ผลของการเสริมฤทธิ์ของ cephatanthine เมื่อให้ร่วมกับ 5-fluorouracil ต่อเซลล์มะเร็งลำไส้ใหญ่และทวารหนักของมนุษย์

นางสาวสุกัญญา อุ่นสนธิ์

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POTENTIATING EFFECT OF CEPHARANTHINE IN COMBINATION WITH 5-FLUOROURACIL ON HUMAN COLORECTAL CANCER CELLS

Miss Sukanya Unson

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Medical Science
Faculty of Medicine
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สุกัญญา อุ่นสนธิ์ : ผลของการเสริมฤทธิ์ของ cepharanthine เมื่อให้ร่วมกับ 5-fluorouracil ต่อเซลล์มะเร็งลำไส้ใหญ่และทวารหนักของมนุษย์ (POTENTIATING EFFECT OF CEPHARANTHINE IN COMBINATION WITH 5-FLUOROURACIL ON HUMAN COLORECTAL CANCER CELLS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ดร.ปิยนุช วงศ์อนันต์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. จันทนี อิทธิพานิชพงศ์, 60 หน้า.

5-Fluorouracil (5-FU) จัดเป็นยาหลักที่นิยมใช้ในการรักษาผู้ป่วยโรคมะเร็งลำไส้ใหญ่และทวารหนัก แต่ปัญหาการดื้อต่อยาของเซลล์มะเร็งและผลข้างเคียงที่รุนแรงยังคงเป็นข้อจำกัดของการใช้ยาชนิดนี้ จากการศึกษาก่อนหน้านี้พบว่า cepharanthine (CEP) ซึ่งเป็นสารในกลุ่ม biscoclaurine alkaloid ไม่เพียงแต่มีฤทธิ์ในการต้านมะเร็ง แต่ยังสามารถเสริมฤทธิ์ของยาต้านมะเร็งได้ ดังนั้นวัตถุประสงค์ของการทดลองนี้คือเพื่อศึกษาผลของการเสริมฤทธิ์ของ CEP เมื่อให้ร่วมกับ 5-FU ในเซลล์มะเร็งลำไส้ใหญ่และทวารหนักของมนุษย์ จากผลการทดลองแสดงให้เห็นว่าการให้ CEP สามารถเสริมความเป็นพิษของ 5-FU ต่อเซลล์มะเร็งลำไส้ใหญ่และทวารหนักได้อย่างมีนัยสำคัญ จากการศึกษาการเหนี่ยวนำให้เซลล์เกิดการตายแบบ apoptosis พบว่าการให้ 5-FU เพียงอย่างเดียวจะเหนี่ยวนำให้เซลล์เกิดการตายแบบ apoptosis ในขณะที่การให้ 5-FU ร่วมกับ CEP จะทำให้เซลล์เกิดการตายแบบ necrosis เพิ่มมากขึ้น นอกจากนี้จากการวิเคราะห์ด้วยเทคนิค real-time PCR พบว่าการแสดงออกของยีน Bcl-2 จะลดลงเมื่อให้ 5-FU และยีน Bcl-2 จะยิ่งมีการแสดงออกที่ลดลงเมื่อให้ 5-FU ร่วมกับ CEP อีกทั้งการให้ 5-FU ร่วมกับ CEP ยังสามารถยับยั้งวัฏจักรของเซลล์ที่ระยะ S ได้ อย่างไรก็ตามพบว่า CEP ไม่มีผลต่อการการทำงานและการแสดงออกของยีน P-gp, MRP1, or BCRP ในเซลล์ HT-29 เซลล์มะเร็งลำไส้ใหญ่และทวารหนัก ได้ จากผลการทดลองข้างต้นชี้ให้เห็นว่า CEP สามารถเสริมฤทธิ์ของ 5-FU โดยเหนี่ยวนำให้เซลล์เกิดการตายแบบ necrosis ลดการแสดงออกของยีน Bcl-2 และทำให้วัฏจักรของเซลล์เกิดการเปลี่ยนแปลง ดังนั้น CEP อาจจะเป็นอีกทางเลือกหนึ่งในการนำมาใช้ร่วมกับ 5-FU เพื่อรักษาผู้ป่วยโรคมะเร็งลำไส้ใหญ่และทวารหนัก

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SUKANYA UNSON: POTENTIATING EFFECT OF CEPHARANTHINE IN COMBINATION WITH 5-FLUOROURACIL ON HUMAN COLORECTAL CANCER CELLS. ADVISOR: PIYANUCH WONGANAN, Ph.D., CO-ADVISOR: ASSOC. PROF. CHANDHANEE ITTHIPANICHPONG, 60 pp.

5-Fluorouracil (5-FU) is widely used as a first line drug for patients with colorectal cancer. The use of this drug has however been limited by drug resistance and serious side effects. Several preclinical studies reported that cepharanthine (CEP), a biscoclaurine alkaloid, possesses not only anticancer activity but also chemopotentiation effect. This study intended to determine the potentiating effect of CEP on anticancer effect of 5-FU in colorectal cancer cells. The results clearly demonstrated that CEP significantly enhanced cytotoxic activity of 5-FU in HT-29 cells. Although treatment with 5-FU alone could trigger cancer cells to undergo apoptosis, an increase in necrotic cell death was detected following treatment with 5-FU in combination with CEP. Real-time PCR analysis indicated that down-regulation of *Bcl-2* gene expression caused by 5-FU alone was further decreased following treatment with 5-FU and CEP. In addition, the combination treatment could significantly inhibit cell cycle progression in S phase. CEP however did not alter mRNA levels and activities of P-gp, MRP1, or BCRP drug efflux transporters in HT-29 cells. Taken together, the results from this study suggest that the chemopotentiation of 5-FU by CEP may be associated with induction of necrosis, down-regulation of Bcl-2, as well as alteration of cell cycle progression and CEP may potentially be used as an adjuvant agent for enhancing 5-FU potency in colorectal cancer treatment.

Field of Study: Medical Science — Student's Signature ....
Academic Year: 2015 — Advisor's Signature ....
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LIST OF ABBREVAITIONS

% = Percentage

μg/ml = Microgram per milliliter

μl = Microliter

μM = Micromolar

5-FU = 5-fluorouracil

ANOVA = Analysis of variance

APC = Adenomatous polyposis coli

ATCC = American Type Culture Collection

Bad = BCL-2 antagonist of cell death

Bak = BCL-2-antagonist/killer-1

Bax = BCL-2-associated X protein

Bcl-2 = B-cell lymphoma-2

Bcl-xL = B-cell lymphoma-extra large

BCRP = breast cancer resistance protein

Caspase = Cysteine aspartic acid specific protease

cDNA = Complementary DNA

CEP = Cepharanthine

CO_2 = Carbon dioxide

COX = Cyclooxygenase

CRC = Colorectal cancer

CT = Cycle threshold

CyA = Cyclosporin A

DEPC = Dietyl pyrocarbonate

DMEM = Dulbecco's modified Eagle's medium

DMSO = Dimethylsulfoxide

DNA = Deoxyribonucleic acid

DPD = Dihydropyrimidine dehydrogenase

ERCC1 = Excisional repair cross complementation1

FAP = Familial adenomatous polyposis

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Fas = Fibroblast associated antigen

FBS = Fetal bovine serum

FdUMP = Fluorodeoxyuridine monophosphate

FdUTP = Fluorodeoxyuridine triphosphate

FUTP = Fluorouridine triphosphate

GAPDH = Glyceraldehyde 3-phosphate dehydrogenase

GST = Glutathione S-transferase

h = Hour

HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IC_{50} = 50% Inhibition concentration

IL = Interleukin

JNK = Jun N-terminal kinase

KRAS = Kirsten Rat Sarcoma

LRP = lipoprotein receptor-related protein

MAPK = Mitogen-activated protein kinase Mcl-1 = Myeloid cell leukemia 1

MDR = Multidrug resistance

mg/ml = Milligram per milliliter

mRNA = Messenger RNA

MRP = Multidrug resistance protein

MSD = Membrane spanning domains

MTT = 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide

NBD = Nucleotide-binding domains

NF-κB = Nuclear factor kappa-light-chain-enhancer of activated B cells

nm = Nanometer

°C = Degree centigrade

OD = Optical density

OPRT = Orotate phosphoribosyl transferase

P53 = Tumor protein 53

P-gp = P-glycoprotein

PBS = Phosphate buffer saline

PG = Prostaglandin

PI = Propidium iodide

PI3K = Phosphoinositide 3-kinase

PUMA = BCL-2 binding component-3

RNA = Ribonucleic acid

RNase A = Ribonuclease A

Rpm = Round per minutes

RT-PCR = Reverse transcription polymerase chain reaction

TS = Thymidylate synthase

VEGF = Vascular endothelial growth factor

CHAPTER I

INTRODUCTION

1.1 Background and rationale

Colorectal cancer (CRC) is the development of cancer in the colon or rectum tissues. Although there are many kinds of cancer, colorectal cancer is the third most common cancer in man and the second in woman worldwide. This cancer ranks the fourth among cancer-associated deaths, accounting for 694,000 deaths and 1.4 million new cases (1). In 2014, the National Cancer Institute of Thailand reported that colorectal cancer is the second most common cancer in male and it ranks the third in female (2).

Colorectal cancer treatment options are surgery, radiotherapy, chemotherapy and targeted therapy. Chemotherapeutic agents commonly used in patients with CRC are 5-fluorouracil (5-FU), oxaliplatin, irinotecan and capectabine (3). Of several chemotherapeutic agents, 5-FU has been used as a first line drug for CRC treatment. It was reported that when 5-FU is used as a single agent to treat advanced CRC, the response rate is approximately 10-15% (4). And when it is used in combination with other chemotherapies such as irinotecan and oxaliplatin for advanced colorectal cancer, the response rate has increased to about 40-50% (5). Although, 5-FU is widely used for patients with CRC, treatment outcomes for many patients remain poor due to drug resistance and serious side effects. Multiple mechanisms have been shown to contribute to 5-FU resistance including enhanced drug efflux, reduced drug influx, decreased drug activation, increased drug inactivation, mutation and overexpression of drug targets such as thymidylate synthase (TS), impairment of apoptosis pathway and alteration of cell cycle checkpoint (6).

Drug efflux transporters play an essential role in extruding drugs or substrates across the cell membrane, leading to reduce level of drug accumulation in tumor cells. It is commonly known that P-glycoprotein (P-gp), multidrug resistance associated protein (MRP) and breast cancer resistance protein (BCRP), members of ABC transporter family, are associated with anticancer drug resistance (7). High

expression of the P-gp in cancer cells has been found to be associated with resistance to various anticancer drugs such as anthracyclines, epipodophyllotoxins, vinca alkaloid and 5-FU (8-10). Yu ZW et al. also reported that chemotherapy resistance in human colorectal cancer is caused by inactivation of *P-53* and up-regulation of *MDR1* gene expression (11). It was recently shown that overexpression of P-gp is involved in COX-2-mediated drug resistance (14). Modulation of 5-FU resistance phenotype by upregulating function of p38 MAPK, leading to downregulation of the expression of LDL receptor-related protein (LRP), glutathione S-transferase (GST-p), excision repair cross-complementing group 1 (ERCC1), MRP1, and function of P-gp (12). Furthermore, silencing of BCRP using RNA interference (RNAi) could result in 5-FU accumulation and 5-FU induced DNA damage in breast cancer cells (13).

Cepharanthine (CEP) is an alkaloid derived from plants of the genus Stephania, mainly *Stephania cepharantha* Hayata. This natural product is approved by the Japanese Ministry of Health to treat radiation-induced leukopenia, alopecia areata and alopecia pityrodes without severe side effect (14-16). It has been shown to have several pharmacological activities such as anti-tumor, apoptosis-inducing and multidrug resistance-reversing effects (17-19). Interestingly, several preclinical studies have demonstrated the anticancer activity of CEP against several different types of cancer, including osteosarcoma, cholangiocarcinoma, oral squamous cell carcinoma, hepatocarcinoma, leukemia and nasopharyngeal carcinoma (20-23). Furthermore, CEP could restore susceptibility of cancer cells to many chemotherapeutic agents such as actinomycin D, docetaxel, vincristine and paclitaxel by inhibiting drug efflux transporters, including P-gp and MRP-7 (24-26). Previously, Rattanawong A et al have found that p53-mutant HT-29 colorectal cancer cells were relatively resistance to anticancer drugs commonly used in CRC than p53-wild-type COLO-205 colorectal cancer cells (27). Therefore, the present study was to determine potentiating effect of CEP on anticancer activity of 5-FU and its underlying mechanism(s) in HT-29 cells. Effects of CEP on expression and function of the most important drug efflux transporters, including P-gp, MRP-1 and BCRP were also investigated.

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1.2 Research questions

1.2.1 Could cepharanthine improve anticancer activity of 5-FU in colorectal cancer cells?

1.2.2 Could cepharanthine reduce the expression and activity of drug efflux transporters in colorectal cancer cells?

1.3 Objective

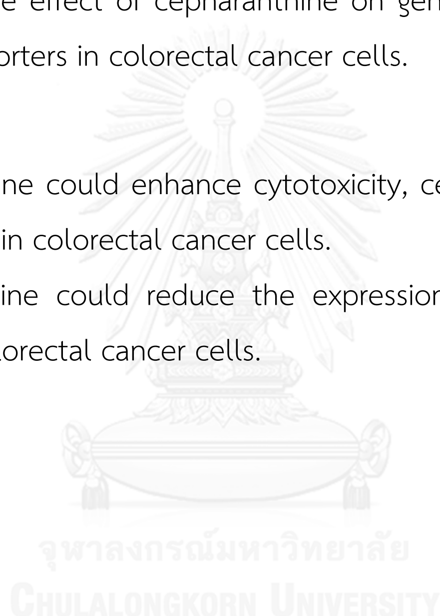
1.3.1. To determine potentiating effects of cepharanthine on cytotoxicity of 5-FU in colorectal cancer cells.

1.3.2 To determine effect of cepharanthine on gene expression and function of drug efflux transporters in colorectal cancer cells.

1.4 Hypothesis

1.4.1. Cepharanthine could enhance cytotoxicity, cell cycle arrest and apoptosis induction of 5-FU in colorectal cancer cells.

1.4.2. Cepharanthine could reduce the expression and activity of drug efflux transporters in colorectal cancer cells.



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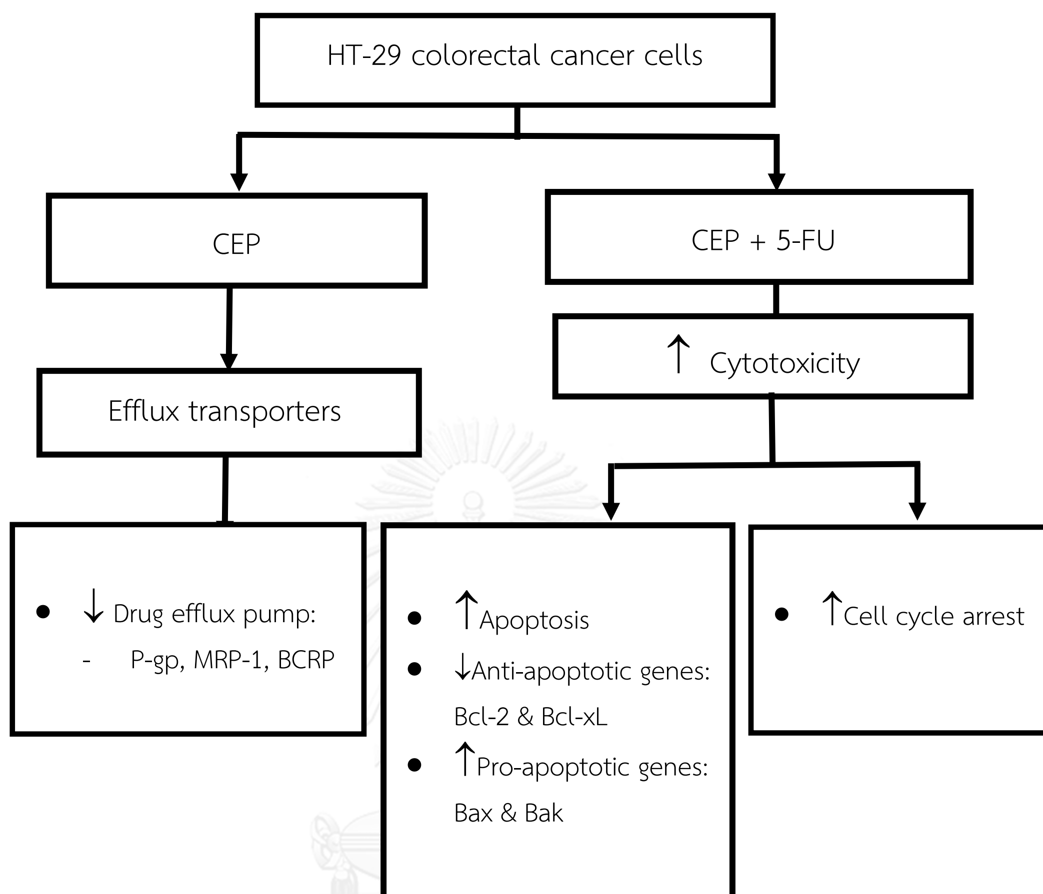
1.5 Conceptual framework



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CHAPTER II

LITERATURE REVIEWS

2.1 Colorectal cancer

Colorectal cancer is the development of cancer in the colon or rectum. It is one of the most common cancers in both sexes worldwide. In 2013, the mortality and incidence rates are highest in Australia, New Zealand, North America and Europe, accounting for 1.4 million new cases and over 694,000 deaths worldwide (1). The National Cancer Institute of Thailand revealed that colorectal cancer is the second most common cancer in male and it ranks the third in female (2).

The etiology and progression of CRC are associated with both environmental factors and genetic factors. Several factors can increase the risk of developing colorectal polyps or colorectal cancer including diet and lifestyle factors, side-effects of medical interventions, and comorbid medical conditions. It is well-known that low intake of dietary fiber and high consumption of red meat have been strongly associated with CRC. Several studies also showed that cigarette smoking, heavy alcohol drinking and lack of exercise are risk factors of CRC. Moreover, medical intervention such as pelvic irradiation, cholecystectomy and ureterocolic anastomosis after surgery can increase the risk of CRC. A strong correlation between CRC and Barrett's esophagus, human immunodeficiency virus infection, or inflammatory bowel disease was also observed (28). In addition to environmental factors, genetic mutation, including upregulation of tumor suppressor genes such as adeomatous polyposis coli (*APC)* and *p53* as well as downregulation of oncogenic genes such as Kirsten Rat Sarcoma (*KRAS)* and v-raf murine sarcoma viral oncogene homolog B1 (*BRAF)* have been involved in CRC initiation and progression (Figure 1) (29). The mutations of the *APC* gene on chromosome 5q21 could cause both of the inheritance of familial adenomatous polyposis (FAP) and Gardner's syndrome (30). Conversely, KRAS or BRAF mutations at approximately 37% and 13%, respectively was detected in patients with CRC (31, 32). Furthermore, cyclooxygenase-2 (COX-2),

which is commonly known to be responsible for inflammation, has also found to be involved in cancer initiation and progression. COX-2 overexpression has been detected about 40-90% in patients with colorectal adenomas. Additionally, COX-2 has recently been found to be related with drug resistance by preventing cancer cells from apoptosis and increasing P-gp expression in tumor cells (33).

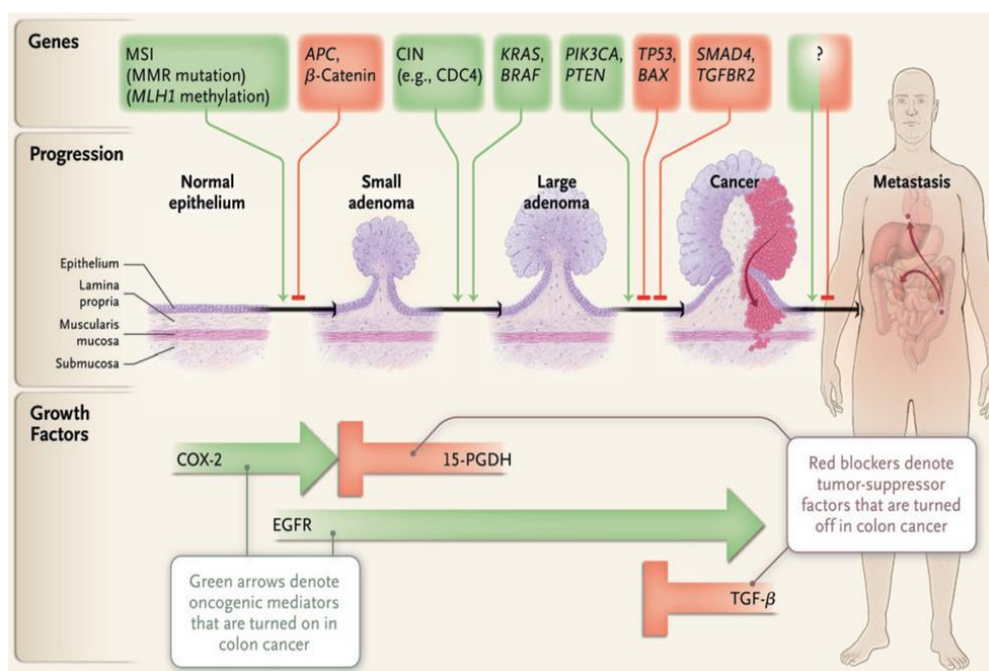

Figure 1. Gene and growth factor pathways that changes in various stages of colorectal cancer progression (29).

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Stages of colorectal cancer

Stage of cancer is used to decide what treatment options are recommended. It is usually expressed as a number on a scale of 0 through IV, which based on four characteristics; tumor size, cancer invasion or non-invasion, lymph node status and distant metastasis (Table 1).

Table 1. Staging of colorectal cancer.

Stage	Description
Stage 0	Abnormal cells are detected in the mucosa of the colon wall
Stage I	Cancer has formed in the mucosa of the colon wall and has spread to the submucosa.
Stage II	Cancer has grown through the muscle layer of the colon wall and invaded nearby organ.
Stage III	Cancer has spread to nearby lymph nodes but not to other parts of the body.
Stage IV	Cancer has spread through the blood and lymph nodes to other parts of the body often lung and liver.

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2.2 5-Fluorouracil

The types of CRC treatment are surgery, radiotherapy, chemotherapy and targeted therapy. Chemotherapeutic agents that are commonly used in patients with CRC are 5-fluorouracil (5-FU), oxaliplatin, irinotecan and capecitabine (34). In addition, several target-based anticancer agents such as bevacizumab, cetuximab and panitumumab have been used as either mono- or combination therapy for management of patients with metastatic CRC (35). Of all these agents, 5-FU has been widely used as a first-line drug for CRC treatment.

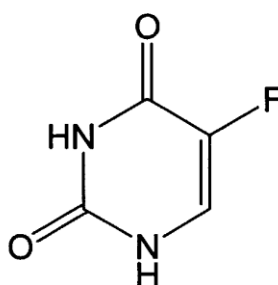

Figure 2. Chemical structure of 5-fluorouracil ($C_4H_3FN_2O_2$) (36).

5-Fluorouracil (5-FU), an analog of uracil with a fluorine atom at the C-5 position (Figure 2), kills cells specifically in S phase of the cell cycle. When get inside the cells, 5-FU can be converted into three main active metabolites, fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP). FdUMP can inhibit thymidylate synthase (TS), a key enzyme involved in DNA synthesis and repair while the other two active metabolites, FdUTP and FUTP can be incorporated into DNA and RNA, leading to DNA and RNA damage, respectively (Figure 3) (37). It has been reported that more than 85 % of administered 5-FU is catabolized by dihydropyrimidine dehydrogenase (DPD) that is an initial rate-limiting enzyme in the catabolism of 5-FU in liver.

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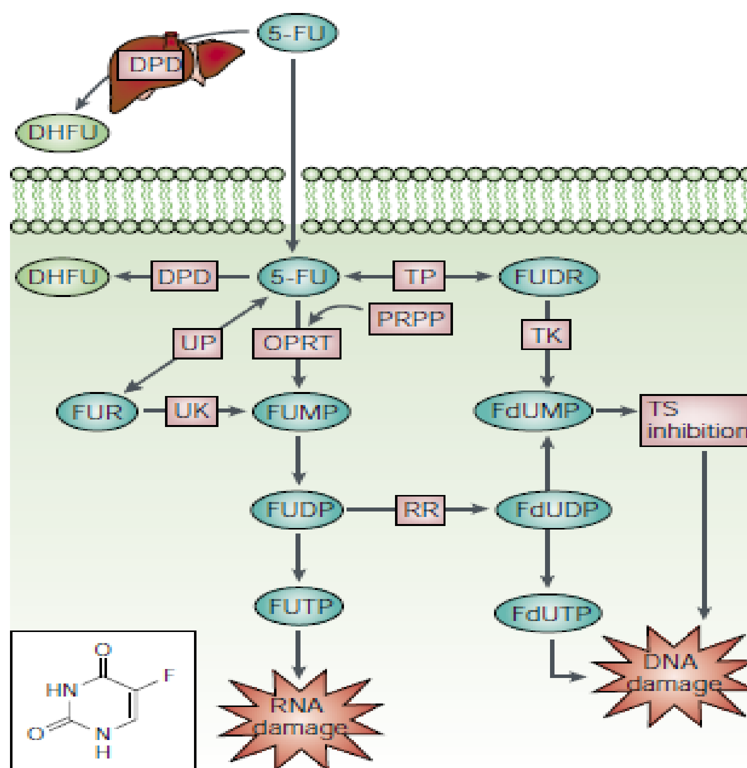

Figure 3. Metabolism and mechanism of action of 5-fluorouracil (37).

Although 5-FU has generally been used as a first-line drug for patients with CRC for a long time but its efficacy has been limited due to drug resistance and serious side effects. It was demonstrated that 5-FU-based chemotherapy has improved response rates and survival of patients with stage III colon cancer; however, the response rates to this drug in patients with advanced disease remain poor (4). And when the drug is used in combination with other chemotherapies such as irinotecan and oxaliplatin, the response rate was increased to only about 40-50% in patients with advanced CRC (5). Serious side effect of 5-FU are chest pain and increases in cardiac enzymes, which may indicate problems with the heart. Therefore, new therapeutic strategies that can improve efficacy and reduce side effects of 5-FU are urgently needed.

2.3 Mechanism of 5-FU resistance

Chemotherapy resistance is still an important clinical problem in the treatment of CRC. Various mechanisms have been associated with 5-FU resistance including evasion of apoptosis , decrease in drug activation, mutation of drug targets, prevention of cell cycle arrest and alteration of drug efflux and influx (Figure 4) (6).

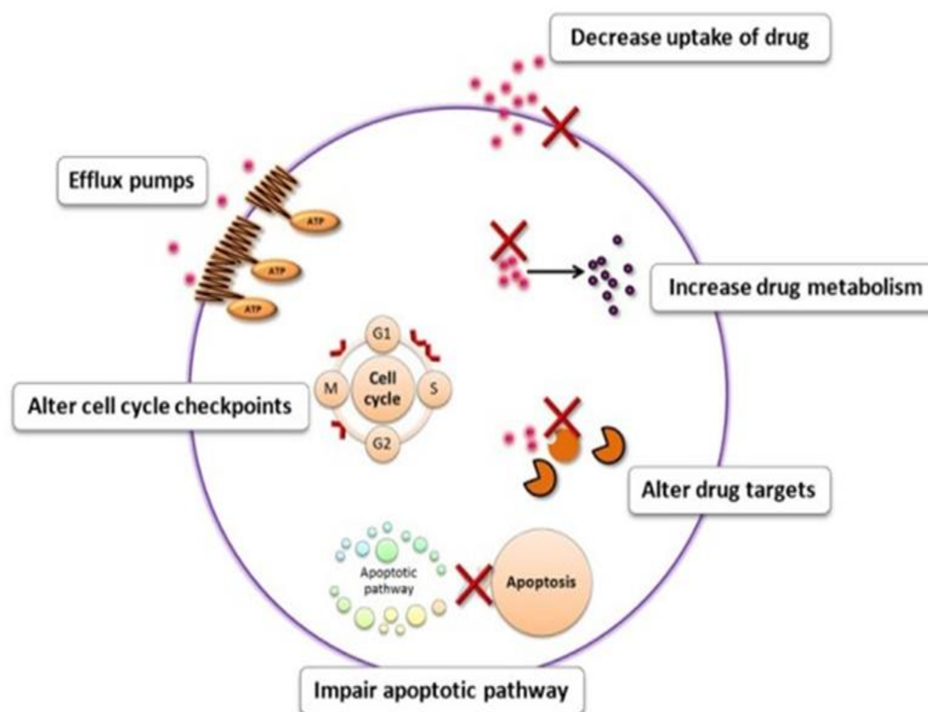

Figure 4. Numerous mechanisms have been involved with 5-FU resistance (38).

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Drug efflux transporters

Drug efflux is most often mediated by members of the ATP-binding cassette (ABC) transporter family, classified into seven groups (ABCA to ABCG) depending on sequence similarly. Most ABC transporters consist of two membrane spanning domains (MSDs) and two nucleotide-binding domains (NBDs) (39). As show in Figure 5 and 6, ligand binding domain (LBD) and NBDs are located in the cytoplasm, whereas MSDs are presented at transmembrane that are specifically recognizes drug or substrate protein. The NBDs contain the ATP-binding site that composed of three signature conserved sequences (the Walker A, B and C). Drug efflux transporters are activated by ATP hydrolysis as an energy source. Binding of ATP to NBD results in

conformational change of MSD leading to translocation of drug or substrates (40, 41). Efflux transporters have been found in several human tissues including liver, kidney, adrenal gland, brain capillaries, small intestine and colon (42, 43).

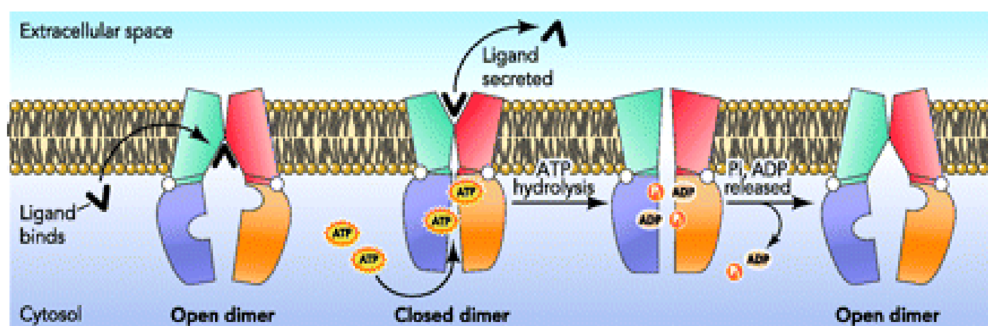

Figure 5. A simple ATP-switch mechanism powers ABC transporters (42)

The major member of efflux transporters have been shown to confer drug resistance in cancer therapy, including P-glycoprotein (P-gp/MDR1), multidrug resistance associated protein (MRP), and breast cancer resistance protein (BCRP). Increased expression of drug efflux transporters lead to decreased drug level within the cells, causing cancer cells highly resistant to chemotherapy (9). P-gp, also called ABCB1, is a 170-kDa transmembrane phosphoglycoprotein encoded by *mdr1* gene (43). It has been reported that over-expression of P-gp in malignant cells decreased accumulation of several anticancer drugs such as anthracyclines, epipodophyllotoxins, vinca alkaloid and 5-FU, resulting in drug resistance (8, 10). Loss of P-53 function has been associated with higher expression of *MRD1* gene leading to 5-FU resistance in human colorectal cancer LoVo/5-FU cells (11). Several natural compounds such as curcumin, sinomenine, tetrandrine and fangchinoline have been found to reverse drug resistance through inactivation of P-gp (44-46).

In addition to P-gp, the 190-kDa multidrug resistance protein 1 (MRP1), also called ABCC1, confers resistance to a broad spectrum of anticancer drugs. It has been reported that the expression of MRP1 in tumors is much higher than in the surrounding normal tissue (47). Metformin was shown to reverse chemotherapy resistance in 5-FU-resistant-human hepatocellular carcinoma Bel-7402 cells by down-regulation of *P-gp* and *MRP1* expression and function (48). Furthermore, a novel

immunomodulatory agent FTY720 enhanced sensitivity of susceptible (HCT-8) and resistant (HCT-8/5-FU) colon cancer cells to 5-FU though suppression of *P-gp* and *MRP1* expression, causing cancer cells undergo apoptosis (49). It has also been reported that inhibition of drug transporter function through downregulation of MRP1 expression could reverse resistance of hepatocellular carcinoma to 5-FU (50).

Unlike P-gp and MRP1, BCRP, called ABCG2, is a half transporter consisting of one of TMD and one of cytosolic NBD which may function as either homodimers or homo-multimers. It is a 72-kDa membrane protein localized to the plasma membrane of the drug resistant cells (51). BCRP plays significant roles in absorption, distribution, and elimination of drugs or BCRP substrates (52). BCRP and P-gp are frequently co-expressed, and their co-expression is related with poor outcomes in acute myeloid leukemia (53). In addition, Ho MM. suggested that BCRP can predict the sensitivity of 5-FU and it might be a target to reverse 5-FU resistance in breast cancer (13).

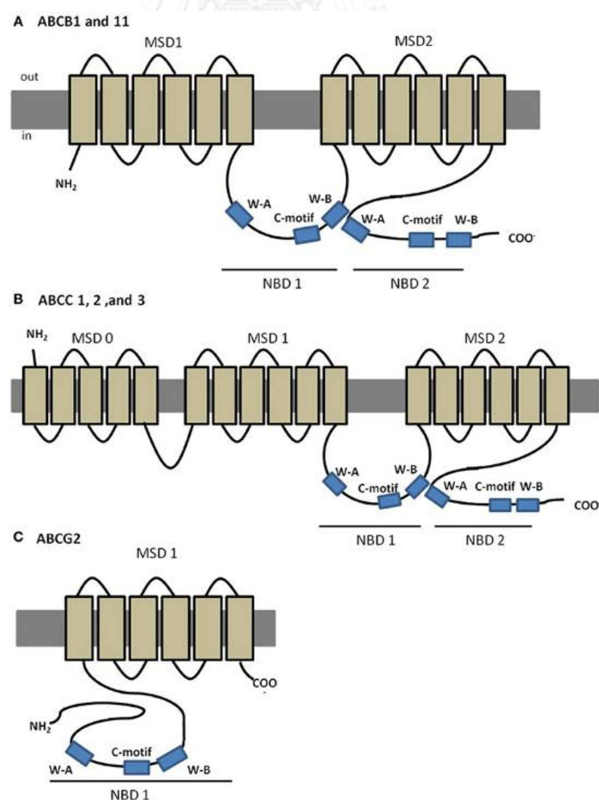

Figure 6. The structures of multidrug resistance of ABC transporters (54).

Apoptosis

The apoptosis-inducing effect is one of the most important mechanisms of chemotherapeutic drugs and radiation in the treatment of cancer. There are two major apoptotic pathways: the extrinsic pathway and the intrinsic pathway. Both pathways play a crucial roles in maintaining tissue homeostasis (55). The extrinsic pathway, also called death receptor pathway, is triggered by binding of an extracellular death ligand such as FasL and TNF-α to its cell surface receptor. The intrinsic or mitochondrial pathway is activated in response to different death stimuli that are generated inside the cells such as endoplasmic reticulum stress, growth factor withdrawal, radiation, and cytotoxic drugs (56). The Bcl-2 family proteins are known as important regulators of the mitochondrial response in the intrinsic apoptotic pathway (Figure 7). Bcl-2 can be classified into three functional groups: anti-apoptotic proteins such as Bcl-2 and Bcl-xl, pro-apoptotic effector proteins such as Bax and Bak and pro-apoptotic activator proteins such as Bid, Bad, Noxa and Puma (57). Previously, modulation of 5-fluorouracil resistance was shown to be related with Bcl-xL to Bax ratio in human colon cancer cell lines (58). Studies demonstrated that overexpression of *Bcl-2* and *Bcl-xL* gene prevent 5-FU-treated HCT116 colorectal cancer cell line undergoing apoptosis (59). Furthermore, 5-FU resistant tumor cells induced by long-term drug exposure displayed high level of Bcl-2 and Bcl-xL proteins and low level of Bax protein, correlated with p53 status in different human colorectal cancer cell lines (HT-29, LS174T, SW480, HCT116, HCT-EB, LoVo and LS513) (60).

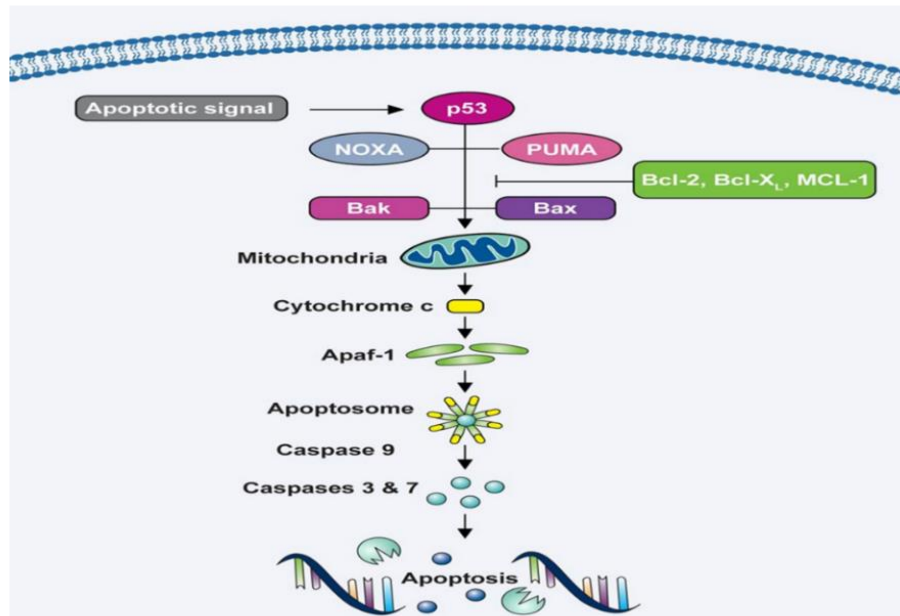

Figure 7. Elements of the intrinsic mitochondrial apoptotic pathway (61).

Cell cycle

In eukaryotes, the cell cycle has two main phases: the interphase and the mitotic phase. Interphase of cell cycle can be divided into three phases: G1, S and G2. The first gap phase, G1, is the first stage of interphase, in which the cell prepares and synthesizes mRNA and proteins required for DNA synthesis. The S phase, occurring between G1 and G2 phases, is the synthesis phase of the cell cycle where DNA is replicated. The G2 phase is a second gap phase in which cell prepare for mitosis. In this phase, the cell continues to grow and synthesize more proteins that are necessary for cell division. The M phase or mitotic phase is a phase of the cell cycle in which a nucleus is divided into two identical nuclei (mitosis) followed by division of cytoplasm (cytokinesis), resulting in formation of two daughter cells (Figure 8) (62).

Alteration of cell cycle distribution has been shown to be involved in acquired 5-FU resistance. The 5-FU resistant cell lines displayed significantly cell cycle delays at G1 and G1/S phase and prolonged period of DNA replication. Moreover, the expression of CDK2 protein, cyclin D3 and cyclin A were noticeably low in the resistant cell lines (63). Previously, studies also discovered that cell-cycle

perturbation or increase in TS protein expression levels are associated with 5-FU resistance in different resistant head and neck squamous carcinoma cell lines (64).

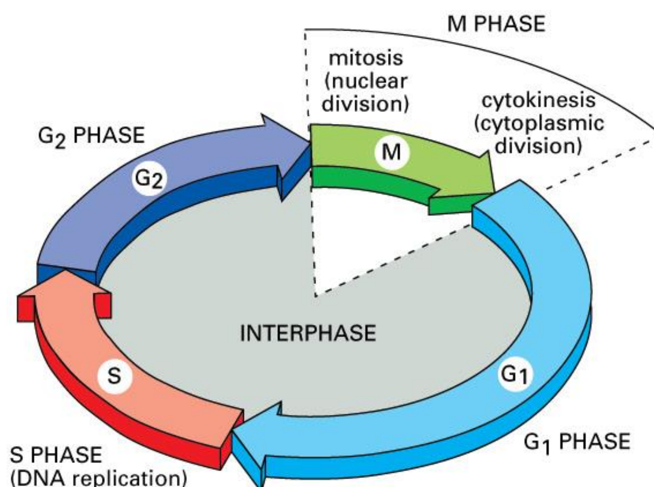

Figure 8. Four phases of cell cycle (62).

2.4 Cepharanthine (CEP)

Cepharanthine (CEP) is an alkaloid isolated from plants of the genus Stephania, mainly *Stephania cepharantha* Hayata. In Japan, CEP has long been used as a potential treatment for many diseases including radiation-induced leukopenia (breast, lung and ovarian cancers), idiopathic thrombocytopenic purpura, alopecia areata, alopecia pityrodes and venomous snake bite without reported serious side effects (14-16). It has also been shown to have several pharmacological activities including antitumor, anti-allergic, anti-inflammatory, antioxidant, anti HIV1, membrane-stabilizing, immunomodulatory, apoptosis-inducing and multidrug resistance-reversing effects (17, 18, 65-70).

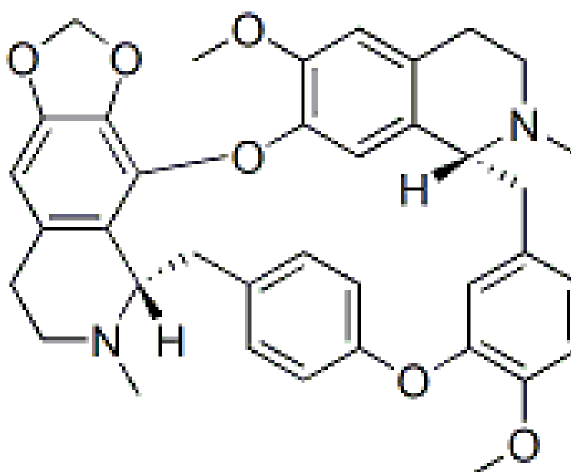

Figure 9. Chemical structure of cepharanthine (CEP) (18)

Anticancer effects of CEP

Several preclinical studies have shown the anticancer activity of CEP against several different types of cancer including lung carcinoma, leukemia, cholangiocarcinoma, oral squamous cell carcinoma, hepatocellular carcinoma, lymphoma, osteosarcoma and nasopharyngeal carcinoma (19, 21, 71-74). This compound was found to inhibit cell growth and induce cell cycle arrest at G1 phase by inhibiting expression of STAT3, anti-apoptotic gene such as *Bcl-xL* and cell cycle regulators such as *c-Myc* and *cyclin D* in human SaOS2 osteosarcoma cell line (20). Additionally, CEP could inhibit the growth of tumor by down-regulating cyclin E leading to induce G1 arrest through a pathway of p27Kip1 induction (75). It was also demonstrated that CEP controlled the growth of cancer cells and vascularization via inactivation of NF-κB resulting in inhibition of VEGF and IL-8 expression both *in vitro* and *in vivo* (22). Moreover, CEP was able to induce primary effusion lymphoma (PEL) undergo apoptosis through inhibition of NF-κB *in vitro*. Similar results were observed in a mouse model (76). Studies in cholangiocarcinoma illustrated that CEP effectively inhibited cancer cell growth in a dose- and time-dependent manner *in vitro*. In animals, this compound was able to decrease tumor size by apoptosis induction through activation of caspase-3 and caspase-9 and inhibition of NF-κB nuclear translocation (21). In addition, apoptosis-inducing effect of CEP was mediated through

reactive oxygen species (ROS) production, leading to upregulation of Bax, downregulation of Bcl-2 and activation of caspase-3 and PARP in non-small lung cancer cells (77). Similarly, Biswas KK reported that apoptosis of human hepatocellular carcinoma cell line (HuH-7) following CEP treatment was associated with production of ROS, activation of JNK1/2, MAPK p38, and ERK p44/42 as well as downregulation of Akt (23). Moreover, inhibition of metastatic human cholangiocarcinoma cell migration and invasion by CEP was related to suppression of ICAM-1 and MMP-2 (78). Kikukawa Y *et al*. also found that CEP exerted anticancer effects on myeloma cell lines by the activation of apoptotic pathways and cell cycle arrest (79). In addition, CEP could induce cell apoptosis in both glioma cells with the wild-type p53 gene and in those with the mutant-type p53 gene, and this compound was found to enhance the anti-proliferative effect of nimustine hydrochloride (ACNU) (80) Moreover, treatment with CEP alone or in combination with interferon-gamma (IFN-γ) inhibited the growth of all four human tumor cell lines (RPMI 4788, PC 10, HeLa, ZR-75-1) *in vitro* and inhibited activity of pulmonary metastasis in a dose-dependent manner in a nude mouse tumor-xenograft model of colon cancer (81). It was also reported that treatment of CEP could enhance tumor radioresponse by inducing cells to undergo apoptosis and inactivating DNA double-strand break repair in oral squamous cell carcinoma (14). Additionally, CEP has been recently shown to enhance the radiosensitization of cancer cells through decrease in expression of *c-Myc, Bcl-2, STAT3* and *COX-2* in HeLa cells (82).

Multidrug resistance-reversing effects of CEP

Several studies have shown that CEP could reverse drug resistance in various types of cancer such as kidney cancer, oral cancer, osteosarcoma, hepatocellular carcinoma, ovarian cancer and myeloid leukemia. Previously, Zehadi *et al.* demonstrated that treatment of ovarian cancer cells with MDR phenotype with docetaxel and CEP using an injectable polymer lipid formulation increased accumulation level of anticancer agent and induced cancer cell apoptosis (26). Mechanistic studies have demonstrated that CEP reversed vinblastine and daunorubicin resistance in LLC-GA5-COL150 cells by inhibiting P-gp transporter

activity (83). Moreover, CEP significantly increased the accumulation of doxorubicin in DOX-resistant hepatocellular carcinoma cell lines by modulating P-gp mediated efflux (24). Studies in squamous cell carcinoma illustrated that CEP could enhance efficacy of anticancer drug S-1 by inducing cell apoptosis, increasing orotate phosphoribosyl transferase (OPRT) and decreasing dihydropyrimidine dehydrogenase (DPD) and thymidylate synthase (TS) expression (19). Treatment with 2-5 μM of CEP in combination with a nontoxic concentration of herbimycin A (a benzoquinoid ansamycin antibiotic) was also found to induce K562 cells undergo apoptosis through degradation of Bcr-Abl and Akt protein (18). In addition, CEP significantly down-regulated the expression of MDR1 gene via increased expression of c-Jun that encoded by the *JUN* gene in adriamycin-tolerance K562 cells (84). Besides P-gp, CEP was able to reverse MRP7-mediated resistance to paclitaxel via inhibition of the drug efflux activity of MRP7 in MRP7-transfected HEK293 cells (85). Similarly, Malofeeva EV et al reported that CEP at a concentration of 5 μM inhibited MRP7 activity, leading to enhanced docetaxel accumulation in ABCC10-overexpressing cells (86).

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CHAPTER III

MATERIALS AND METHODS

3.1 Equipments

- Autopipette (Gilson, USA)
- Autoclave (Sanyo, Japan)
- Biohazard laminar flow hood (Science, Germany and Labconco, USA)
- Controller pipette (Gilson, USA)
- CO_2 incubator (Thermo, USA)
- Centrifuge (Hettich, USA and Eppendrof, Germany)
- Fluorescence flow cytometer (BD Biosciences, USA)
- Light microscope (Nikon, Japan)
- Microplate reader (Thermo, Finland)
- Fluorescence microplate reader (Thermo, Finland)
- PCR thermal cycler (Eppendorf, Germany)
- pH meter (Mettler Toledo, Switzerland)
- StepOne Plus^{TM} Real-Time PCR system (Applied Biosystems, USA)
- Vortex mixer (Scientific Industries, USA)

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3.2 Materials

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

3.3 Reagents

- 0.25% trypsin-EDTA (Gibco, USA)
- 0.4% trypan blue dye (Sigma, USA)
- 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Sigma, USA)
- 5(6)-carboxyfluoresceindiacetate (Sigma, Switzerland)
- 5-fluorouracil (5-FU)(Sigma, USA)
- Annexin V, Fluorescein (FITC) (Gibco,USA)
- Cepharanthine (Abcam, UK)
- Cyclosporin A (Sigma, China)
- DEPC-treated water (Ambion, USA)
- Dimethyl sulfoxide (DMSO) (Sigma, USA)
- Dulbecco's modified eagle medium (DMEM) (Gibco, New Zealand)
- Ethanol (Merk, Germany)
- Express SYBER Green qPCR supermix universal (Invitrogen, USA)
- Fetal bovine serum (Gibco, New Zealand)
- Hank balanced salt solution (HBSS)(Sigma, USA)
- ImProm-II^{TM} Reverse Transcription system (Promega, USA)
- Ko143 (Sigma, USA)
- Mitoxantrone (Sigma, USA)
- MK571 (Sigma, USA)
- Penicillin-streptomycin (Gibco, New Zealand)
- Propidium iodide (Santa Cruz Biotechnology, USA)

3.4 Methods

3.4.1 Preparation of cepharanthine (CEP) and 5-fluorouracil (5-FU) stock solution.

A stock solution was prepared by dissolving CEP and 5-FU in dimethyl sulfoxide (DMSO) and storing at 4°C. The stock solution was diluted with culture medium to give appropriate final concentration. The 0.2% DMSO was used as a vehicle control.

3.4.2 Cell culture

The human colorectal cancer cell line HT-29 (COX-2 positive and p53 mutation) was purchased from American Type Culture Collection (ATCC) (Rockville, MD). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin and maintained under an atmosphere of 5% CO_2 at 37 $^{\circ}C$.

3.4.3 Cytotoxicity assay

The effects of CEP and 5-FU on cell viability were evaluated by MTT assay. Briefly, HT-29 cells were seeded in a 96-well plate at a density of $5x10^3$ cells/well and incubated overnight at 37 $^{\circ}C$ and 5% CO_2. The cells were then treated with 0.01, 0.1, 1, 10, or 100 μM of CEP or 5-FU or 0.2% DMSO (vehicle control) for 24, 48 or 72 h. After that, 15 μL of MTT (0.05 mg/mL in PBS) was added to each well and further incubated for 4 h. The medium was removed and 150 μL of 100% DMSO was added to solubilize the MTT formazan crystals. The optical density was determined at 570 nm using a microplate reader (Thermo, Finland). The percent of cell viability was calculated using the following formula: percent of cell viability = (Abs. sample/Abs. control) x 100. The IC_{50} value was estimated using the fitted line by GraphPad prism software.

To assess whether CEP enhances anticancer activity of 5-FU, the HT-29 cells were seeded at a density of $5x10^3$ cells/well in 96 well plates. After overnight incubation, the cell were either pre-treated with 2.5 μM of CEP for 4 h, followed by treatment with various concentrations of 5-FU (0.01, 0.1, 1, or 100 μM) or treated with various concentrations of 5-FU (0.01, 0.1, 1, 10, or 100 μM) in the absence or presence of 2.5 μM of CEP and incubated for another 48 h. Cell viability was evaluated by MTT assay as mentioned above.

3.4.4 Determination of Bcl-2 gene family and drug efflux transporters mRNA expression by quantitative real-time RT-PCR

Effect of CEP and/or 5-FU on expression level of Bcl-2 family members such as anti-apoptosis (Bcl-2 and Bcl-xl) and pro-apoptosis (BAX and BAK) were quantified by quantitative real-time RT-PCR using SYBR Green Real-time super mix universal (Invitrogen, USA). Briefly, HT-29 cells were seeded in 6-well plates at a density of $5x10^5$ cells/well. After overnight incubation, the cells were treated with 2.5 μM of CEP alone, 50 μM of 5-FU alone or 2.5 μM of CEP combined with 50 μM of 5-FU. Bcl-2 family genes were assessed at 24 h following treatment. To evaluate effect of CEP on drug efflux transporters (MRD1, MRP1 and BCRP), the cells were treated with CEP at a concentration of 1.25, 2.5 or 5 μM for 24 h and mRNA levels of drug efflux transporters were evaluated.

Total RNA was isolated using TRIzol reagent (Invitrogen, USA), according to the manufacturer's instructions. PCRs were performed using the oligonucleotide primers noted in Table 2. Real-time reactions were run on StepOne Plus™ Real-Time PCR (Thermo Fisher Scientific, USA) with the following cycling conditions: 50 °C for 2 min, 95 °C for 2 min, and 40 cycles of 95 °C for 30s, 60 °C for 30 s and 72 °C for 30s. The expression of target gene was calculated by the$2^{-\Delta\Delta CT}$ method. The mRNA level for each target gene was normalized to GAPDH and relative expression ratio of a target gene was compared with vehicle control.

Table 2. The sequences of forward and reverse primers used for quantitative real-time RT-PCR.

Primer name	Primer sequences
GAPDH	Forward: 5'-AAGGTCGGAGTCAACGGATTTGGT-3' Reverse: 5'-ATGGCATGGACTGTGGTCATGAGT-3'
Bcl-2	Forward: 5'-TCATGTGTGTGGAGAGCGTCAA-3' Reverse: 5'-CTACTGCTTTAGTGAACCTTTTGC-3'
Bcl-xl	Forward: 5'-TTGGACAATGGACTGGTTGA-3' Reverse: 5'-GTAGAGTGGATGGTCAGTG-3'
BAX	Forward: 5'-GACGAACTGGACAGTAACATG-3' Reverse: 5'-AGGAAGTCCAATGTCCAGCC-3'
BAK	Forward: 5'-AGCTGCCATGGTAATCTAACTCA-3' Reverse: 5'-GATGTGGAGCGAAGGTCACT-3'
P-gp (MDR1)/ABCB1	Forward: 5'-TGTTCAAACTTCTGCTGCTCCTGA-3' Reverse: 5'-CCCATCATTGCAATAGCACG-3'
MRP1/ABCC1	Forward: 5'- AGC TTT ATG CCT GGG AGC TGG -3' Reverse: 5'- CGG CAA ATG TGC ACA AGG CCA C -3'
BCRP/ABCG2	Forward: 5'- CAC AAC CAT TGC ATC TTG GCT G -3' Reverse: 5'- TGA GAG ATC GAT GCC CTG CTT T -3'

Bcl-2 gene family: *Bcl-2, Bcl-xl, BAX and BAK*

Drug efflux transporters: *P-gp (MDR1)/ABCB1, MRP1/ABCC1, BCRP/ABCG2*

Endogenous control: *GAPDH*

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3.4.5 Flow cytometry for analysis of apoptosis

The potentiation effect of CEP on 5-FU-induced apoptosis was evaluated using flow cytometry. The cells were plated on 6-well plates at a density of 5×10^5 cells/well. After 24 h incubation, the media were discarded and cells were treated with 2.5 µM of CEP alone, 50 µM of 5-FU alone or 2.5 µM of CEP combined with 50 µM of 5-FU for 24 and 48 h. After that, the cells were trypsinized, washed twice with ice-cold PBS and collected by centrifugation at 1500 rpm for 5 min. The cell pellet was re-suspended with 500 µl of assay buffer and stained with 1 µl of Annexin V FIT-C (Invitrogen, USA) and 1 µl of 0.05 µg/ml propidium iodide (PI) (Santa Cruz Biotechnology, USA) for 15 min at room temperature in dark. The stained cells were classified into 4 populations, including viable cells (annexin V^-, PI^-), early apoptotic cells (annexin V^+,PI^-), late apoptotic cells, (annexin V^+, PI^+), and necrotic cells, (annexin V^-, PI^+) using flow cytometry (BD LSR II, Biosciences).

3.4.6 Detection of the cell cycle destitutions by flow cytometry

The DNA content of cells accumulated in each phase of cell cycle was detected by flow cytometry using PI staining. Briefly, HT-29 cells were seeded in 6-well plates at a density of 5×10^5 cells/well. The cells were then incubated with 2.5 µM of CEP alone, 50 µM of 5-FU alone or 2.5 µM of CEP combined with 50 µM of 5-FU for 24 h. The end of incubation time, cells were washed with PBS, trypsinized and then harvested by centrifugation at 1500 rpm for 5 min. Cells were washed twice with ice-cold PBS and fixed with 70% ethanol for 15 min at -20 °C. The cells were then washed again with ice-cold PBS and incubated with 500 µl of Hanks' Balanced Salt Solution (HBSS) containing 5 µl of RNase (4 mg/ml) for 30 min at room temperature. Finally, 5 µl of PI stain solution (0.05 µg/ml) was added and incubated for 30 min in the dark at room temperature. The cell cycle distributions were measured using a BD LSR II flow cytometer. The proportion of cells in different phase, G1, S and G2/M phase were analyzed by FCS Express 5 Image Cytometry software (De Novo Software, CA).

3.4.7 Evaluation of drug efflux transporters functional activity.

The activity of drug efflux transporters including P-gp (MDR1), MRP1, BCRP were determined by microplate reader using fluorescent substrates. For determination of drug accumulation, rhodamine 123, 5(6)-carboxyfluoresceindiacetate and mitoxantrone were used as substrates for P-gp, MRP1 and BCRP respectively. And cyclosporin A, MK571 and Ko143 were used as an inhibitor of P-gp, MRP1 and BCRP, respectively. It should be noted that the non-toxic concentrations of fluorescent substrates or inhibitors were used to examine the effect of CEP on activity of drug efflux transporters. The cells were plated in 24-well plates at a density of $4x10^5$ cells/well and incubated overnight. After that, the medium was decanted and the cells were pre-treated with either 1.25, 2.5 or 5 μM of CEP, 20 μM of cyclosporine A (P-gp inhibitor), 100 μM of MK571 (MRP1 inhibitor), 20 μM of Ko143 (BCRP inhibitor) or HBSS (negative control) for 30 min at 37 °C in 5% CO_2 incubator. After 30 min incubation, rhodamine 123, 5(6)-carboxyfluorescein diacetate, and mitoxantrone, a specific fluorescent substrate for P-gp, MRP1 and BCRP, respectively, was added into each well and incubated for additional 60 min. The cells were then washed twice with ice-cold HBSS and lysed with 1% triton X 100. The fluorescence intensity of substrate was measured using a fluorescence microplate reader (Thermo, Finland) with the excitation wavelength at 488, 492 or 488 nm, and the emission wavelengths 525, 517 or 530 nm for P-gp, MRP1 and BCRP, respectively (87, 88).

3.4.8 Statistical analysis

Data are expressed as mean ± standard error of mean (SEM). Statistical analysis of data was performed using one-way analysis of variance (ANOVA) followed by LSD post hoc test for multiple group comparison. An unpaired two-tailed Student’s t-test was used to compare the differences between two groups.

CHAPTER IV

RESULTS

The cytotoxic effect of 5-fluorouracil (5-FU) and cepharanthine (CEP) on cell viability of HT-29 cells.

The cytotoxic effects of 5-FU and CEP on HT-29 human colorectal cancer cell line were evaluated using MTT assay. As shown in Figure 10A, 5-FU significantly inhibited the growth of cancer cells in a dose- and time-dependent manner. The IC_{50} of 5-FU were 69.83 ± 9.67 μM for 72 h of treatment. It however should be noted, at 24 or 48 h of incubation, this anticancer drug was not effective in controlling cancer cell growth. Similar to 5-FU, CEP effectively inhibited cancer cell growth in a dose- and time-dependent manner (Figure 10B). The compound; however, displayed much higher anticancer activity than 5-FU in human colorectal cancer cells. The IC50 of CEP were 7.10 ± 0.11, 5.16 ± 0.18 and 4.63 ± 0.52 μM for 24, 48 or 72 h, respectively (Table 3).

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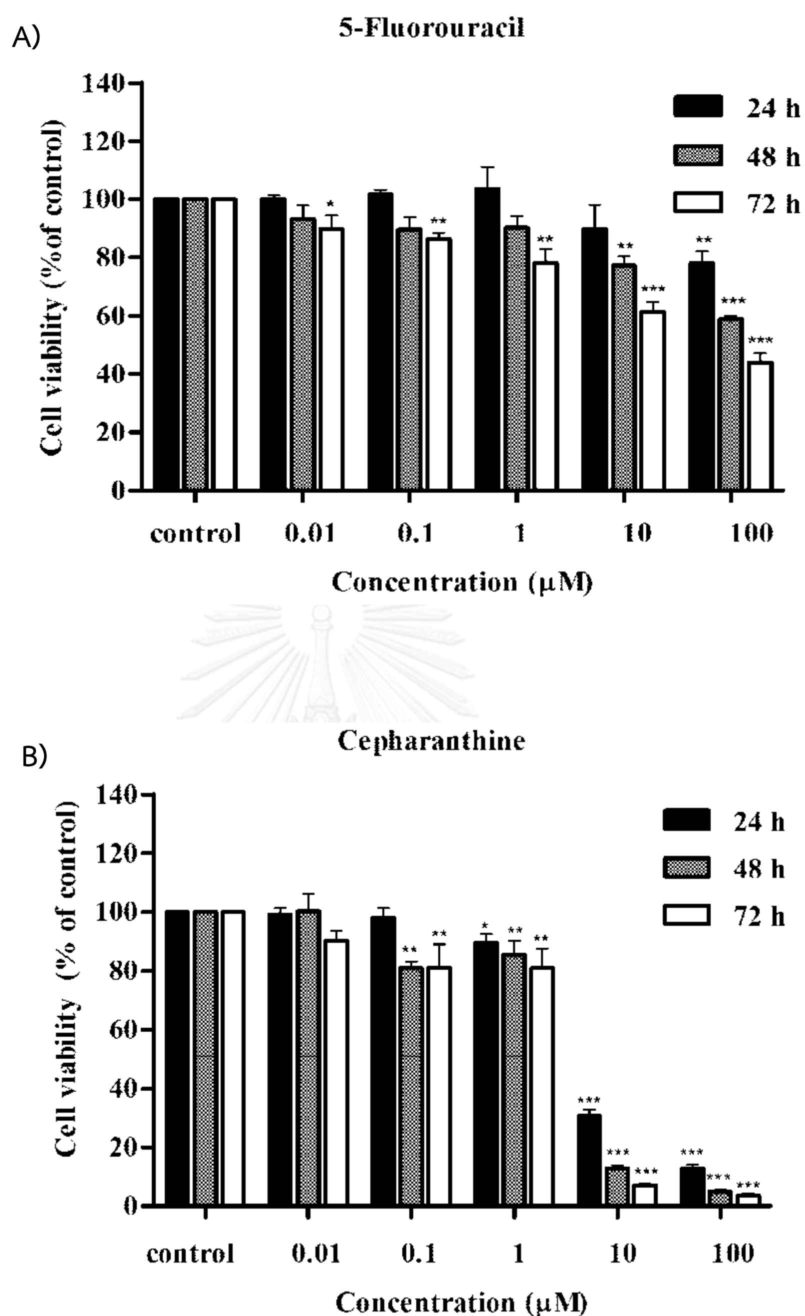

Figure 10. The cytotoxicity effects of 5-fluorouracil (5-FU) and cepharanthine (CEP) in HT-29 cells. The cells were treated with different concentrations of 5-FU (A) or CEP (B) for 24, 48, or 72 h and cell viability was evaluated by MTT assay. Data are presented as mean ± SEM (n=3). $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ indicated statistically significant difference when compared with vehicle control (0.2% DMSO).

Table 3. The IC_{50} values of 5-FU and CEP on HT-29 cells after 24, 48, or 72 h of incubation.

Time (h)	IC_{50} values (μM)	
	5-FU	CEP
24	N/A	7.10 ± 0.11
48	N/A	5.16 ± 0.18
72	69.83 ± 9.67	4.63 ± 0.52

N/A (Not applicable)

The effect of CEP on cytotoxicity of 5-FU in HT-29 cells.

The effects of pre- and co-treatment of CEP on cytotoxicity of 5-FU in colorectal cancer cells were therefore evaluated. For the pre-treatment condition, the HT-29 cells were pretreated with CEP at a concentration of 2.5 μM, a non-toxic concentration, for 4 h, followed by the incubation with various concentrations (0.01, 0.1, 1, 10, 100 μM) of 5-FU for 48 h. And for the co-treatment condition, the cells were treated with various concentrations of 5-FU (0.01, 0.1, 1, 10, 100 μM) in the presence or absence of CEP at a concentration of 2.5 μM for 48 h. Cell viability was then assessed. As shown in Figure 11A, pre-treatment of HT-29 cells with 2.5 μM of CEP for 4 h significantly increased cell toxicity of 5-FU when compared with treatment with 5-FU alone. Similar results were observed when the cells were treated with CEP in combination with 5-FU (Figure 11B). Therefore, mechanistic studies underlying potentiating effect of CEP on anticancer activity of 5-FU were further investigated in the co-treatment condition.

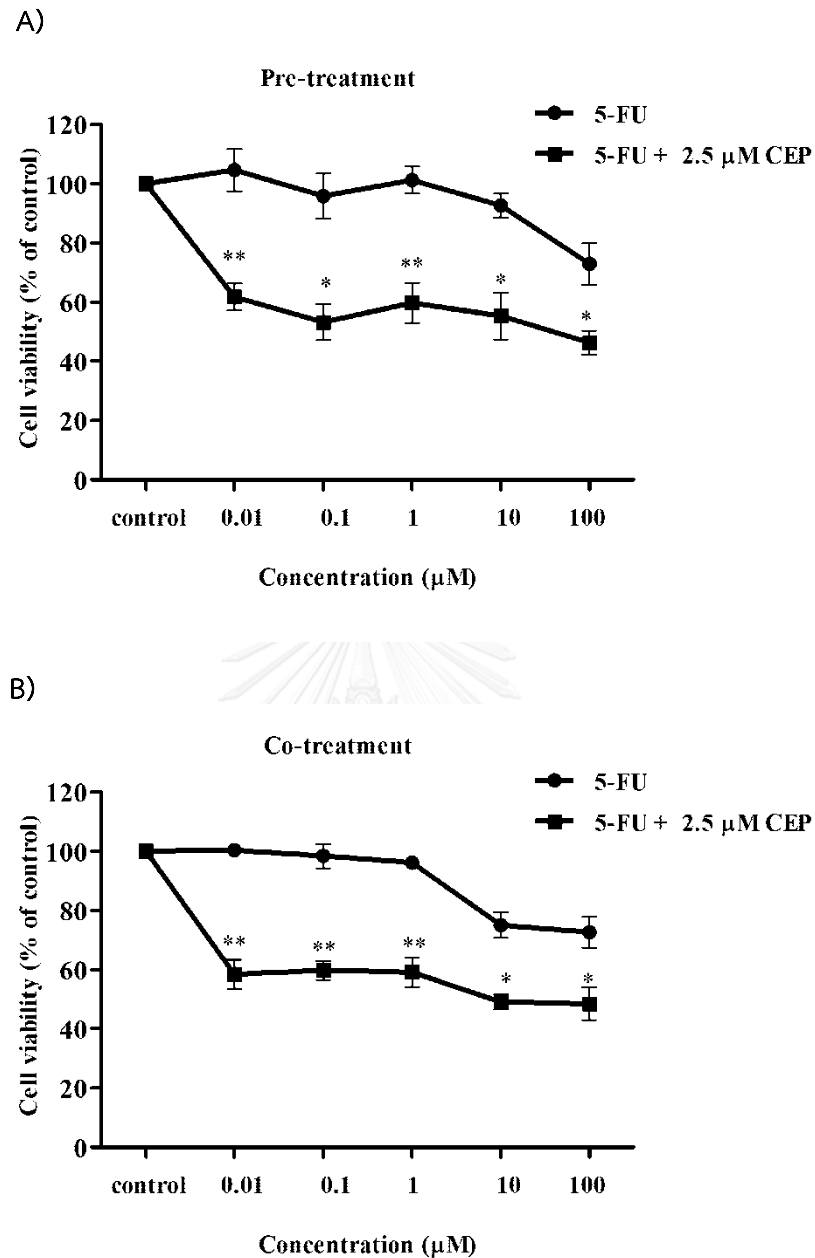

Figure 11. The effect of pre- or co-treatment with CEP on cytotoxicity of 5-FU at 48 h of incubation. The cells were pre- (A) and co-treated (B) with 2.5 µM of CEP and various concentrations of 5-FU for 48 h and cell viability was determined using MTT assay. Data are presented as mean ± SEM (n=3). $^*P < 0.05$ and $^{**}P < 0.01$ indicated statistically significant difference when compared with 5-FU alone.

The effect of CEP or 5-FU alone or combination of CEP and 5-FU on apoptosis induction in HT-29 cells.

Induction of apoptosis is one of the important mechanisms underlying anticancer activities of several chemotherapeutic agents, including 5-FU. The effect of CEP on apoptosis-inducing effect of 5-FU in HT-29 cells was therefore determined. The cells were treated with either 2.5 μM of CEP, 50 μM of 5-FU, or 2.5 μM of CEP in combination with 50 μM of 5-FU for 24 or 48 h, and Annexin V and PI staining was performed to differentiate cells at different apoptosis stages using flow cytometry. As shown in Figures 12A and B, treatment with 50 μM of 5-FU for 48 h increased early apoptotic cell death in HT-29 cells by approximately 3 fold with respect to untreated cells. In contrast to 5-FU, as compared with control group, treatment with 2.5 μM of CEP induced cancer cells undergo necrosis and late apoptosis approximately 3 times at 48 h of incubation. However, it is worth mentioning that number of apoptotic cells following treatment with 2.5 μM of CEP in combination with 50 μM of 5-FU were not significantly different from that upon exposure with 5-FU alone (Figure 13). On the other hand, HT-29 cells undergoing necrosis following the combination treatement was almost double those treated with 5-FU alone. Taken together, these results suggest that, in HT-29 cells, CEP induces cells undergoing necrosis and apoptosis.

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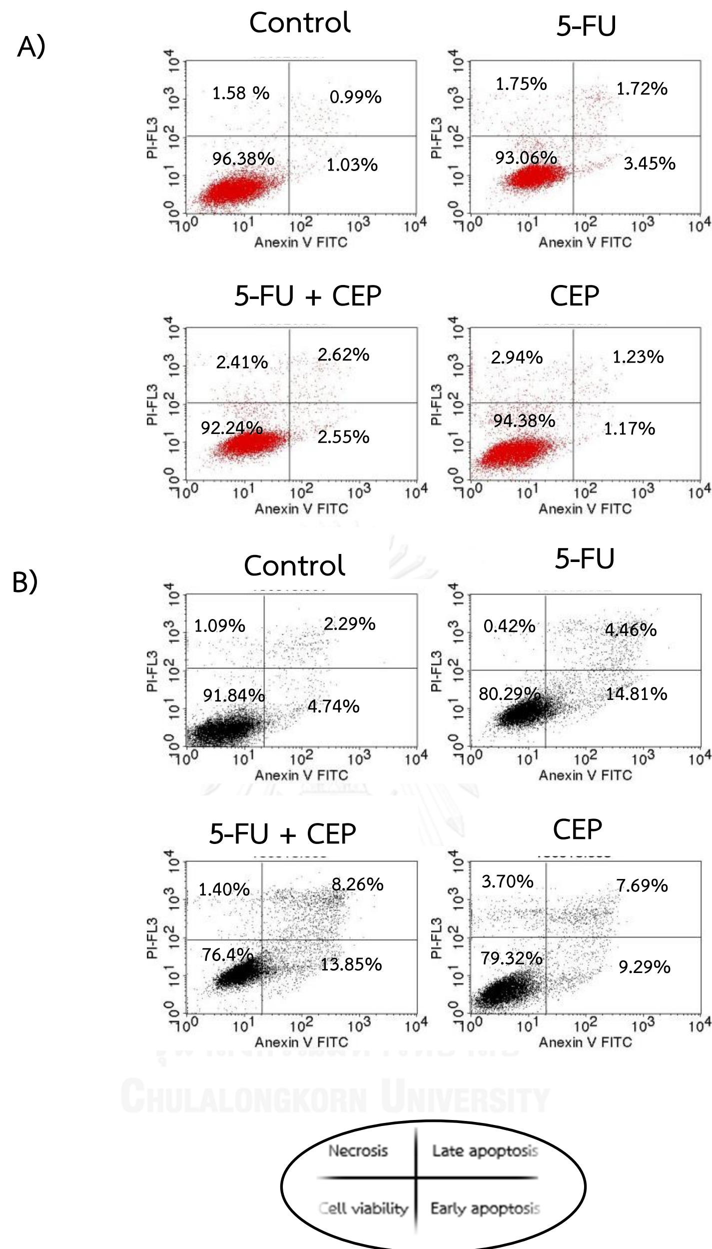

Figure 12. The effects of CEP and/or 5-FU on apoptotic cell death in HT-29 cells. The cells were treated with either 2.5 μM of CEP or 50 μM of 5-FU alone or 2.5 μM of CEP combined with 50 μM of 5-FU for 24 h and 48 h. Apoptosis and necrosis were determined by Annexin V-FITC and PI staining followed by flow cytometry analysis. Representative cytograms of cell apoptosis analysis of HT-29 cells for 24 (A) or 48 h (B).

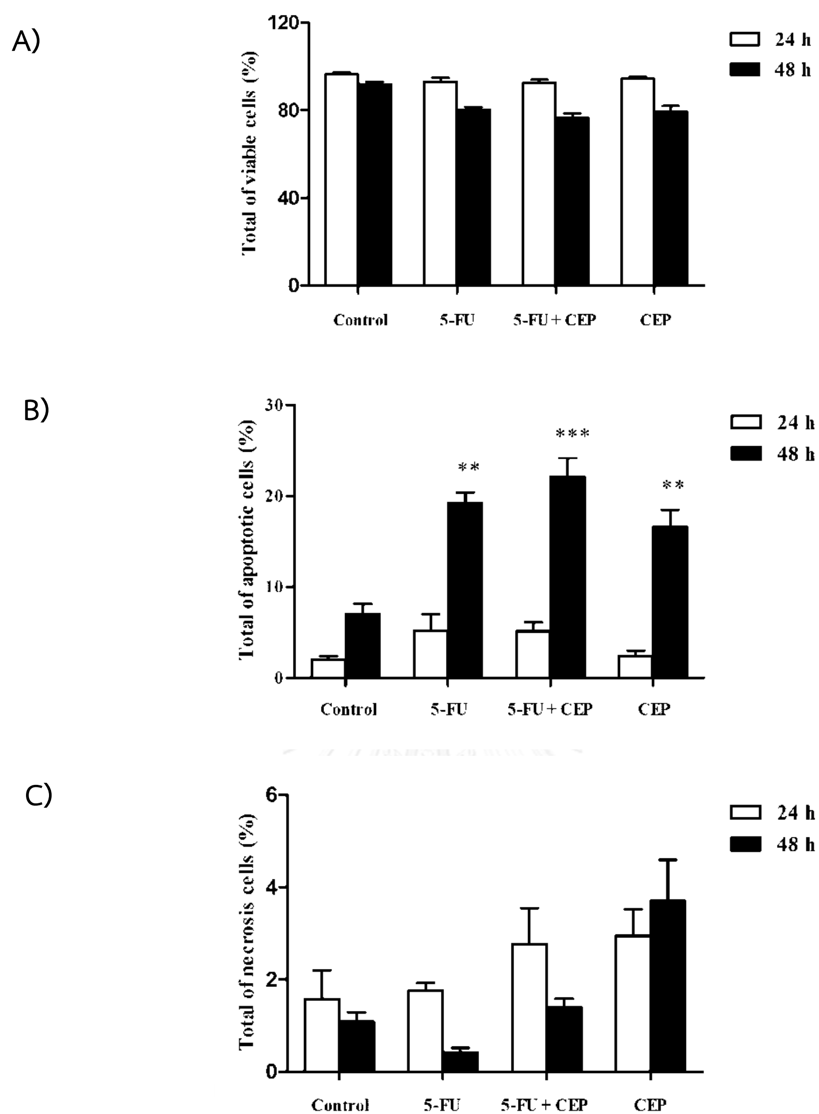

Figure 13. The percent of total of viable cells (A), apoptosis cells (B) and necrosis cells (C) for 24 or 48 h. Data are presented mean ± SEM (n=3). The number of events assessed for each condition was 10,000. $^{**}P < 0.01$ and $^{***}P < 0.001$ indicated statistically significant difference when compared with vehicle control (0.2% DMSO).

The effect of CEP or 5-FU alone or combination of CEP and 5-FU on the expression levels of Bcl-2 family genes in HT-29 cells.

Chemotherapy induces cancer cell to undergo apoptosis, primary through the intrinsic pathway. Thus, the expression levels of anti-apoptotic, Bcl-2 and Bcl-xL, and pro-apoptotic, Bax and Bak, were evaluated following treatment with either CEP or 5-FU alone or CEP in combination with 5-FU. As illustrated in Figure 14A, 2.5 μM of CEP alone did not alter the *Bcl-2* mRNA level but the *Bcl-2* gene expression was significantly decreased after treatment with 50 μM of 5-FU alone. Interestingly, co-treatment of cells with CEP 2.5 μM and 5-FU 50 μM could further down-regulated the *Bcl-2* mRNA level by approximately 3 fold with respected to 5-FU-treated cells. In contrast to *Bcl-2*, the *Bcl-xL* gene expression was unaffected following treatment with either CEP or 5-FU alone or CEP combined with 5-FU (Figure 14B). For pro-apoptotic genes, although treatment with CEP did not change expression of *Bax* gene compared to vehicle control group, a significant down-regulation of *Bax* mRNA expression was detected following treatment with 5-FU alone or in combination with CEP (Figure 15A). Similar to *BAX*, mRNA level of the other pro-apoptotic gene, *Bak* was significantly decreased when cells were treated with 5-FU alone, whereas treatment with either CEP alone or in combination with 5-FU did not alter the *Bak* gene expression (Figure 15B). Taken together, it is likely that down-regulation of *Bcl-2* mRNA expression may be related to chemopotentiating activity of CEP.

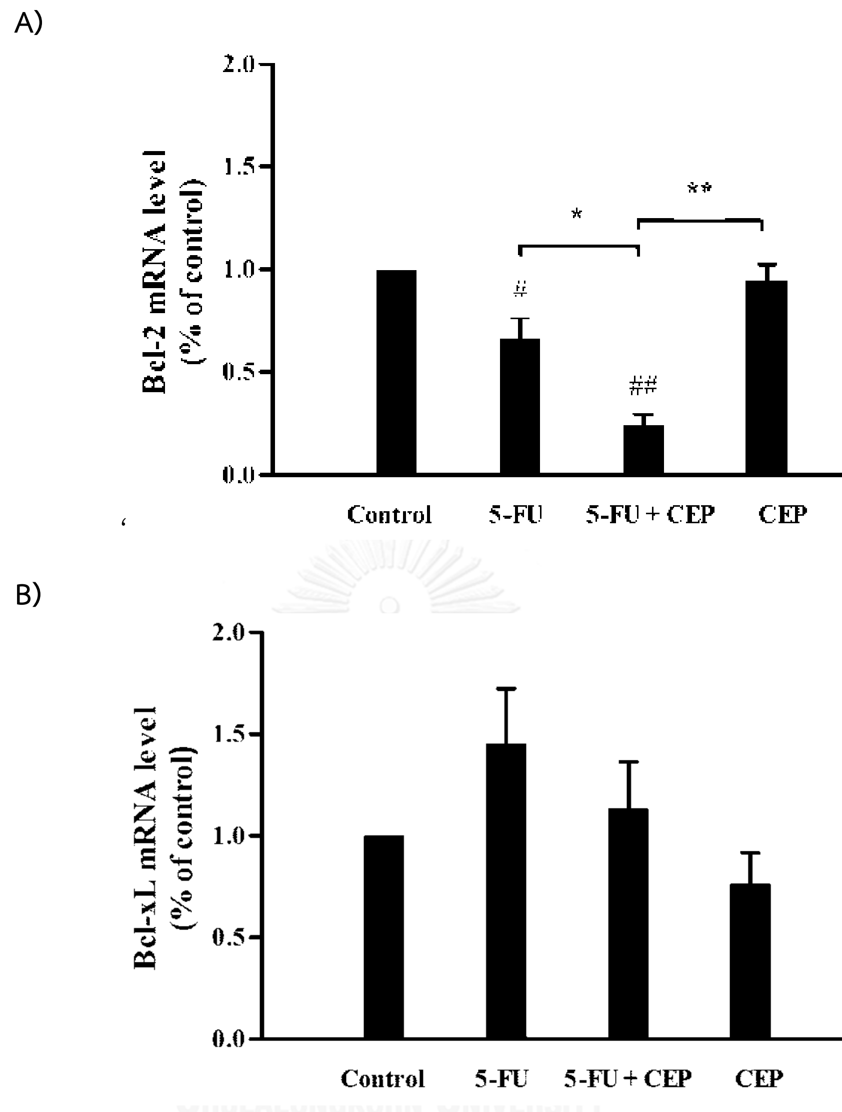

Figure 14. The effect of CEP and/or 5-FU on the expression of anti-apoptotic genes in HT-29 cells. Cells were treated with 2.5 μM of CEP alone, 50 μM of 5-FU alone or 2.5 μM of CEP in combination with 50 μM of 5-FU for 24 h and the expression levels of anti-apoptotic genes, including *Bcl-2* (A) and *Bcl-xL* (B) were analyzed by real-time RT-PCR analysis. Data were performed as means ± SEM (N=3). $^{\#}P<0.05$ and $^{\#\#}P<0.01$ indicated statistically significant difference when compared to control (0.2 % DMSO). $^{*}P<0.05$ and $^{**}P<0.01$ indicated statistically significant difference when compared to a single treatment.

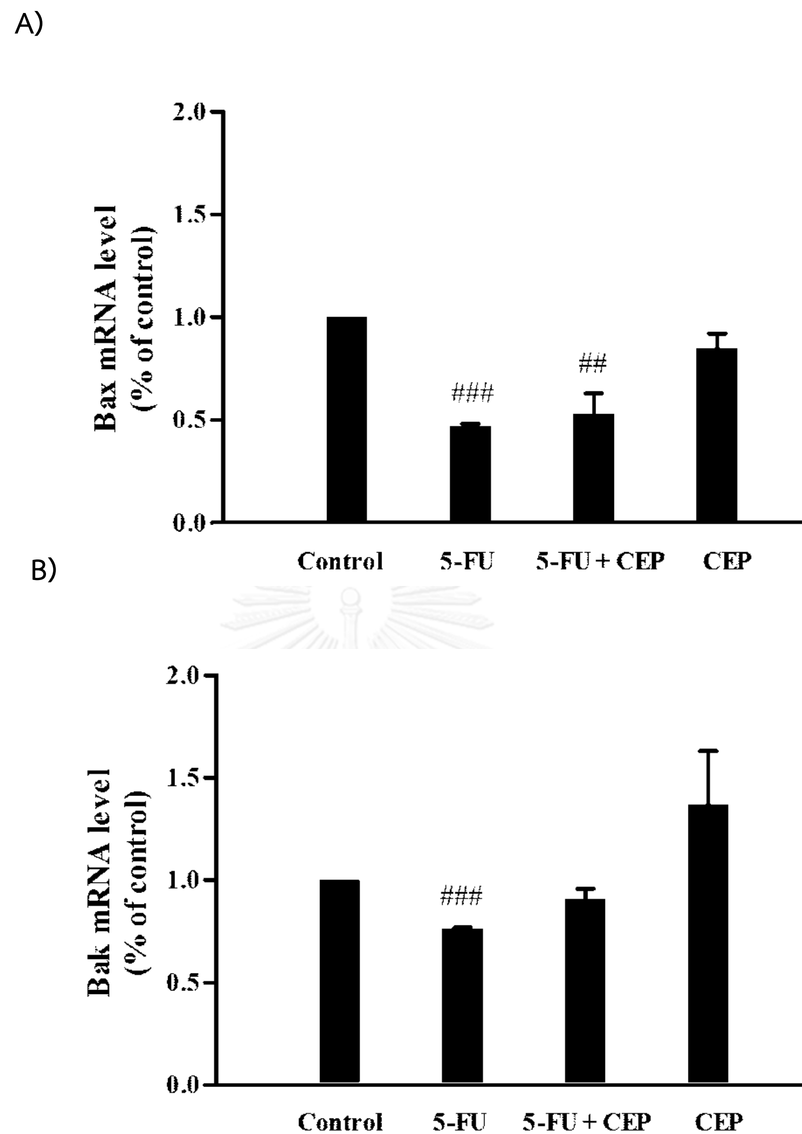

Figure 15. The effect of CEP and/or 5-FU on the expression of pro-apoptotic genes in HT-29 cells. Cells were treated with either 2.5 μM of CEP or 50 μM of 5-FU alone or 2.5 μM of CEP in combination with 50 μM of 5-FU for 24 h and the expression levels of anti-apoptotic genes, including *Bax* (A) and *Bak* (B) were analyzed by real-time RT-PCR analysis. Data were performed as means ± SEM (N=3). The expression level of control was set at 1 and the relative expression levels of other group were assessed. $^{\#}P<0.05$ and $^{\#\#}P<0.01$ indicated statistically significant difference when compared to control (0.2 % DMSO).

The effect of CEP or 5-FU alone or combination of CEP and 5-FU on cell cycle distribution of HT-29 cells.

To understand the mechanisms underlying the chemopotentiating effets of 5-FU by CEP in HT-29 cells, cell cycle distribution was analyzed. The cells were treated with either 2.5 μM of CEP, 50 μM of 5-FU, or 2.5 μM of CEP in combination with 50 μM of 5-FU for 24 h. Cellular DNA content at different stages of the cell cycle was tested by flow cytometry after staining with propidium iodide (PI). As shown in the Figure 16, treatment with either CEP or 5-FU alone or CEP in combination with 5-FU significantly induced accumulation of HT-29 cells in the S phase which was associated with a reduction of cells accumulated in the G1 phase when compared with untreated cells. Interestingly, treatment with 5-FU alone resulted in cell cycle arrest predominantly at the S phase whereas cells were accumulated at the G1 phase upon exposure to CEP alone. It however should be noted that numbers of cells at the S phase were similar to those at the G1 phase following co-treatment of cells with CEP and 5-FU. These results demonstrated that treatment with either CEP or 5-FU alone or CEP in combination with 5-FU could alter cell cycle distribution in different manner.

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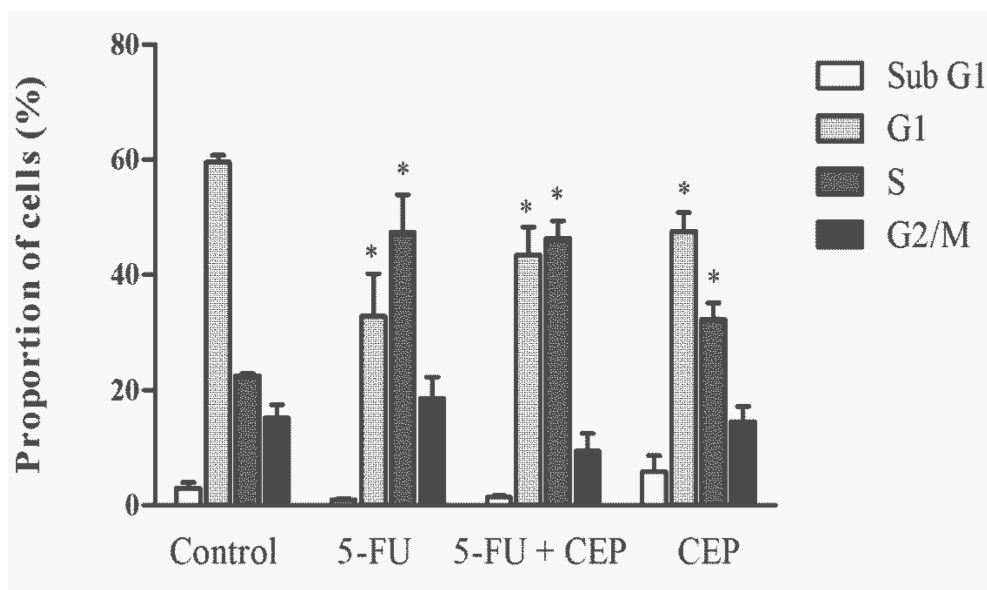

Figure 16. The effect of CEP and/or 5-FU on cell cycle distribution of HT-29 cells. The cells were treated with either 2.5 µM of CEP or 50 µM of 5-FU alone or a combination of 2.5 µM CEP and 50 µM 5-FU for 24 h. After treatment, the cells were harvested, stained with PI and evaluated by flow cytometry. The percentage of cells in sub-G1, G1, S and G2/M phases are reported as means ± SEM. (n=3). The number of events assessed for each condition was 10,000. *$P < 0.05$ indicated statistically significant difference compared with vehicle control (0.2% DMSO).

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Effect of CEP on gene expression and function of drug efflux transporters in HT-29 cells.

It is well-known that up-regulation of efflux membrane transporters can decrease cellular drug level, leading to failure of cancer chemotherapy. The influence of CEP on the mRNA levels of three major drug efflux transporters of the ABC family, including *P-gp* (*MDR1*), *MRP1* and *BCRP* was determined. The cells were treated with various concentrations of CEP (1.25, 2.5, 5 μM) and the mRNA expression levels were evaluated using quantitative real-time RT-PCR. As illustrated in Figure 17, CEP did not significantly change the *P-gp* mRNA level. Similar to P-gp, the expression of *MRP1* and *BCRP* genes were not affected by CEP treatment (Figure 18 and 19).

Effect of CEP on activity of drug efflux transporters was then investigated using drug accumulation analysis. As shown in Figure 20, cyclosporine A, a P-gp inhibitor significantly enhanced accumulation of rhodamine, a fluorescent substrate of P-gp whereas CEP did not alter rhodamine level in HT-29 cells compared to HBSS-treated cells. Similarly, 5(6)-carboxyfluorescein diacetate (CFDA, a fluorescent substrate of MRP1) level in cells treated with MK571, a MRP1 inhibitor, was approximately doubled that in untreated cells while there was no significant change in CFDA level following CEP treatment (Figure 21). Similar results were also observed with BCRP transporter (Figure 22). These results indicated that neither gene expression nor function of the most important drug efflux transporters, P-gp, MRP1 and BCRP was altered by CEP treatment.

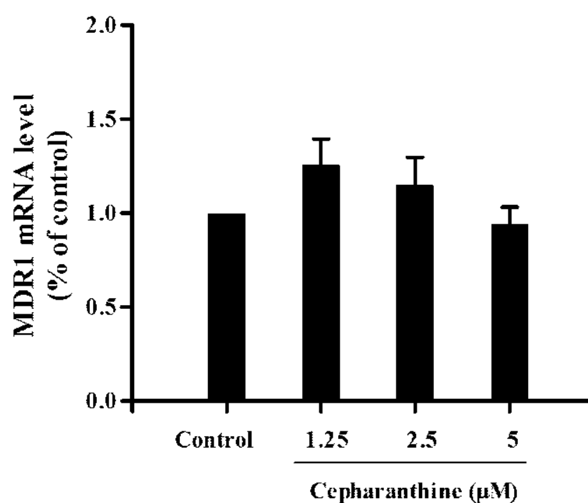

Figure 17. The effect of CEP on mRNA level of MDR1 transporter in HT-29 cells. The cells were treated with CEP (1.25, 2.5, 5 μM) and expression of MDR1 gene was analyzed using quantitative real-time RT-PCR. Data were presented as means ± SEM. (n=3). Each experimental group was compared with control group (0.2 % DMSO).

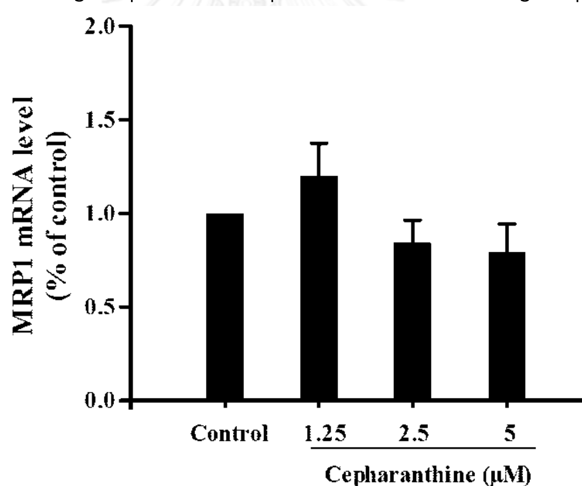

Figure 18. The effect of CEP on mRNA level of MRP1 transporter in HT-29 cells. The cells were treated with CEP (1.25, 2.5, 5 μM) and expression of MRP1 gene was analyzed using quantitative real-time RT-PCR. Data were presented as means ± SEM. (n=3). Each experimental group was compared with control group (0.2 % DMSO).

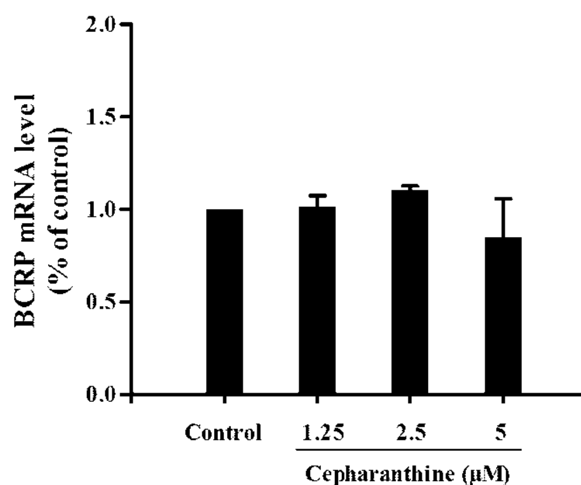

Figure 19. The effect of CEP on mRNA level of BCRP transporter in HT-29 cells. The cells were treated with CEP (1.25, 2.5, 5 µM) and expression of BCRP gene was analyzed using quantitative real-time RT-PCR. Data were presented as means ± SEM. (n=3). Each experimental group was compared with control group (0.2 % DMSO).

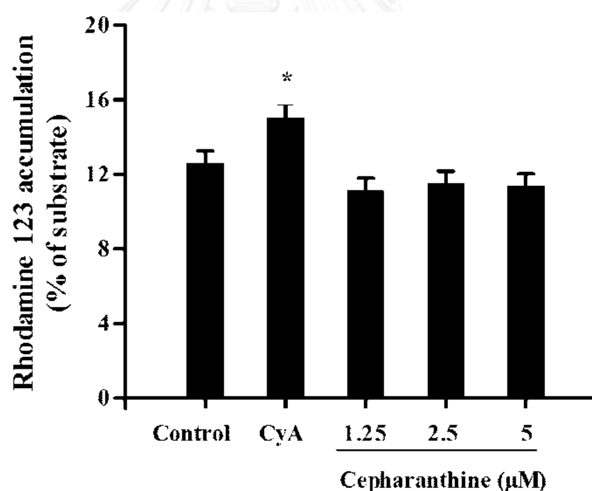

Figure 20. The effect of CEP on activity of P-gp drug efflux transporter in HT-29 cells. The cells were treated with 1.25, 2.5, or 5 µM of CEP, 20 µM of cyclosporine A (P-gp inhibitor) or HBSS (negative control) for 30 min. Rhodamine 123 as a specific fluorescent substrate of P-gp was added into each well for 60 min. Drug accumulation was then assessed. Data were presented as means ± SEM. (n=3). Each experimental group was compared with control group (HBSS).

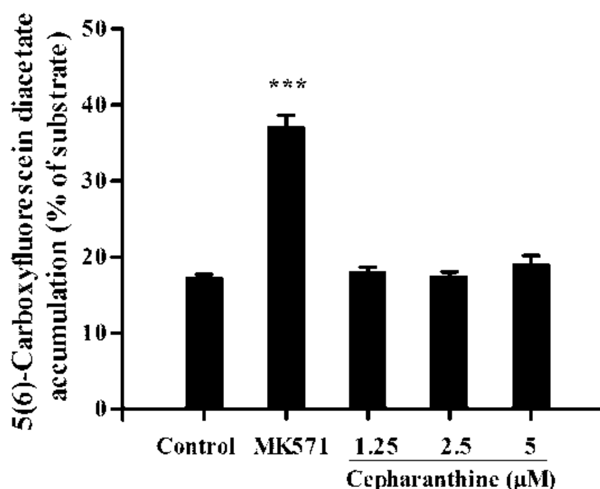

Figure 21. The effect of CEP on activity of MRP1 drug efflux transporters in HT-29 cells. The cells were treated with 1.25, 2.5, or 5 μM of CEP, 100 μM of MK571 (MRP1 inhibitor) or HBSS (negative control) for 30 min. CFDA as a specific fluorescent substrate of MRP1 was added into each well for 60 min. Drug accumulation was then assessed. Data were presented as means ± SEM. (n=3). Each experimental group was compared with control group (HBSS).

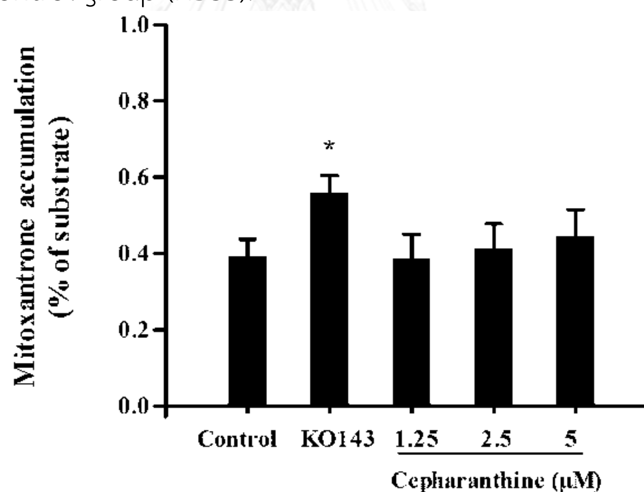

Figure 22. The effect of CEP on activity of BCRP drug efflux transporter in HT-29 cells. The cells were treated with 1.25, 2.5, or 5 μM of CEP, 20 μM of Ko143 (BCRP inhibitor) or HBSS (negative control) for 30 min. Mitoxantrone as a specific fluorescent substrate of BCRP was added into each well for 60 min. Drug accumulation was then assessed. Data were presented as means ± SEM. (n=3). Each experimental group was compared with control group (HBSS).

CHAPTER V

DISCUSSION AND CONCLUSION

Colorectal cancer (CRC) is one of the most frequently diagnosed cancers in both man and women worldwide. Drugs for CRC treatment are chemotherapeutic agents such as 5-fluorouracil (5-FU), oxaliplatin, irrinotacan and capecitabine or target-based agents such as bevacizumab, cetuximab and panitumumab (34, 35). Of all anticancer agents, 5-FU has been widely used as a first-line drug in both metastatic and advanced CRC. Clinical studies demonstrated that the combination chemotherapy regimens are more effective than single agent and it is a standard treatment for advanced CRC (4, 5). Nowadays, the drugs of CRC have not improved patient survival due to drug resistance or severe side effect. Therefore, non-toxic compounds with potentiating activities are urgently needed for use in combination with commonly used chemotherapeutic agents.

Cepharanthine (CEP) has been approved by the Japanese Ministry of Heath to treat several diseases, including radiation-induced leucopenia in patients with breast, lung and ovarian cancer without any serious side effects (14-16). Interestingly, several pre-clinical studies revealed that CEP exerts antitumor activity against various type of cancers including lung carcinoma, leukemia, cholangiocarcinoma, oral squamous cell carcinoma, hepatocellular carcinoma, lymphoma, osteosarcoma and nasopharyngeal carcinoma (20-23, 74). Moreover, CEP was shown to potentially enhance the sensitivity of cancer cells to radiation and many anticancer drugs such as doxorubicin, docetaxel and vinca alkaloids (19, 24-26). Recently, it has been reported that CEP was able to control the growth of p53-mutant HT-29 colorectal cancer cells which are relatively resistance to commonly used anticancer drugs (27). Thus, the goal of this study was to investigate whether CEP could enhance anticancer activity of 5-FU in HT-29 cancer cells. The results in the present study clearly illustrated that either pre- or co-treatment of 2.5 μM CEP significantly potentiate cytotoxicity of 5-FU (Figure 11). In addition to commonly used chemotherapeutic drugs, Ono M et al. reported that CEP enhanced anti-proliferative effect of interferon-gamma (IFN-γ) in

different human tumor cells lines (RPMI 4788, PC 10, HeLa, ZR-75-1) (81). Similarly, increases in cytotoxic activity of onconase (Onc), a ribonuclease isolated from oocytes or early embryos of the Northern Leopard frog, by CEP were detected in human promyelocytic leukemia (HL-60), histiomonocytic lymphoma (U937), multiple myeloma (RPMI-8228), prostate carcinoma (DU 145) and prostate adenocarcinoma (LNCaP) (89). Although studies in human malignant glioma cell lines revealed that CEP at concentrations above 15 μg/ml could increase cytotoxicity of nimustine hydrochloride (ACNU), this compound at low concentrations (1 to 10 μg/ml) was found to promote cancer cell proliferation (80). However, this findings demonstrated that CEP was able to control the growth of HT-29 colorectal cancer cells more effectively than 5-FU (Figure 10). Therefore, it is possible that anticancer effect of CEP may depend on cell type.

Most chemotherapeutic agents induce cancer cells to undergo apoptosis (90). The present study illustrated that 5-FU significantly induced apoptotic cell death in HT-29 cells, CEP however did not significantly enhance apoptosis-induced effect of 5-FU (Figure 12). Several lines of evidence indicated that CEP induces apoptotic cell death in several types of cancer (20, 21, 26, 79, 82). CEP was shown to induce cholangiocarcinoma and primary effusion lymphoma to undergo apoptosis via inhibition of NF-κB (21, 76). Additional studies in a human hepatocellular carcinoma cell line indicated that apoptosis-inducing effect of CEP is mediated through production of reactive oxygen species (ROS), activation of JNK1/2, MAPK p38, ERk p44/42 and downregulation of Akt (23). It has also been reported that treatment with CEP in combination with herbimycin A was able to activate apoptotic cell death by down-regulation of Bcr-Abl and Akt protein (19). The results of this study were however somewhat different from many previous studies. CEP was found to induce HT-29 cells undergoing necrosis and apoptosis in the present study. It was previously reported that HT-29 cells carry APC, BRAF, PIK3CA and p53 mutations (91). Mutations of p53 and BRAF have been shown to be associated with apoptosis resistance (92). However, 5-FU induced cells undergo apoptosis had not associated with p53 status in CRC (58). Thus, it is possible that genetic mutations may modulate apoptotic response of HT-29 cells to CEP.

It is commonly known that both pro-apoptotic proteins (Bax and Bak) and anti-apoptotic proteins (Bcl-2 and Bcl-xL) of the Bcl-2 family regulate sensitivity of cancer cells to radiation and anticancer drugs, including 5-FU (58, 93). Previous studies demonstrated 5-FU-resistant colorectal cancer cells upon long-term exposure to the drug displayed high level of Bcl-2 and Bcl-xL proteins but low level of Bax protein (60). Similarly, high Bcl-2 expression and decreased spontaneous apoptosis were found in colorectal cancer cell line with p53 mutation (94). In contrast to Bcl-2, down-regulation of Bax as well as inactivation of p53 allowed cancer cells to evade apoptosis, resulting in poor treatment outcome in most colorectal cancer cell lines studied (95, 96). It was reported that treatment with CEP inhibited the expression of *Bcl-xL*, target genes of STAT3, in human osteosarcoma SaOS2 cells (20). CEP was also found to exert apoptosis-inducing effect by upregulation of Bax, downregulation of Bcl-2 and activation of caspase-3 and PARP in non-small lung cancer cells (77). Moreover, CEP has been shown to enhance sensitivity of HeLa cells to radiation by decreasing expression of STAT3, COX-2, c-myc and Bcl-2 (82). Similarly, the results of this study revealed that treatment of HT-29 cells with 5-FU significantly decreased *Bcl-2* mRNA level and co-treatment of the cells with 5-FU in combination with CEP could further down-regulate *Bcl-2* gene expression (Figure 14A). Taken together, it is likely that decrease in *Bcl-2* mRNA level may be related to chemopotentiation of 5-FU by CEP.

In addition, suppression of cancer cell growth can also be associated with blocking of cell cycle progression (97). Previously, Flis S and Splawinski J reported that 5-FU significantly caused cell accumulation at the S phase in several colorectal cancer cell lines (98). Similarly, the present study revealed that treatment of HT-29 cells with 5-FU alone significantly increased the numbers of cells in the S phase while cells were accumulated predominantly in the G1 phase upon exposure to CEP alone (Figure 16). Therefore, a combination treatment resulted in cell accumulation in both G1 and S phase. Previously, CEP was shown to cause cell cycle arrest at G1 phase in different cancer cells, including adenosquamous cell carcinoma, cholangiocarcinoma and myeloma (17, 21, 75, 79). Mechanistic studies revealed that CEP disrupt cell cycle progression by downregulating expression of cyclin D1, cyclin-

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dependent kinase (CDK)-6 and c-myc as well as inhibiting $p21^{WAF1}$ expression (17). Additional studies to determine effect of a combination treatment of CEP and 5-FU on cell cycle regulators are warranted.

The ABC efflux transporters such as P-gp, MRP1 and BCRP play a critical role in drug resistance by extruding drug out of the cell, limiting drug efficacy. Previously, CEP was found to be an effective agent for the reversal of P-gp mediated resistance to STI571 in P-gp-overexpressed K562/ MDR cell (99). Further mechanistic studies illustrated that CEP decreased the expression of P-gp in a time- and concentration-dependent manner through activation of c-Jun/JNK pathway in K562/ADR cells (84). However, Abe T et al. showed that CEP only partially reversed P-gp-mediated drug resistance but did not reverse MRP-mediated drug resistance in both T98G and IN500 human glioma cell lines overexpressing MDR or MRP (99). Additionally, it was demonstrated that CEP enhanced sensitivity of daunomycin by binding to phosphatidylserine in the plasma membrane and perturbing membrane function but the compound did not alter the cellular uptake or influx of anticancer agents in the KB human cancer cells (70). This study showed that CEP did not affect both mRNA expression level and activity of P-gp, MRP1 and BCRP (Figure 17-22), suggesting that chemopotentiation of 5-FU by CEP in HT-29 colorectal cancer cells may not be associated with three major drug efflux transporters. Previous studies revealed that CEP could reverse paclitaxel resistance in human embryonic kidney 293 cells by inhibiting MRP7 drug efflux transporters (85). It was also demonstrated that MRP3, MRP4, and MRP5 were up-regulated in 5-fluorouracil-resistant cells and MRP5 was found to contribute to 5-FU resistance in pancreatic carcinoma cells (100). Therefore, it is likely that, CEP may inhibit the expression and function of other members of ABC drug efflux transporters such as MRP-7 and MRP-5, leading to increased intracellular drug accumulation and enhancing drug efficacy. Additional studies to determine effect of CEP on other efflux transporters and effect of CEP at higher concentrations on P-gp, MRP-1 and BCRP require further investigation.

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Conclusion

This study clearly illustrated that CEP could effectively potentiate anticancer activity of 5-FU in human HT-29 colorectal cancer cells, which are normally insensitive to the drug. Treatment of CEP and 5-FU was able to significantly alter cell cycle progression. In addition, expression of anti-apoptotic *Bcl-2* gene was reduced by 5-FU and further decreased following treatment with 5-FU in combination with CEP. Therefore, this investigation suggests that combination of CEP with 5-FU can potentially be used as an alternative strategy to increase anticancer activity of the drug, thereby decreasing dose while increasing safety profile of the chemotherapeutic drug for colorectal cancer treatment.

REFERENCES

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. CA: a cancer journal for clinicians. 2013;63(1):11-30.

2. T NCI. HOSPITAL BASED CANCER REGISTRY ANNUAL REPORT. 2014.

3. Shukla RK. Recent advances in oral anticancer agents for colon cancer. Future Oncology (London, England). 2013;9(12):1893-908.

4. Johnston PG, Kaye S. Capecitabine: a novel agent for the treatment of solid tumors. Anti-cancer Drugs. 2001;12(8):639-46.

5. Giacchetti S, Perpoint B, Zidani R, Le Bail N, Faggiuolo R, Focan C, et al. Phase III multicenter randomized trial of oxaliplatin added to chronomodulated fluorouracil-leucovorin as first-line treatment of metastatic colorectal cancer. Journal of Clinical Oncology : official journal of the American Society of Clinical Oncology. 2000;18(1):136-147.

6. Zhang N, Yin Y, Xu SJ, Chen WS. 5-Fluorouracil: mechanisms of resistance and reversal strategies. Molecules (Basel, Switzerland). 2008;13(8):1551-69.

7. Borst P, Evers R, Kool M, Wijnholds J. A family of drug transporters: the multidrug resistance-associated proteins. Journal of the National Cancer Institute. 2000;92(16):1295-302.

8. Katayama K, Noguchi K, Sugimoto Y. Regulations of P-Glycoprotein/ABCB1/MDR1 in Human Cancer Cells. New Journal of Science. 2014;2014:10.

9. Cole SP, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, et al. Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. Science (New York, NY). 1992;258(5088):1650-4.

10. Choi JH, Lim HY, Joo HJ, Kim HS, Yi JW, Kim HC, et al. Expression of multidrug resistance-associated protein1,P-glycoprotein, and thymidylate synthase in gastric cancer patients treated with 5-fluorouracil and doxorubicin-based adjuvant chemotherapy after curative resection. British Journal of Cancer. 2002;86(10):1578-85.

11. Yu ZW, Zhao P, Liu M, Dong XS, Tao J, Yao XQ, et al. Reversal of 5-flouroucial resistance by adenovirus-mediated transfer of wild-type p53 gene in multidrug-resistant human colon carcinoma LoVo/5-FU cells. World Journal of Gastroenterology : WJG. 2004;10(13):1979-83.

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12. Lu F, Hou YQ, Song Y, Yuan ZJ. TFPI-2 downregulates multidrug resistance protein in 5-FU-resistant human hepatocellular carcinoma BEL-7402/5-FU cells. Anatomical Record. 2013;296(1):56-63.

13. Yuan J, Lv H, Peng B, Wang C, Yu Y, He Z. Role of BCRP as a biomarker for predicting resistance to 5-fluorouracil in breast cancer. Cancer Chemotherapy and Pharmacology. 2009;63(6):1103-10.

14. Ohta T, Morita K. Effect of cepharanthin on radiotherapy induced leukopenia. Rinsho Hoshasen Clinical Radiography. 1990;35(4):471-4.

15. Morita K, Nakamura M, Nagamachi M, Kishi T, Miyachi Y. Seventeen cases of alopecia areata: combination of SADBE topical immunotherapy with other therapies. The Journal of Dermatology. 2002;29(10):661-4.

16. Sato T, Ohnishi ST. In vitro anti-sickling effect on cepharanthine. European Journal of Pharmacology. 1982;83(1-2):91-5.

17. Harada K, Bando T, Yoshida H, Sato M. Characteristics of antitumour activity of cepharanthin against a human adenosquamous cell carcinoma cell line. Oral Oncology. 2001;37(8):643-51.

18. Wu J, Suzuki H, Zhou YW, Liu W, Yoshihara M, Kato M, et al. Cepharanthine activates caspases and induces apoptosis in Jurkat and K562 human leukemia cell lines. Journal of Cellular Biochemistry. 2001;82(2):200-14.

19. Harada K, Ferdous T, Itashiki Y, Takii M, Mano T, Mori Y, et al. Effects of cepharanthine alone and in combination with fluoropyrimidine anticancer agent, S-1, on tumor growth of human oral squamous cell carcinoma xenografts in nude mice. Anticancer Research. 2009;29(4):1263-70.

20. Chen Z, Huang C, Yang YL, Ding Y, Ou-Yang HQ, Zhang YY, et al. Inhibition of the STAT3 signaling pathway is involved in the antitumor activity of cepharanthine in SaOS2 cells. Acta Pharmacologica Sinica. 2012;33(1):101-8.

21. Seubwai W, Vaeteewoottacharn K, Hiyoshi M, Suzu S, Puapairoj A, Wongkham C, et al. Cepharanthine exerts antitumor activity on cholangiocarcinoma by inhibiting NF-kappaB. Cancer Science. 2010;101(7):1590-5.

22. Harada K, Ferdous T, Itashiki Y, Takii M, Mano T, Mori Y, et al. Cepharanthine inhibits angiogenesis and tumorigenicity of human oral squamous cell carcinoma cells by suppressing expression of vascular endothelial growth factor and interleukin-8. International Journal of Oncology. 2009;35(5):1025-35.

23. Biswas KK, Tancharoen S, Sarker KP, Kawahara K, Hashiguchi T, Maruyama I. Cepharanthine triggers apoptosis in a human hepatocellular carcinoma cell line (HuH-

จุฬาลงกรณ์มหาวิทยาลัย
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7) through the activation of JNK1/2 and the downregulation of Akt. FEBS Letters. 2006;580(2):703-10.

24. Nakajima A, Yamamoto Y, Taura K, Hata K, Fukumoto M, Uchinami H, et al. Beneficial effect of cepharanthine on overcoming drug-resistance of hepatocellular carcinoma. International Journal of Oncology. 2004;24(3):635-45.

25. Hotta T, Tanimura H, Yamaue H, Iwahashi M, Tani M, Tsunoda T, et al. Modulation of multidrug resistance by cepharanthine in fresh human gastrointestinal tumor cells. Oncology. 1997;54(2):153-7.

26. Zahedi P, De Souza R, Huynh L, Piquette-Miller M, Allen C. Combination drug delivery strategy for the treatment of multidrug resistant ovarian cancer. Molecular Pharmaceutics. 2011;8(1):260-9.

27. Rattanawong A. LW, and Wonganan P. Anti-cancer effects of cepharanthine in human colon cancer cells. KKU Journal Graduate Studies. 2015;15(3).

28. Lin OS. Acquired risk factors for colorectal cancer. Methods in Molecular Biology (Clifton, NJ). 2009;472:361-72.

29. Markowitz SD, Bertagnolli MM. Molecular origins of cancer: Molecular basis of colorectal cancer. The New England Journal of Medicine. 2009;361(25):2449-60.

30. Nishisho I, Nakamura Y, Miyoshi Y, Miki Y, Ando H, Horii A, et al. Mutations of chromosome 5q21 genes in FAP and colorectal cancer patients. Science (New York, NY). 1991;253(5020):665-9.

31. Bos JL, Fearon ER, Hamilton SR, Verlaan-de Vries M, van Boom JH, van der Eb AJ, et al. Prevalence of ras gene mutations in human colorectal cancers. Nature. 1987;327(6120):293-7.

32. Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, et al. Mutations of the BRAF gene in human cancer. Nature. 2002;417(6892):949-54.

33. Zhang DQ, Guo Q, Zhu JH, Chen WC. Increase of cyclooxygenase-2 inhibition with celecoxib combined with 5-FU enhances tumor cell apoptosis and antitumor efficacy in a subcutaneous implantation tumor model of human colon cancer. World Journal of Surgical Oncology. 2013;11:16.

34. Engstrom PF, Arnoletti JP, Benson AB, 3rd, Chen YJ, Choti MA, Cooper HS, et al. NCCN Clinical Practice Guidelines in Oncology: colon cancer. Journal of the National Comprehensive Cancer Network : JNCCN. 2009;7(8):778-831.

35. Hoyle M, Crathorne L, Peters J, Jones-Hughes T, Cooper C, Napier M, et al. The clinical effectiveness and cost-effectiveness of cetuximab (mono- or combination chemotherapy), bevacizumab (combination with non-oxaliplatin chemotherapy) and panitumumab (monotherapy) for the treatment of metastatic colorectal cancer after

Chulalongkorn University

first-line chemotherapy (review of technology appraisal No.150 and part review of technology appraisal No. 118): a systematic review and economic model. Health Technology Assessment. 2013;17(14):1-237.

36. Hohneker JA. Clinical development of eniluracil: current status. Oncology (Williston Park, NY). 1998;12(10 Suppl 7):52-6.

37. Longley DB, Harkin DP, Johnston PG. 5-fluorouracil: mechanisms of action and clinical strategies. Nature Reviews Cancer. 2003;3(5):330-8.

38. Chai S, To KK, Lin G. Circumvention of multi-drug resistance of cancer cells by Chinese herbal medicines. Chinese Medicine. 2010;5:26.

39. Dean M, Rzhetsky A, Allikmets R. The human ATP-binding cassette (ABC) transporter superfamily. Genome Research. 2001;11(7):1156-66.

40. Kerb R. Implications of genetic polymorphisms in drug transporters for pharmacotherapy. Cancer Letters. 2006;234(1):4-33.

41. Ho RH, Kim RB. Transporters and drug therapy: implications for drug disposition and disease. Clinical Pharmacology and Therapeutics. 2005;78(3):260-77.

42. Linton KJ. Structure and function of ABC transporters. Physiology. 2007;22:122-130.

43. Endicott JA, Ling V. The biochemistry of P-glycoprotein-mediated multidrug resistance. Annual Review of Biochemistry. 1989;58:137-71.

44. Sun YF, Wink M. Tetrandrine and fangchinoline, bisbenzylisoquinoline alkaloids from *Stephania tetrandra* can reverse multidrug resistance by inhibiting P-glycoprotein activity in multidrug resistant human cancer cells. Phytomedicine : international journal of phytotherapy and phytopharmacology. 2014;21(8-9):1110-9.

45. Neerati P, Sudhakar YA, Kanwar JR. Curcumin regulates colon cancer by Inhibiting P-Glycoprotein in cancerous colon perfusion rat model. Journal of Cancer Science & Therapy. 2013;5:313-9.

46. Liu Z, Duan ZJ, Chang JY, Zhang ZF, Chu R, Li YL, et al. Sinomenine sensitizes multidrug-resistant colon cancer cells (Caco-2) to doxorubicin by downregulation of MDR-1 expression. PloS One. 2014;9(6):e98560.

47. Rosenberg MF, Mao Q, Holzenburg A, Ford RC, Deeley RG, Cole SP. The structure of the multidrug resistance protein 1 (MRP1/ABCC1). crystallization and single-particle analysis. The Journal of Biological Chemistry. 2001;276(19):16076-82.

48. Ling S, Tian Y, Zhang H, Jia K, Feng T, Sun D, et al. Metformin reverses multidrug resistance in human hepatocellular carcinoma Bel7402/5fluorouracil cells. Molecular Medicine Reports. 2014;10(6):2891-7.

49. Xing Y, Wang ZH, Ma DH, Han Y. FTY720 enhances chemosensitivity of colon cancer cells to doxorubicin and etoposide via the modulation of P-glycoprotein and multidrug resistance protein 1. Journal of Digestive Diseases. 2014;15(5):246-59.
50. Gu W, Liu L, Fang FF, Huang F, Cheng BB, Li B. Reversal effect of bufalin on multidrug resistance in human hepatocellular carcinoma BEL-7402/5-FU cells. Oncology Reports. 2014;31(1):216-22.
51. Xu J, Liu Y, Yang Y, Bates S, Zhang JT. Characterization of oligomeric human half-ABC transporter ATP-binding cassette G2. The Journal of Biological Chemistry. 2004;279(19):19781-9.
52. Mao Q, Unadkat JD. Role of the breast cancer resistance protein (BCRP/ABCG2) in drug transport--an update. The AAPS Journal. 2015;17(1):65-82.
53. Ho MM, Hogge DE, Ling V. MDR1 and BCRP1 expression in leukemic progenitors correlates with chemotherapy response in acute myeloid leukemia. Experimental Hematology. 2008;36(4):433-42.
54. Ferreira M, Costa J, Reis-Henriques MA. ABC transporters in fish species: a review. Frontiers in Physiology. 2014;5:266.
55. Solary E. Apoptosis: molecular mechanisms. Comptes Rendus Des Seances De La Societe De Biologie et de ses filiales. 1998;192(6):1065-76.
56. Reed JC, Pellecchia M. Apoptosis-based therapies for hematologic malignancies. Blood. 2005;106(2):408-18.
57. Leibowitz B, Yu J. Mitochondrial signaling in cell death via the Bcl-2 family. Cancer Biology & Therapy. 2010;9(6):417-22.
58. Nita ME, Nagawa H, Tominaga O, Tsuno N, Fujii S, Sasaki S, et al. 5-Fluorouracil induces apoptosis in human colon cancer cell lines with modulation of Bcl-2 family proteins. British Journal of Cancer. 1998;78(8):986-92.
59. Sasaki S, Watanabe T, Kobunai T, Konishi T, Nagase H, Sugimoto Y, et al. hRFI overexpressed in HCT116 cells modulates Bcl-2 family proteins when treated with 5-fluorouracil. Oncology Reports. 2006;15(5):1293-8.
60. Violette S, Poulain L, Dussaulx E, Pepin D, Faussat AM, Chambaz J, et al. Resistance of colon cancer cells to long-term 5-fluorouracil exposure is correlated to the relative level of Bcl-2 and Bcl-X(L) in addition to Bax and p53 status. International Journal of Cancer Journal International Du Cancer. 2002;98(4):498-504.
61. Ashkenazi A. Targeting death and decoy receptors of the tumour-necrosis factor superfamily. Nature Reviews Cancer. 2002;2(6):420-30.

62. Vermeulen K, Van Bockstaele DR, Berneman ZN. The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. Cell Proliferation. 2003;36(3):131-49.
63. Abe Y, Nakagawa O, Yamaguchi R, Sasaki S. Synthesis and binding properties of new selective ligands for the nucleobase opposite the AP site. Bioorganic & Medicinal Chemistry. 2012;20(11):3470-9.
64. Guo X, Goessl E, Jin G, Collie-Duguid ES, Cassidy J, Wang W, et al. Cell cycle perturbation and acquired 5-fluorouracil chemoresistance. Anticancer Research. 2008;28(1a):9-14.
65. Kohno H, Inoue H, Seyama Y, Yamashita S, Akasu M. Mode of the anti-allergic action of cepharanthine on an experimental model of allergic rhinitis. Nihon yakurigaku zasshi Folia Pharmacologica Japonica. 1987;90(4):205-11.
66. Goto M, Zeller WP, Hurley RM. Cepharanthine (biscoclaurine alkaloid) treatment in endotoxic shock of suckling rats. The Journal of Pharmacy and Pharmacology. 1991;43(8):589-91.
67. Kogure K, Goto S, Abe K, Ohiwa C, Akasu M, Terada H. Potent antiperoxidation activity of the bisbenzylisoquinoline alkaloid cepharanthine: the amine moiety is responsible for its pH-dependent radical scavenge activity. Biochimica et Biophysica acta. 1999;1426(1):133-42.
68. Okamoto M, Ono M, Baba M. Potent inhibition of HIV type 1 replication by an antiinflammatory alkaloid, cepharanthine, in chronically infected monocytic cells. AIDS Research and Human Retroviruses. 1998;14(14):1239-45.
69. Kondo Y, Imai Y, Hojo H, Hashimoto Y, Nozoe S. Selective inhibition of T-cell-dependent immune responses by bisbenzylisoquinoline alkaloids in vivo. International Journal of Immunopharmacology. 1992;14(7):1181-6.
70. Shiraishi N, Akiyama S, Nakagawa M, Kobayashi M, Kuwano M. Effect of bisbenzylisoquinoline (biscoclaurine) alkaloids on multidrug resistance in KB human cancer cells. Cancer Research. 1987;47(9):2413-6.
71. Ito H, Ito H, Amano H, Noda H. Inhibitory effect of a biscoclaurine alkaloid, cepharanthin, on lung metastasis of Lewis lung carcinoma. Japanese Journal of Pharmacology. 1991;56(2):195-202.
72. Ikeda R, Che XF, Yamaguchi T, Ushiyama M, Zheng CL, Okumura H, et al. Cepharanthine potently enhances the sensitivity of anticancer agents in K562 cells. Cancer Science. 2005;96(6):372-6.

73. Wu J, Suzuki H, Akhand AA, Zhou YW, Hossain K, Nakashima I. Modes of activation of mitogen-activated protein kinases and their roles in cepharanthine-induced apoptosis in human leukemia cells. Cellular Signalling. 2002;14(6):509-15.

74. Liu G, Wu D, Liang X, Yue H, Cui Y. Mechanisms and in vitro effects of cepharanthine hydrochloride: Classification analysis of the drug-induced differentially-expressed genes of human nasopharyngeal carcinoma cells. Oncology Reports. 2015;34(4):2002-10.

75. Harada K, Supriatno, Yamamoto S, Kawaguchi S, Yoshida H, Sato M. Cepharanthine exerts antitumor activity on oral squamous cell carcinoma cell lines by induction of p27Kip1. Anticancer Research. 2003;23(2b):1441-8.

76. Takahashi-Makise N, Suzu S, Hiyoshi M, Ohsugi T, Katano H, Umezawa K, et al. Biscoclaurine alkaloid cepharanthine inhibits the growth of primary effusion lymphoma in vitro and in vivo and induces apoptosis via suppression of the NF-kappaB pathway. International Journal of Cancer Journal International Du Cancer. 2009;125(6):1464-72.

77. Hua P, Sun M, Zhang G, Zhang Y, Tian X, Li X, et al. Cepharanthine induces apoptosis through reactive oxygen species and mitochondrial dysfunction in human non-small-cell lung cancer cells. Biochemical and Biophysical Research Communications. 2015;460(2):136-42.

78. Uthaisar K, Seubwai W, Srikoon P, Vaeteewoottacharn K, Sawanyawisuth K, Okada S, et al. Cepharanthine suppresses metastatic potential of human cholangiocarcinoma cell lines. Asian Pacific journal of cancer prevention : APJCP. 2012;13 Suppl:149-54.

79. Kikukawa Y, Okuno Y, Tatetsu H, Nakamura M, Harada N, Ueno S, et al. Induction of cell cycle arrest and apoptosis in myeloma cells by cepharanthine, a biscoclaurine alkaloid. International Journal of Oncology. 2008;33(4):807-14.

80. Kono K, Takahashi JA, Ueba T, Mori H, Hashimoto N, Fukumoto M. Effects of combination chemotherapy with biscoclaurine-derived alkaloid (Cepharanthine) and nimustine hydrochloride on malignant glioma cell lines. Journal of Neuro-oncology. 2002;56(2):101-8.

81. Ono M, Tanaka N, Orita K. Positive interactions between human interferon and cepharanthin against human cancer cells in vitro and in vivo. Cancer Chemotherapy and Pharmacology. 1994;35(1):10-6.

82. Fang ZH, Li YJ, Chen Z, Wang JJ, Zhu LH. Inhibition of signal transducer and activator of transcription 3 and cyclooxygenase-2 is involved in radiosensitization of

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cepharanthine in HeLa cells. International Journal of Gynecological cancer : official journal of the International Gynecological Cancer Society. 2013;23(4):608-14.

83. Hirai M, Tanaka K, Shimizu T, Tanigawara Y, Yasuhara M, Hori R, et al. Cepharanthin, a multidrug resistant modifier, is a substrate for P-glycoprotein. The Journal of Pharmacology and Experimental Therapeutics. 1995;275(1):73-8.

84. Han L, Wang Y, Guo X, Zhou Y, Zhang J, Wang N, et al. Downregulation of MDR1 gene by cepharanthine hydrochloride is related to the activation of c-Jun/JNK in K562/ADR cells. BioMed Research International. 2014;2014:164391.

85. Zhou Y, Hopper-Borge E, Shen T, Huang XC, Shi Z, Kuang YH, et al. Cepharanthine is a potent reversal agent for MRP7(ABCC10)-mediated multidrug resistance. Biochemical Pharmacology. 2009;77(6):993-1001.

86. Malofeeva EV, Domanitskaya N, Gudima M, Hopper-Borge EA. Modulation of the ATPase and transport activities of broad-acting multidrug resistance factor ABCC10 (MRP7). Cancer Research. 2012;72(24):6457-67.

87. Li L, Pan Q, Sun M, Lu Q, Hu X. Dibenzocyclooctadiene lignans: a class of novel inhibitors of multidrug resistance-associated protein 1. Life Sciences. 2007;80(8):741-8.

88. Shi LX, Ma R, Lu R, Xu Q, Zhu ZF, Wang L, et al. Reversal effect of tyroservatide (YSV) tripeptide on multi-drug resistance in resistant human hepatocellular carcinoma cell line BEL-7402/5-FU. Cancer Letters. 2008;269(1):101-10.

89. Ita M, Halicka HD, Tanaka T, Kurose A, Ardelt B, Shogen K, et al. Remarkable enhancement of cytotoxicity of onconase and cepharanthine when used in combination on various tumor cell lines. Cancer biology & therapy. 2008;7(7):1104-8.

90. Ricci MS, Zong WX. Chemotherapeutic approaches for targeting cell death pathways. The Oncologist. 2006;11(4):342-57.

91. Aurelio ON, Kong XT, Gupta S, Stanbridge EJ. p53 mutants have selective dominant-negative effects on apoptosis but not growth arrest in human cancer cell lines. Molecular and Cellular Biology. 2000;20(3):770-8.

92. Ikehara N, Semba S, Sakashita M, Aoyama N, Kasuga M, Yokozaki H. BRAF mutation associated with dysregulation of apoptosis in human colorectal neoplasms. International Journal of Cancer Journal International Du Cancer. 2005;115(6):943-50.

93. Kim R. Unknotting the roles of Bcl-2 and Bcl-xL in cell death. Biochemical and Biophysical Research Communications. 2005;333(2):336-43.

94. De Angelis PM, Stokke T, Thorstensen L, Lothe RA, Clausen OP. Apoptosis and expression of Bax, Bcl-x, and Bcl-2 apoptotic regulatory proteins in colorectal

carcinomas, and association with p53 genotype/phenotype. Molecular Pathology : MP. 1998;51(5):254-61.

95. Ogura E, Senzaki H, Yamamoto D, Yoshida R, Takada H, Hioki K, et al. Prognostic significance of Bcl-2, Bcl-xL/S, Bax and Bak expressions in colorectal carcinomas. Oncology Reports. 1999;6(2):365-9.

96. Sturm I, Kohne CH, Wolff G, Petrowsky H, Hillebrand T, Hauptmann S, et al. Analysis of the p53/BAX pathway in colorectal cancer: low BAX is a negative prognostic factor in patients with resected liver metastases. Journal of Clinical Oncology : official journal of the American Society of Clinical Oncology. 1999;17(5):1364-74.

97. Sherr CJ. Cell cycle control and cancer. Harvey Lectures. 2000;96:73-92.

98. Flis S, Gnyszka A, Flis K, Splawinski J. MS275 enhances cytotoxicity induced by 5-fluorouracil in the colorectal cancer cells. European Journal of Pharmacology. 2010;627(1-3):26-32.

99. Mukai M, Che XF, Furukawa T, Sumizawa T, Aoki S, Ren XQ, et al. Reversal of the resistance to STI571 in human chronic myelogenous leukemia K562 cells. Cancer Science. 2003;94(6):557-63.

100. Hagmann W, Jesnowski R, Faissner R, Guo C, Lohr JM. ATP-binding cassette C transporters in human pancreatic carcinoma cell lines. Upregulation in 5-fluorouracil-resistant cells. Pancreatology : official journal of the International Association of Pancreatology (IAP). 2009;9(1-2):136-44.

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APPENDIX A

PREPARATION OF REAGENTS

DMEM stock solution 1 liter

DMEM powder	10.4 g
Sodium bicarbonate	3.7 g
ddH_2O	850 ml

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

Add ddH_2O to 1 liter and sterilized by filtering through a 0.2 sterile membrane filter

Complete DMEM medium 200 ml

DMEM stock	178 ml
Fetal Bovine Serum	20 ml
Penicillin/streptomycin	2 ml

1x Phosphate Buffered Saline (PBS)

NaCl	8.065 g
KCl	0.2 g
KH2PO4	0.2 g
Na2HPO4	1.15 g
ddH_2O	850 ml

Add ddH_2O to 1 liter and sterilized by filtering through a 0.2 sterile membrane filter

Hank balance salt solution (HBSS) 1 liter

Hank balance salt powder	9.8 g
Sodium bicarbonate	0.35 g
ddH_2O	850 ml

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

Add ddH_2O to 1 liter and sterilized by filtering through a 0.2 sterile membrane filter

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1x Assay Buffer 100 ml

HEPES (1M)	1.0 ml
CaCl2 (0.1M)	2.8 ml
NaCl (5M)	2.5 ml
ddH2O	93.7 ml

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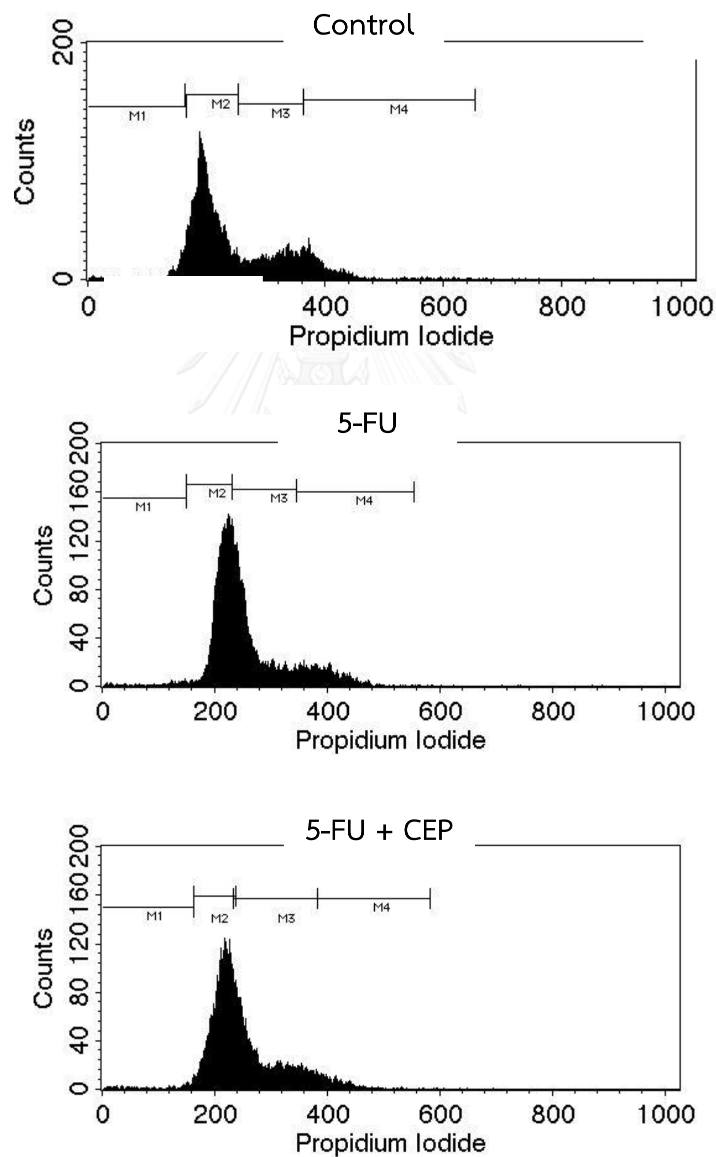
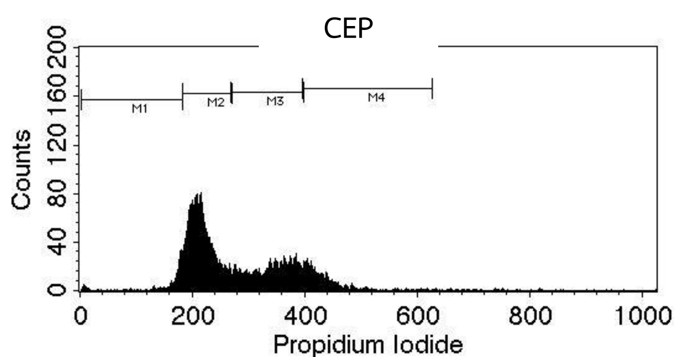
APPENDIX B

RESULTS

APPENDIX B-1 Representative histogram from flow cytometric analysis of HT-29 cell cycle pattern after treatment with CEP or 5-FU alone or combination of CEP and 5-FU for 24 h.





Appendix B-2: Alteration of HT-29 cells in the cell cycle phases after treatment with CEP or 5-FU alone or combination of CEP and 5-FU for 24 h.

	Proportion of cells (%)			
Sample (μM)	**Sub-G1**	**G1**	**S**	**G2/M**
control	2.99 ± 0.98	59.54 ± 1.25	22.54 ± 0.37	15.17 ± 2.30
5-FU	3.99 ± 0.29	32.83 ± 7.41	47.44 ± 6.50	18.55 ± 3.67
5-FU + CEP	4.99 ± 0.26	43.49 ± 4.90	46.37 ± 3.06	9.42 ± 3.05
CEP	5.99 ± 2.67	47.55 ± 3.28	32.33 ± 2.83	14.55 ± 2.65

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Name	Miss Sukanya Unson
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