figures 17A-D). Taken together, the results of this study suggest that up-regulation of Bak may be responsible for cepharanthine-mediated apoptosis induction in OVCAR-3 cells.

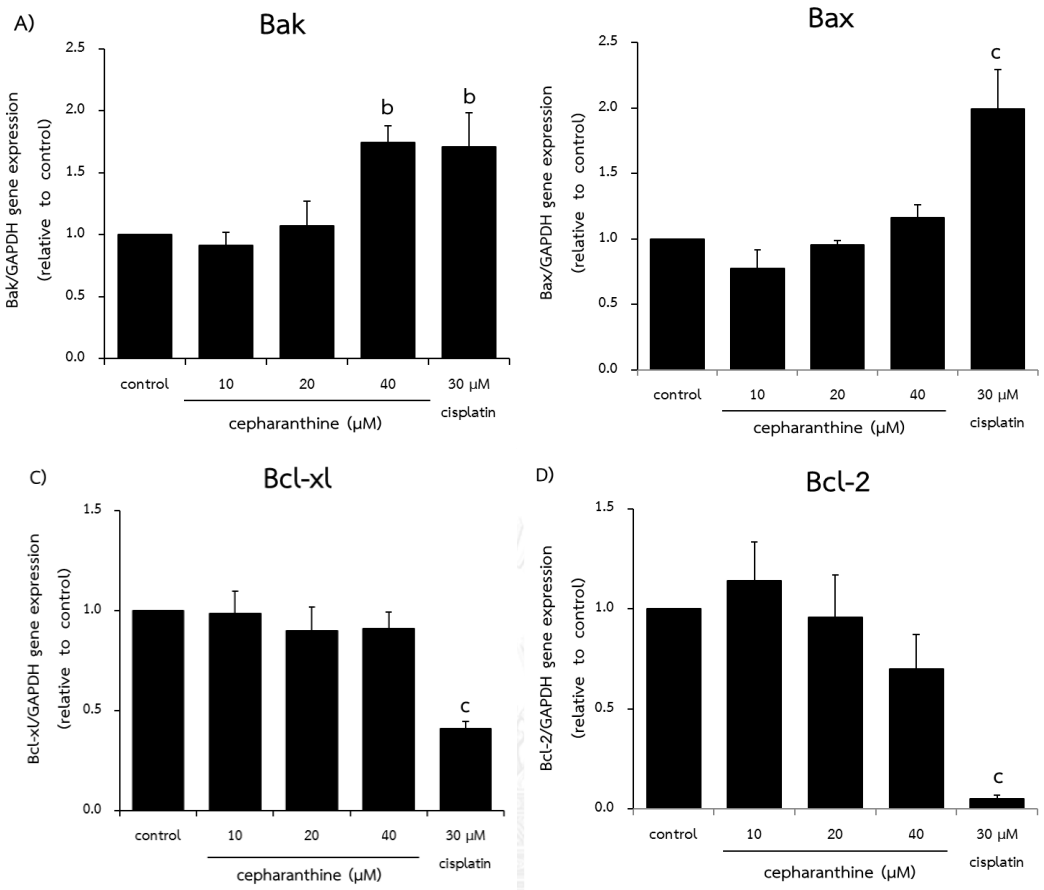


Figure 17 The effect of cepharanthine on the mRNA levels of (A) Bak, (B) Bax, (C) Bcl-xl, and (D) Bcl-2 in OVCAR-3 cells after treatment with 10, 20 and 40 μM of cepharanthine, 30 μM of cisplatin (positive control) or 0.2% DMSO (vehicle control) for 24 h. The mRNA levels were assessed using real-time RT PCR analysis. The values were normalized to GAPDH and revealed as fold changes relative to the vehicle control. Data are presented as means ± SEM of three independent experiments. $^{b}P<0.01$ and $^{c}P<0.001$ compared to the vehicle control.

CHAPTER V
DISCUSSION AND CONCLUSION

Ovarian cancer is one of the most lethal malignancies in women worldwide. The combination treatment of a platinum-based drug and a taxane remains the first line chemotherapy for ovarian cancer. Although ovarian cancer initially responds to chemotherapy, later patients often develop chemoresistance. It was reported that more than half of ovarian cancer patients receiving platinum/taxane combination therapy will relapse within 18 months of diagnosis [91]. Additionally, the dose-dependent toxic side effects of most chemotherapeutic agents have also limited their uses in cancer treatment [6-9]. Therefore, it is necessary to find novel compounds that have efficient anticancer activity with minimal side effects for ovarian cancer treatment.

Currently, natural compounds have been extensively investigated for use either alone or in combination with conventional chemotherapeutic agents to overcome drug resistance and minimize severe side effects [92]. Cepharanthine, a naturally occurring alkaloid, has displayed anticancer activity against various different types of cancer, including cholangiocarcinoma, leukemia, hepatocellular carcinoma, cervical adenocarcinoma, myeloma, osteosarcoma, adenosquamous cell carcinoma and oral squamous cell carcinoma both *in vitro* and *in vivo* [18-23, 25, 27, 28]. To the best of my knowledge, the anticancer activity of this compound has never been tested in ovarian cancer. Thus, the anticancer effects of cepharanthine have been evaluated in two human ovarian cancer cell lines, CaOV-3 and OVCAR-3 in this study. The OVCAR-3 cells which were derived from a patient refractory to cisplatin and doxorubicin have been classified as

chemoresistant cells while the CAOV-3 cells have been classified as chemosensitive cells. As expected, all three commonly used chemotherapeutic drugs, cisplatin, doxorubicin and oxaliplatin, inhibited the growth of CaOV-3 cells more effective than OVCAR-3 cells. Similarly, cytotoxicity of cepharanthine was found to be higher in CaOV-3 cells than OVCAR-3 cells (Figures 8 and 9). The IC_{50} value of cepharathine in CaOV-3 cells at 48 h incubation (10.93±0.14 μM) was approximately 3 times lower than the IC_{50} value of cepharanthine in OVCAR-3 cells (31.20±1.27 μM). Several studies have revealed that mutations in tumor suppressor genes or proto-oncogenes are highly associated with drug resistance in cancer cells [85, 93, 94]. Katsui et al. demonstrated that the resistance to adriamycin (doxorubicin) in osteosarcoma cells was associated with p53 mutation [29]. Similarly, Perego et al. have reported mutation of p53 gene causes the development of cisplatin-resistance in ovarian cancer cells such as OVCAR-3 [41]. Interestingly, recent studies have shown that cepharanthine was more effective in controlling the growth of a p53-mutated colorectal cancer cell line, HT-29, than a p53 wild-type colorectal cancer cell line, COLO-205 [80]. The results from the present study however illustrated that cepharanthine was more toxic to CaOV-3 cells than to OVCAR-3 cells. Previously, several studies reported that both CaOV-3 and OVCAR-3 ovarian cancer cell lines contain p53 mutation [37, 95] but oncogenic mutations in the PI3K/AKT pathway are only detected in OVCAR-3 cells [46, 96]. Previous study demonstrated that the activation of the PI3K signaling pathway through mutation of PIK3CA or AKT has been found to be involved the resistance to cisplatin in ovarian cancer [35, 47, 48]. In addition to oncogenic pathway, amplification of cell cycle regulators, including cyclin E and cyclin D1, which are associated with chemoresistance, were also found in OVCAR-3 cells [37, 97-100]. Therefore, it is

likely oncogenic mutations, besides p53, are involved in the differences in sensitivity to cepharanthine of CaOV-3 cells and OVCAR-3 cells. It, however, should be noted that cepharanthine exerted more potent anticancer activity than cisplatin in both CAOV-3 and OVCAR-3 cells (Tables 2 and 3), suggesting that cepharanthine has potential to be a novel chemotherapeutic drug for cancer therapy.

Several antineoplastic drugs exhibit their anticancer activities via induction of cell cycle arrest and apoptosis [88]. It was reported that cepharanthine could induce cell cycle arrest at G1, S and G2/M phase in myeloma, osteosarcoma, adenosquamous cell carcinoma and oral squamous carcinoma [23, 25, 27, 28]. In this study, treatment of CaOV-3 cells with cepharanthine at 5 μM induced cell cycle arrest at G1 phase while at higher concentrations (10 and 20 μM) of cepharanthine caused significant accumulation of CaOV-3 cells at sub G1 phase (Figure 10). The results are consistent with other recent finding that high concentration of cepharanthine induced sub-G1 peak in adenosquamous carcinoma TYS cells [27]. In contrast to CaOV-3 cells, cepharanthine did not induce cell cycle arrest at any phase of the cell cycle in a chemoresistant cancer cell line, OVCAR-3. Cepharanthine at the highest concentration tested (40 μM) induced OVCAR-3 cells to dramatically accumulate at sub-G1 phase (Figure 11). This was in agreement with previous study showing that tetradrine, a bisbenzylisoquinoline alkaloid extracted from the root of the *Stephania tetrandrae*, did not alter cell cycle distribution of OVCAR-3 cells [101]. Beaufort et al. previously reported that high-level of cyclin E amplification was detected in OVCAR-3 cells [37] and these cells also overexpressed cyclin D [99, 102]. Since both cyclin E and D play important roles in cell cycle progression, it is possible that amplification and/or overexpression of these cell cycle

regulators are responsible for resistance to cepharanthine-mediated cell cycle arrest in OVCAR-3 cells.

The cell cycle is controlled by the activation of cyclin-CDKs specific complexes and cyclin-dependent kinase inhibitors (CKIs) [67, 69]. The present study clearly illustrated that cepharanthine decreased the mRNA level of cyclin A and increased the mRNA level of p21 in CaOV-3 cells in a concentration-dependent manner (Figure 12), suggesting that changes in cyclin A and p21 are associated with cepharanthine-induced cell cycle arrest. It was previously shown that cepharanthine induced TYS cells carrying p53 mutation, to undergo cell cycle arrest by up-regulating of $p21^{WAF1}$ protein [27]. Thus, cepharanthine may be able to induce cell cycle arrest in CaOV-3 cells in a p53-independent manner. Although the results from this study demonstrated that cepharanthine did not induce cell cycle arrest in OVCAR-3 cells, real-time PCR analysis indicated that this compound significantly down-regulated mRNA levels of cyclin A and cyclin E and markedly up-regulated mRNA levels of cyclin D and p21 in OVCAR-3 cells (Figure 13). It is possible that increase in cyclin D mRNA counteracts with decrease in cyclin A and cyclin E mRNA, making no significant change in cell cycle progression of OVCAR-3 cells. Additionally, previous study reported that activation of oncogenic MEK/MAPK pathway led to p27 deregulation and antiestrogen resistance in human breast cancer cells [103]. Therefore, mutations in tumor suppressor genes or proto-oncogenes may be related to resistance to cell cycle arrest in OVCAR-3 cells. The results of this study indicated that cepharanthine decreased mRNA levels of cyclin A and cyclin E but increased cyclin D mRNA. The findings are similar to other previous evidence illustrating that suberoylanilide hydroxamic acid markedly down-regulated the expression of cyclin A but up-regulated the expression of cyclin B in breast

cancer cells [104]. Though, the mechanism underlying opposing effect of cepharanthine on mRNA expression of cyclins is still unknown, it is likely that cyclin D up-regulation contributes to resistance of OVCAR-3 cells to cepharantine-mediated cell cycle arrest. Indeed, cyclin D play an important role in controlling cell cycle arrest. It was reported that cyclin D1 degradation is sufficient to induce G1 cell cycle arrest in ovarian cancer cells [105]. Down-regulation of cyclin D was also associated with cell cycle arrest in breast cancer cells [106], osteosarcoma [25], prostate cancer [107] and colon cancer [108]. Previously, numerous studies reported that cepharanthine down-regulated the expression of cyclin D1, cyclin E and c-Myc as well as up-regulated the expression of CKIs, $p21^{WAF1}$, $p27^{Kip1}$ and $p15^{INK4B}$, in several human cancer cells such as myeloma, adenosquamous cell carcinoma, oral squamous cell carcinoma and osteosarcoma cells [23, 25, 27, 28]. However, one of the most surprising finding of this study is that cepharanthine was able to decrease cyclin A in both human ovarian cancer cells (CaOV-3 and OVCAR-3), suggesting that cyclin A may be a therapeutic target of cepharanthine in human ovarian cancer cells. The mechanism responsible for cepharanthine-mediated cyclin A down-regulation still needs further investigation.

Apoptosis is related to anticancer activity of many anticancer drugs [70, 71]. Similarly, cepharanthine has been reported to inhibit the growth of cancer cells by promoting apoptosis in various human cancer cells such as human adenosquamous cell carcinoma TYS cells [27], human oral squamous cell carcinoma B88 cells [28], human leukemia Jurkat and K562 cells [18, 79], human hepatocellular carcinoma HuU-7 cells [21], human cervical adenocarcinoma HeLa cells [22] and human non-small-cell lung cancer A549 and H1299 cells [10]. To the best of my knowledge, this is the first report of cepharanthine-induced apoptosis in ovarian cancer cells. In CaOV-3 cells, treatment with

cepharanthine at the lowest concentration (5 μM) resulted in late apoptosis whereas necrotic cell death was detected at the higher concentration of cepharanthine (10 and 20 μM) (Figure 14). In contrast, cepharanthine only at the highest concentration (40 μM) triggered OVCAR-3 cells to undergo apoptotic cell death at both early- and late-apoptosis (Figure 15). The results from annexin V/PI staining were somewhat different from results in cell cycle analysis using PI staining. This may be due to different methodology used. It was reported that a sub-G1 DNA content, identified by propidium iodide staining, does not distinguish between apoptotic and necrotic cells as well as using annexin V/PI staining [109, 110]. Cells undergo apoptosis through two major pathways, *the extrinsic pathway* and *the intrinsic pathway*. Most chemotherapeutic agents induce cancer cells to undergo intrinsic apoptosis pathway. The intrinsic pathway or mitochondrial pathway is tightly regulated by member of the Bcl-2 family proteins [74, 90]. Several studies reported that cepharanthine induces apoptosis by induction of Bax and reduction of Bcl-2 and Bcl-xl in many cancer cells [10, 22, 23, 25]. This study revealed that high concentration of cepharanthine (20 μM) down-regulated the mRNA level of anti-apoptotic member Bcl-xl and up-regulated the mRNA levels of both pro-apoptotic members, Bax and Bak, in CaOV-3 cells (Figure 16). Similarly, cepharanthine could induce mRNA level of Bak in OVCAR-3 cells (Figure 17). Taken together, the results from these studies suggest that changes in the expression of Bcl-2 family members are responsible for cepharanthine-induced apoptosis in ovarian cancer cells. Interestingly, cepharanthine at high concentrations increased Bax and Bak, reduced of Bcl-xl, and induced necrotic cell death in CaOV-3 cells. Recent studies demonstrated that programmed necrosis (or necroptosis) is another type of regulated cell death. This cell death modality maintains some apoptotic features but also

possesses necrotic morphology and Bcl-2 family members have been shown to play important roles in necroptosis [111, 112]. Taken together, it is likely that changes in pro- and anti-apoptotic proteins are associated with necrotic cell death following treatment of CaOV-3 cells with cepharanthine at high concentrations.

Biswas et al. reported that cepharanthine induced human hepatocellular carcinoma HuH-7 cells death by activation of JNK, which may be up-stream of mitochondrial-associated event, involved in down-regulation of Bcl-2 expression and up-regulation of Bax expression, leading to cytochrome C release [21]. Moreover, recent study demonstrated that cepharanthine decreased the expression of anti-apoptotic Bcl-xl gene, resulting in apoptosis through down-regulation of STAT3 protein in human osteosarcoma SaOS2 cells [25]. It was also shown that resveratrol induced apoptosis in CaOV-3 and OVCAR-3 cells by down-regulating expression of Bcl-xl, Bcl-2 or c-myc through STAT3 signaling pathway [113]. Therefore, it is possible that the growth inhibitory and apoptosis inducing effects of cepharanthine in human ovarian cancer cells may be mediated through inhibition of oncogenic signaling pathways. However, the effects of cepharanthine on STAT3 and JNK signaling pathways require further investigation.

Additionally, several studies have reported a significant benefit effect of this alkaloid in reversing multi-drug resistance (MDR) by down-regulating the expression of drug efflux transporters such as P-gp and multidrug resistance protein-7 (MRP-7) in human leukemia cell lines and human embryonic kidney cell lines [77, 78]. MDR is one of the main causes of treatment failure in ovarian cancer. Recent study demonstrated that cepharanthine reversed docetaxel resistance in a taxane-resistant ovarian

cancer cell line (HeyA8-MDR) both *in vitro* and *in vivo*, illustrating the benefit effect of cepharanthine when used in combination with other drugs. Therefore, in the consideration of its high anticancer activity and safety profile, cepharanthine is a promising compound to be used either as a single agent or in combination with other chemotherapeutic drugs for ovarian cancer treatment. Further investigation of anticancer activities of cepharanthine in human ovarian cancer cells both *in vitro* and *in vivo* are clearly warranted.

Conclusion

This is the first study to investigate the anticancer effects of cepharanthine in human ovarian cancer cells. The results clearly demonstrated that cepharanthine possesses potent anticancer activity against two human ovarian cancer cell lines. Mechanistic studies revealed that cepharanthine potentially induced cell cycle arrest and apoptosis which may be mediated via modulation of mRNA levels of cell cycle regulators, cyclin A and p21 and Bcl-2 family members, Bcl-xl, Bax and Bak, in chemosensitive CaOV-3 cells. In chemoresistant OVCAR-3 cells, induction of apoptosis which may be associated through up-regulation of Bak gene is mainly responsible for anticancer activity of cepharanthine. These finding suggest that cepharanthine could potentially be used as a novel anticancer drug for ovarian cancer which are frequency resistant to commonly used chemotherapeutic drug.

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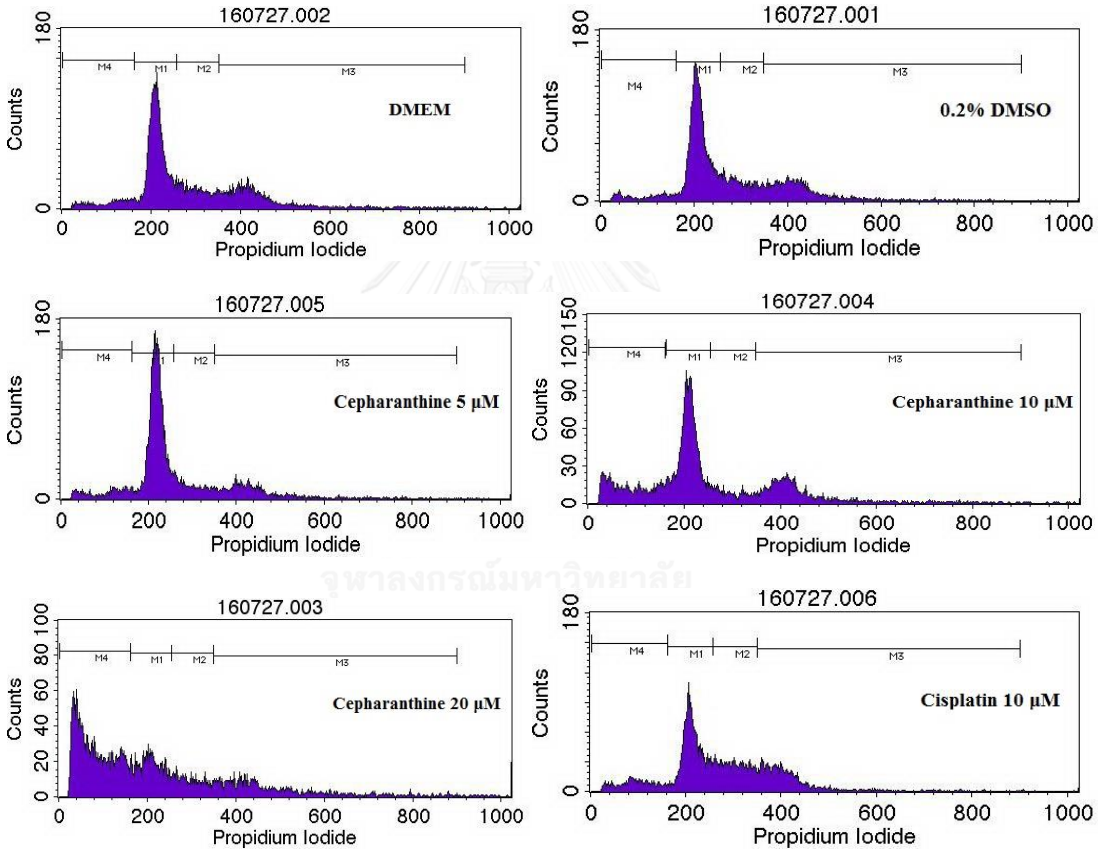
APPENDIX

APPENDIX A

RESULTS

Appendix A-1: The cytograms of cell cycle distribution in chemosensitive ovarian cancer cells (CaOV-3) after treatment with 5, 10 and 20 μM of cepharanthine, 10 μM of cisplatin (positive control), 0.2% DMSO (vehicle contol) or DMEM (untreated control) for 24 h.

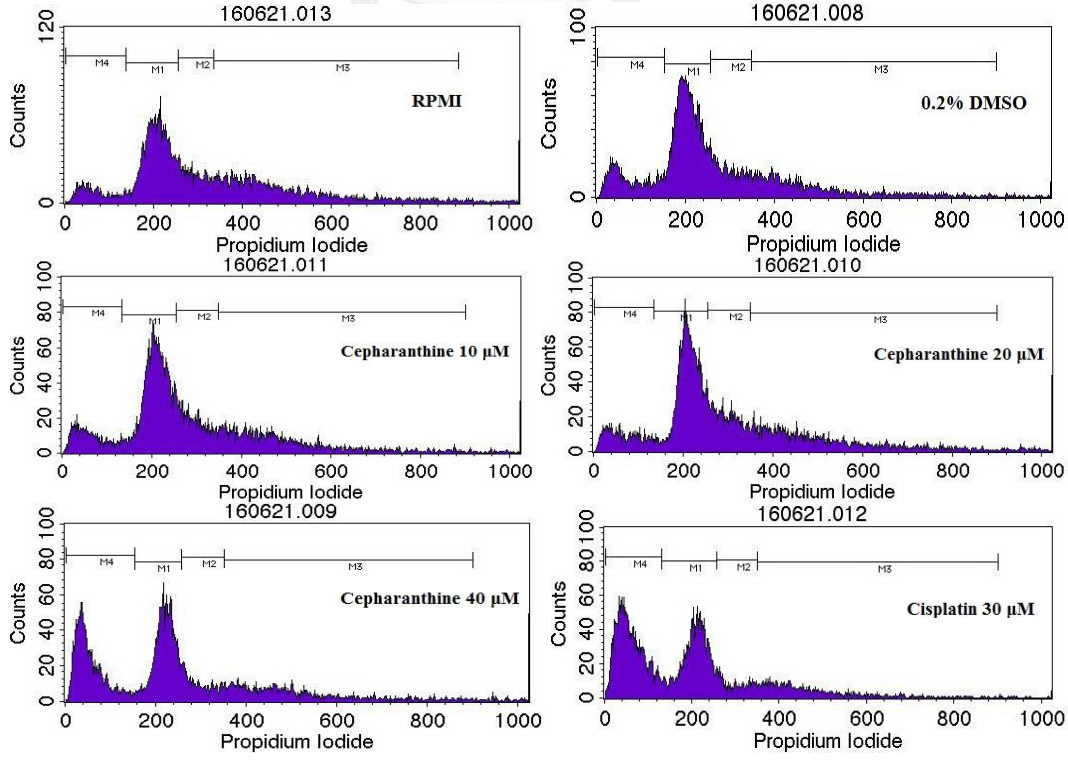


Appendix A-2: Distribution of CaOV-3 cells in each phase of cell cycle after treatment with various concentrations of cepharanthine (CEP) or 10 μM of cisplatin for 24 h.

Treatments	Cell population (%)			
	Sub-G1	G1 phase	S phase	G2/M phase
Untreated	8.14±1.72	52.47±0.12	17.60±0.24	22.21±1.24
0.2% DMSO	6.04±0.70	52.65±3.44	17.03±0.70	24.53±3.40
CEP 5 μM	8.15±0.72	64.10±0.66	11.34±0.51	16.53±0.74
CEP 10 μM	24.07±1.66	47.03±3.31	9.30±1.31	19.80±1.58
CEP 20 μM	54.61±2.29	21.78±0.21	10.51±0.57	13.40±1.90
Cisplatin 10 μM	11.30±1.31	40.53±0.48	27.25±0.96	21.56±1.21

Data represent means ± SEM from three independent experiments.

Appendix A-3: The cytograms of cell cycle distribution in chemoresistant ovarian cancer cells (OVCAR-3) after treatment with 10, 20 and 40 μM of cepharanthine, 30 μM of cisplatin (positive control), 0.2% DMSO (vehicle contol), or RPMI (untreated control) for 48 h.

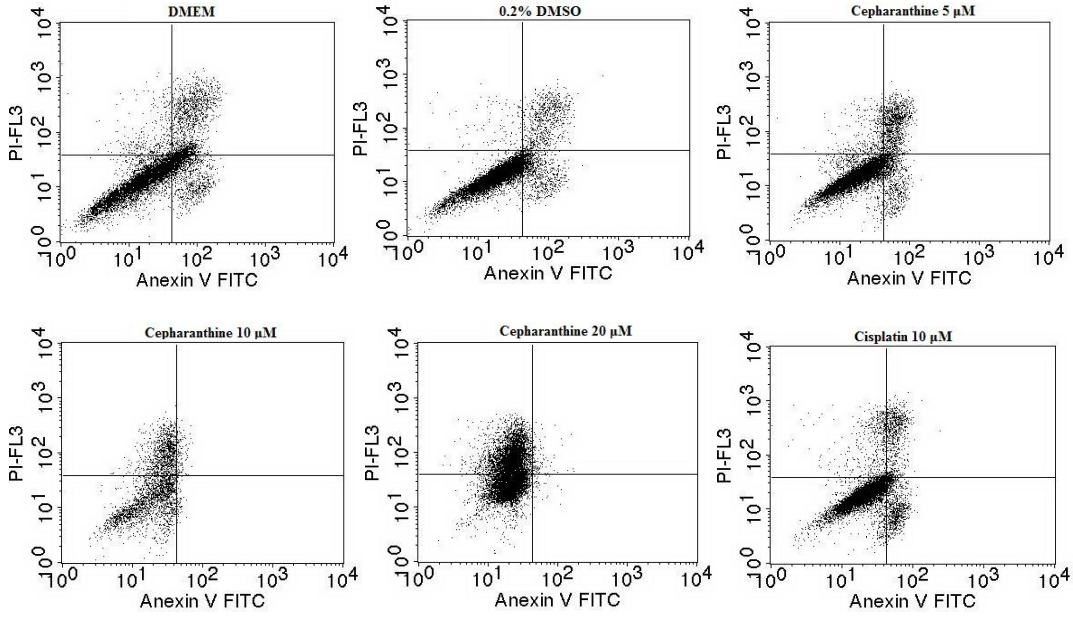


Appendix A-4: Distribution OVCAR-3 cells in each phase of cell cycle after treatment with various concentrations of cepharanthine (CEP) or 30 μM of cisplatin for 48 h.

Treatments	Cell population (%)			
	Sub-G1	G1 phase	S phase	G2/M phase
Untreated	7.32±1.57	47.88±4.24	17.29±1.17	27.71±3.99
0.2% DMSO	8.12±0.65	48.95±4.73	16.03±1.07	26.17±3.50
CEP 10 μM	11.33±2.81	48.54±3.91	16.61±0.66	23.24±2.18
CEP 20 μM	17.36±5.21	46.34±3.31	15.13±2.18	20.01±1.43
CEP 40 μM	27.11±6.28	41.04±5.27	11.76±1.31	18.02±2.96
Cisplatin 30 μM	29.95±7.14	40.96±2.85	11.03±1.30	18.32±5.80

Data represent means ± SEM from three independent experiments.

Appendix A-5: The cytograms of apoptotic and necrotic cell death in CaOV-3 cells after treatment with cepharanthine (5, 10 and 20 μM), 10 μM of cisplatin (positive control), 0.2% DMSO (vehicle control) or DMEM (untreated control) for 24 h.

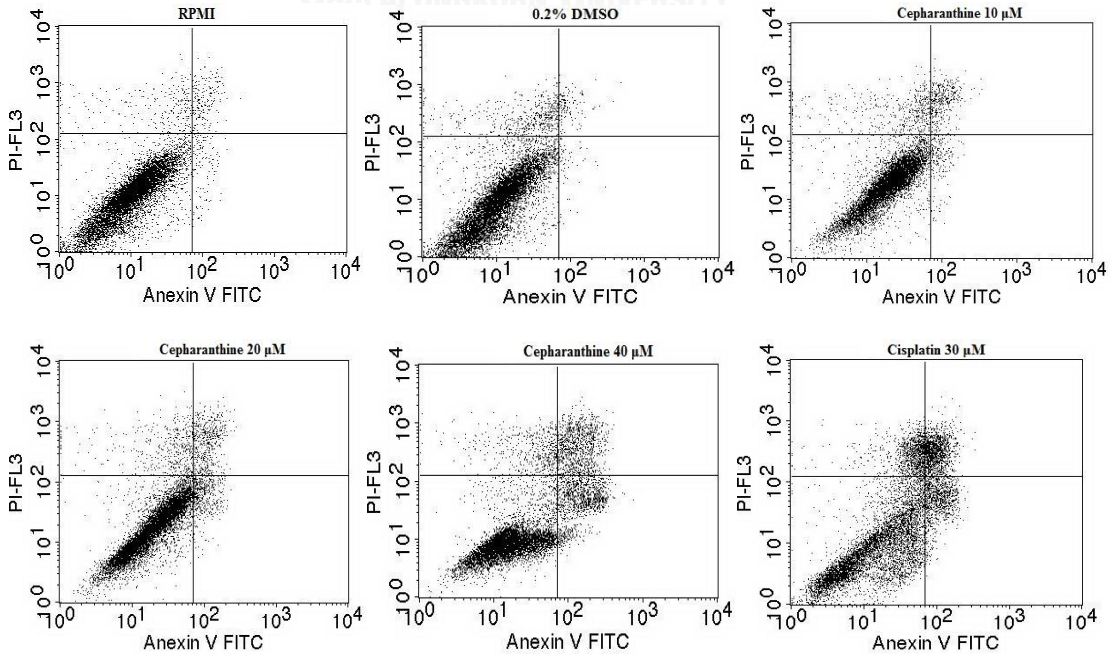


Appendix A- 6: The percentages of apoptotic and necrotic cells after treatment of CaOV-3 cells with various concentrations of cepharanthine (CEP) or 10 μM of cisplatin for 24 h.

Treatments	Cell population (%)			
	Alive	Early-apoptosis	Late-apoptosis	Necrosis
Untreated	75.75±5.01	10.28±4.75	10.79±3.92	3.19±0.51
0.2% DMSO	84.52±3.29	7.65±2.03	4.49±1.99	3.34±2.05
CEP 5 μM	80.68±2.62	6.12±1.65	8.98±0.94	4.23±0.41
CEP 10 μM	66.70±5.93	1.17±0.47	3.06±1.16	29.27±6.74
CEP 20 μM	47.18±3.78	0.42±0.15	1.85±0.56	50.54±3.09
Cisplatin 10 μM	82.28±2.10	6.29±2.50	5.99±0.75	5.44±0.93

Data represent means ± SEM from three independent experiments.

Appendix A-7: The cytograms of apoptotic and necrotic cell death of in OVCAR-3 after treatment with cepharanthine (10, 20 and 40 μM), 30 μM of cisplatin (positive control), 0.2% DMSO (vehicle contol), or RPMI (untreated control) for 48 h.



Appendix A- 8: The percentages of apoptotic and necrotic cells after treatment of OVCAR-3 cells with various concentrations of cepharanthine (CEP) or 30 μM cisplatin for 48 h.

Treatments	Cell population (%)			
	Alive	Early-apoptosis	Late-apoptosis	Necrosis
Untreated	90.15±2.34	3.13±1.10	3.00±0.60	3.73±1.07
0.2% DMSO	88.21±2.48	3.81±1.63	4.45±1.66	3.53±0.83
CEP 10 μM	81.97±3.65	5.86±1.90	7.13±1.59	5.05±1.33
CEP 20 μM	79.54±3.41	8.42±1.86	8.60±1.74	3.44±0.87
CEP 40 μM	64.04±4.51	12.79±0.77	13.39±2.09	9.78±3.20
Cisplatin 30 μM	61.73±1.24	7.63±1.24	14.25±0.95	16.38±2.43

Data represent means ± SEM from three independent experiments.

APPENDIX B

PREPARATION OF REAGENTS

Incomplete DMEM medium stock solution (1 L)

DMEM powder (1 pack)	10.4 g
Sodium bicarbonate ($NaHCO_3$)	3.7 g
ddH_2O	900 ml

Mix and stir until dissolve

Adjust the pH to 7.1-7.2 using 1N NaOH or 1N HCl while stirring

Adjust final volume to 1 L with ddH_2O

Sterilize medium by filtering through a 0.2 sterile membrane filter

Transfer into sterile cell culture bottle and keep at 4ºC in the refrigerator

Complete DMEM medium (100 ml)

Incomplete DMEM medium	89 ml
Heat-inactivated fetal bovine serum (FBS)	10 ml
100 U/ml penicillin and 100 µg/ml streptomycin	1 ml

Incomplete RPMI-1640 medium stock solution (1 L)

RPMI powder (1 pack)	10.4 g
Sodium bicarbonate ($NaHCO_3$)	1.5 g
Glucose	4.5 g
Sodium pyruvate	0.11 g
1M HEPES	10 ml
ddH_2O	900 ml

Mix and stir until dissolve

Adjust the pH to 7.1-7.2 using 1N NaOH or 1N HCl while stirring

Adjust final volume to 1 L with ddH_2O

Sterilize medium by filtering through a 0.2 sterile membrane filter

Transfer into sterile cell culture bottle and keep at 4ºC in the refrigerator

Complete RPMI-1640 medium (100 ml)

Incomplete RPMI-1640 medium	79 ml
Heat-inactivated fetal bovine serum (FBS)	20 ml
100 U/ml penicillin and 100 µg/ml streptomycin	1 ml

1X Phosphate Buffered Saline (PBS) (1 L)

Potassium chloride (KCl)	0.2 g
Sodium chloride (NaCl)	8 g
Potassium dihydrogenphosphate (KH_2PO_4)	0.24 g
Sodium dihydrogenphosphate (Na_2HPO_4)	1.44 g
ddH_2O	900 ml

Mix and stir until dissolve

Adjust the pH to 7.4 with 1N NaOH or 1N HCl while stirring

Adjust final volume to 1 L with ddH_2O

Transfer into cell culture bottle and sterilize by autoclaving

Store at room temperature

1X Assay Buffer (100 ml)

1M HEPES	1 ml
0.1 M Calcium chloride ($CaCl_2$)	2.8 ml
5M Sodium chloride (NaCl)	2.5 ml
ddH_2O	90 ml

Mix and stir until dissolve

Adjust final volume to 100 ml with ddH_2O

Transfer into bottle and keep at 4°C in the refrigerator

VITA

Miss. Vilawan Payon was born on March 15, 1989 in Chonburi, Thailand. In 2010, she received Bachelor degree of Biochemistry, from Burapha University. After graduation, she worked as a quality control technician at Krungthai Food Public Co, Ltd. from 2011 to 2014. In 2015, she entered the Master's degree program in Pharmacology at the Graduate School, Chulalongkorn University.