ผลของเซฟฟราแรนทีนเมื่อให้ร่วมกับ 5-ฟลูออโรยูราซิล ต่อเซลล์มะเร็งลำไส้ใหญ่
ที่ปลูกถ่ายในหนูเม้าส์

นางสาวชนาภรณ์ กงสะเด็น

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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COMBINATION EFFECT OF CEPHARANTHINE AND 5-FLUOROURACIL ON HUMAN COLON CANCER IN A MOUSE XENOGRAFT MODEL

Miss Chanaporn Kongsaden

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Medical Science
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Thesis Title	COMBINATION EFFECT OF CEPHARANTHINE AND 5-FLUOROURACILON HUMAN COLON CANCER IN A MOUSE XENOGRAFT MODEL
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Field of Study	Medical Science
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ชนาภรณ์ กงสะเด็น : ผลของเซฟฟราแรนทีนเมื่อให้ร่วมกับ 5-ฟลูออโรยูราซิล ต่อเซลล์มะเร็งลำไส้ใหญ่ที่ปลูกถ่ายในหนูเม้าส์ (COMBINATION EFFECT OF CEPHARANTHINE AND 5-FLUOROURACILON HUMAN COLON CANCER IN A MOUSE XENOGRAFT MODEL) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ปิยนุช วงศ์อนันต์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: จันทนี อิทธิพานิชพงศ์, หน้า.

Cepharanthine (CEP) เป็นสารสกัดที่ได้จากรากของ *Stephania cepharantha* Hayata ซึ่งมีรายงานถึงฤทธิ์ต้านมะเร็งในมะเร็งหลายชนิดรวมถึงมะเร็งลำไส้ใหญ่ จากการศึกษาเมื่อเร็ว ๆ นี้พบว่า CEP สามารถเสริมฤทธิ์ต้านเซลล์มะเร็งลำไส้ใหญ่ของ 5-fluorourcail (5-FU) ในระดับหลอดทดลอง ดังนั้นงานวิจัยนี้จึงมีวัตถุประสงค์ที่จะศึกษาผลของ CEP เพียงอย่างเดียว และเมื่อให้ร่วมกับ 5-FU ต่อเซลล์มะเร็งลำไส้ใหญ่ในระดับสัตว์ทดลอง โดยการปลูกถ่ายเซลล์มะเร็งลำไส้ใหญ่ HT-29 เข้าสู่ชั้นใต้ผิวหนังทางด้านขวาของสัตว์ทดลอง สัตว์ทดลองจะได้รับ 5-FU หรือ CEP เพียงอย่างเดียว หรือได้รับ 5-FU ร่วมกับ CEP โดยการฉีดเข้าช่องท้อง ทุก 2 วันเป็นเวลา 3 สัปดาห์ ผลการทดลองแสดงให้เห็นว่า CEP เพียงอย่างเดียวหรือเมื่อให้ร่วมกับ 5-FU สามารถชะลอการเจริญเติบโตของก้อนมะเร็งเมื่อเปรียบเทียบกับกลุ่มควบคุม และจากการวิเคราะห์ด้วยเทคนิค real time PCR และ western blot แสดงให้เห็นว่าการให้ CEP เพียงอย่างเดียวสามารถเพิ่มการแสดงออกของ p21 และ Bak ทั้งในระดับ mRNA และ protein และยิ่งไปกว่านั้นการให้ CEP ร่วมกับ 5-FU สามารถเพิ่มการแสดงออกของ Bak และp21 mRNAs และ proteins และลดการแสดงออกของ Bcl-xl mRNA นอกจากนี้การให้ 5-FU ร่วมกับ CEP สามารถป้องกันการเพิ่มการแสดงออกของ MRP1 และ BCRP mRNAs ซึ่งเกิดจากการเหนี่ยวนำของ 5-FU ได้ นอกจากนี้ยังตรวจพบการลดลงของ COX-2 mRNA ในก้อนมะเร็งของสัตว์ทดลองที่ได้รับ CEP เพียงอย่างเดียว หรือเมื่อให้ร่วมกับ 5-FU ดังนั้นเป็นไปได้ว่าการเสริมฤทธิ์ต้านมะเร็งของ 5-FU และ CEP อาจผ่านการเพิ่มแสดงออกของ p21 และ Bax และ ลดการแสดงออกของ COX-2 ในระดับสัตว์ทดลอง ดังนั้นผลของการศึกษานี้ชี้ให้เห็นว่า CEP อาจมีประสิทธิภาพในการนำมาใช้เป็นยาเดี่ยวหรือใช้ร่วมกับ 5-FU ในการรักษามะเร็งลำไส้ใหญ่

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

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CHANAPORN KONGSADEN: COMBINATION EFFECT OF CEPHARANTHINE AND 5-FLUOROURACILON HUMAN COLON CANCER IN A MOUSE XENOGRAFT MODEL. ADVISOR: ASST. PROF. PIYANUCH WONGANAN, Ph.D., CO-ADVISOR: ASSOC. PROF. CHANDHANEE ITTHIPANICHPONG, pp.

Cepharanthine (CEP), an alkaloid isolated from the root of *Stephania cepharantha* Hayata, was reported to possess anticancer activity in various cancers, including colon cancer. Recently, it has been shown to enhance anti-cancer activity of 5-fluorouracil (5-FU) against human colon cancer *in vitro*. The present study aimed to evaluate the effect of CEP alone and CEP in combination with 5-FU in a colon cancer xenograft model. HT-29 tumors were established in the right flank of nude mice. Then, HT-29 tumor bearing mice were treated with either 5-FU or CEP alone or 5-FU combined with CEP by intraperitoneal injection every other day for three weeks. The results showed that CEP alone or in combination with 5-FU significantly delayed tumor growth when compared with the control group. Real-time RT-PCR and western blot analysis revealed that treatment with CEP alone significantly up-regulated the expression of p21 and Bak at both mRNA and protein levels. Remarkably, the combination treatment of CEP and 5-FU increased expression of Bak and p21 mRNAs and proteins as well as reduced expression of Bcl-xl mRNA. In addition, treatment with CEP and5-FU could prevent 5- FU- induced expression of MRP1 and BCRP mRNAs. Moreover, downregulation of COX-2 mRNA was also detected in tumor tissues of animals receiving CEP alone or CEP combined with 5-FU. Taken together, it is likely that synergistic antitumor effect of 5-FU and CEP is mediated through upregulation of p21 and Bak and downregulation of COX-2 in a mouse xenograft model. Therefore, the result of this study suggest that CEP may potentially be used as a single agent or in combination with 5-FU for colon cancer treatment.

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Student's Signature .....
Advisor's Signature .....
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Last but not the least, I would like to thank my family: my parents, my brothers and sister who have supported me spiritually throughout the writing of this thesis and my life in general.

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LIST OF ABBREVAITIONS

µg/ml	Microgram per milliliter
µl	Microliter
µM	Micromolar
5-FU	5-fluorouracil
AA	Arachidonic acid
ABC	ATP-binding cassette
ABTS	2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)
ANOVA	Analysis of variance
Apaf-1	Apoptotic protease-activating factor-1
APC	Adenomatous polyposis coli
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
Bid	BH3-interacting domain death agonist
Bim	BCL-2-like-11
CAA	Constitutive ATM activation
CAK	Cdk-activating kinase
Caspase	Cysteine aspartic acid specific protease
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA

CEP	Cepharanthine
CIP/KIP	CDK interacting protein/Kinase inhibitory protein
c-JNK	c-Jun N-terminal protein kinases
CKI	Cyclin-dependent kinase inhibitor
CO_2	Carbon dioxide
COX-2	Cyclooxygenase-2
DISC	Death-inducing signaling complex
DMEM	Dulbeco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPD	Dihydropyrimidine dehydrogenase
DPPH	1, 1-diphenyl-2-picryl-hydrazyl
DR3	Death receptor3
E-cad	E-cadherin
EGF	Epidermal growth factor
EP receptors	Prostaglandin E receptors
FADD	Fas-associated death domain
FAP	Familial adenomatous polyposis
Fas	Fibroblast associated antigen
FDA	Food and Drug Administration
FdUDP	Fluorodeoxyuridine diphosphate
FdUMP	Fluorodeoxyuridine monophosphate
FdUTP	Fluorodeoxyuridine triphosphate
FGF1	Fibroblast growth factor 1
FGF2	Fibroblast growth factor 2
FUDP	Fluorouridine diphosphate
FUMP	Fluorouridine monophosphate

GAPDH	Glyceraldehydes-3-phosphate dehydrogenase
GISTs	Gastrointestinal stromal tumors
h	Hour
H2AX	Histone H2AX phosphorylation
H_2O_2	Hydrogen peroxide
HtrA2	High-temperature requirement protein A2
IBD	Inflammatory bowel disease
IL-1α	Interleukin-1 alpha
IL-1β	Interleukin-1 beta
INK4	Inhibitor of CDK4
iNOS	Nitric oxide synthase inducible
K-RAS	Kirsten rat sarcoma virus oncogene
LPS	Lipopolysaccharide
MDR1	Multidrug resistance 1
mg/ml	Milligram per milliliter
mRNA	Messenger RNA
MRP	Multidrug resistance protein
NBDs	Nucleotide-binding domains
NF-κB	Nuclear factor kappa-light-chain-enhance of activated B
NO	Nitric oxide
NSAIDs	Nonsteroidal anti-inflammatory drugs
OPRT	Orotate phosphoribosyl transferase
p53	Tumor suppressor protein 53
PARP	Poly (ADP-ribose) polymerase
PGG2	Prostaglandin G2
P-gp	P-glycoprotein

PI3K	Phosphatidylinositol-3-kinase
PKB	Protein kinase B (or AKT)
PLA2	Phospholipase A2
PTGS	Endoperoxide synthases
PUMA	p53 upregulated modulator of apoptosis
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RT-PCR	Reverse transcription polymerase chain reaction
S.C.	Subcutaneously
SEM	Standard error of mean
STAT3	Signal transducer and activator of transcription 3
TCF	Transcriptional activation of T cell factor
TMDs	Transmembrane domains
TNF-α	Tumor necrosis factor-α
TNF-R	Tumor necrosis factor receptor
TP53	Tumor protein 53
TRAIL	Tumor necrosis factor - related apoptosis including ligand
TrpC5	Transient receptor potential channel 5
TS	Thymidylate synthase
TXA2	Thromboxane A2
UTP	Uridine triphosphate
VEGF	Vascular endothelial growth factor

CHAPTER I

INTRODUCTION

1.1 Background and rationale

Colon cancer is one of the most common cancers and a major cause of cancer–related death in several countries such as Australia, New Zealand, Canada, the United States, and Thailand[1]. Colon cancer is the third most common cancer in both men (after lung and prostate cancers) and women (after breast and lung cancer) worldwide[2-4]. In Thailand, there is an increased incidence rate of colon cancer due to rapidly changing eating habits, lifestyle and long-term smoking. The National Cancer Institute of Thailand reported in 2014 that colon cancer was the first most common cancer in men and the third most common cancer in women (after breast and cervix cancer) with a prevalence of 17.21 % and 8.78 %, respectively[5].

There are several treatment options for colon cancer including surgery, radiotherapy, chemotherapy and targeted therapy. Types of treatment chosen depend on stage of the disease. A combination therapy is commonly used to treat the disease and one of the most common treatments of colon cancer is chemotherapy. Chemotherapeutic agents commonly used in colon cancer patients are 5-fluorouracil (5-FU), oxaliplatin, irinotecan and capecitabine. Of all chemotherapeutic drugs, 5-FU has been the mainstay drug for colon cancer treatment for many years[6-8]. Although 5-FU has been widely used but its clinical application has been limited due to low efficacy[9]. Combination treatment of 5-FU with other chemotherapeutic agents such as irinotecan and oxaliplatin has also shown to increase response rates form 10-15% to 40-50% in patients with advanced colon cancer but it has caused serious side-effects such as hematological toxicities, skin reactions, trouble breathing and severe allergic reaction[10, 11]. In addition to serious side effects,

clinical application of the chemotherapeutic agents has been hampered by drug resistance. Several mechanisms have been shown to be involved in resistance to chemotherapy including alteration of drug influx and efflux, enhancement of drug inactivation, overexpression of thymidylate synthase (TS), and evasion of apoptosis[12, 13].

One of the most common causes of chemotherapy resistance is overexpression of ATP-binding cassette (ABC) transporters. Several ABC efflux transporters have been shown to be associated with 5-FU resistance including P-glycoprotein (P-gp/MDR1), multidrug resistance associated protein (MRP), and breast cancer resistance protein (BCRP). Previously, it was reported that high expression of P-gp led to resistance to many chemotherapeutic agents such as daunomycin and 5-fluorouracil[12, 14]. Recently, Wang *et al*. reported that suppression of P-glycoprotein (P-gp/MDR1) via inhibition of transient receptor potential channel 5 (TrpC5) could reverse the resistance of human colon cancer to 5-fluorouracil (5-FU)[14]. In addition, MRP8 (ABCC11) was shown to confer resistance to 5-FU and certain fluoropyrimidines when ectopically expressed into LLC-PK1 cells[15]. Furthermore, silencing of BCRP using RNA interference (RNAi) could result in 5-FU accumulation and 5-FU induced DNA damage in breast cancer cells[16].

Cyclooxygenase (COX)-2, induced by inflammatory stimuli, is involved in pathological processes. Overexpression of COX-2 has also been detected in 80% of colon carcinoma. The expression of COX-2 is correlated with increase of PGE_2 level, which associates with cell proliferation, survival, and angiogenesis[17, 18]. Previous studies also showed that HT-29 colon cancer cells overexpressing COX-2 were resistant to chemotherapeutic agents such as cisplatin and 5-fluorouracil[19, 20] and apoptotic cell death[21]. Therefore, the approach of using natural compounds that can inhibit expression of COX-2

and drug efflux transporter in combination with conventional chemotherapeutic agents is highly promising because the natural compounds may enhance the potency of chemotherapeutic drugs and minimize side effects associated with chemotherapy.

Cepharanthine (CEP) is a biscoclaurine alkaloid isolated from the roots of *Stephania cepharantha* Hayata. It is also found in *Stephania suberusa* L.L forman and *Stephania erecta* Craib in Thailand. CEP has been widely used in Japan to treat a wide variety of acute and chronic diseases. It is approved by the Japanese Ministry of Health for the treatment of radiation-induced leukopenia, alopecia areata and alopecia pityrodes[22-24]. In addition, several clinical benefits of CEP including, management of HIV have been documented[25, 26]. Although it has been extensively used, severe side effects have never been reported. Some common side effects of this drug are anorexia, rash, diarrhea, and headache[22, 23]. Several pharmacological activities of CEP have been reported such as anti-allergic, anti-oxidant, anti-malaria, immunomodulatory, multidrug resistance reversal and anticancer[24, 27, 28]. CEP has demonstrated to inhibit the growth of several types of cancer including leukemia, lymphoma, lung carcinoma, myeloma, cholangiocarcinoma, oral squamous cell carcinoma, hepatocellular carcinoma[24, 29-34]. It was shown that CEP exerted antitumor effects by inhibiting many signaling pathways such as STAT[34, 35] MAPK/ERK[34] and NF-κB[33]. In addition, CEP could induce cancer cells to undergo apoptosis by down-regulating anti-apoptotic genes such as *Bcl-2* and *Bcl-xl*, up-regulating pro-apoptotic genes such as *Bax* and *Bak*, activating caspase-3, and triggering cytochrome C release. Moreover, CEP was found to induce cell cycle arrest through modulation of cell cycle regulators such as cyclin D, cyclin E and p21[31, 36, 37]. It has also been reported that CEP could increase efficiency of

many chemotherapeutic agents such as adriamycin, docetaxel, vincristine, paclitaxel by activity of ATP-binding cassette transporters such as P-gp and MRP7[38-40]. Additionally, CEP could decrease COX-2 expression in HeLa cells *in vitro* and *in vivo*, leading to increase radiosensitization[36]. Recently, Sukanya *et al* has reported that CEP enhanced the anticancer effect of 5-FU in human colon cancer cells in *vitro*[41]. In the present study, the combination effect of CEP and 5-fluorouracil was evaluated in a xenograft mouse model of human colon cancer.

1.2 Research questions

1.2.1 Can CEP enhance the anticancer activity of 5-FU in a nude mouse colon cancer xenograft model?

1.2.2 What are the mechanisms underlying the synergistic effect of CEP on anticancer activity of 5-FU in a mouse xenograft model?

1.3 Objectives

1.3.1 To determine the synergistic antitumor effect of CEP and 5-FU in a nude mouse colon cancer xenograft model.

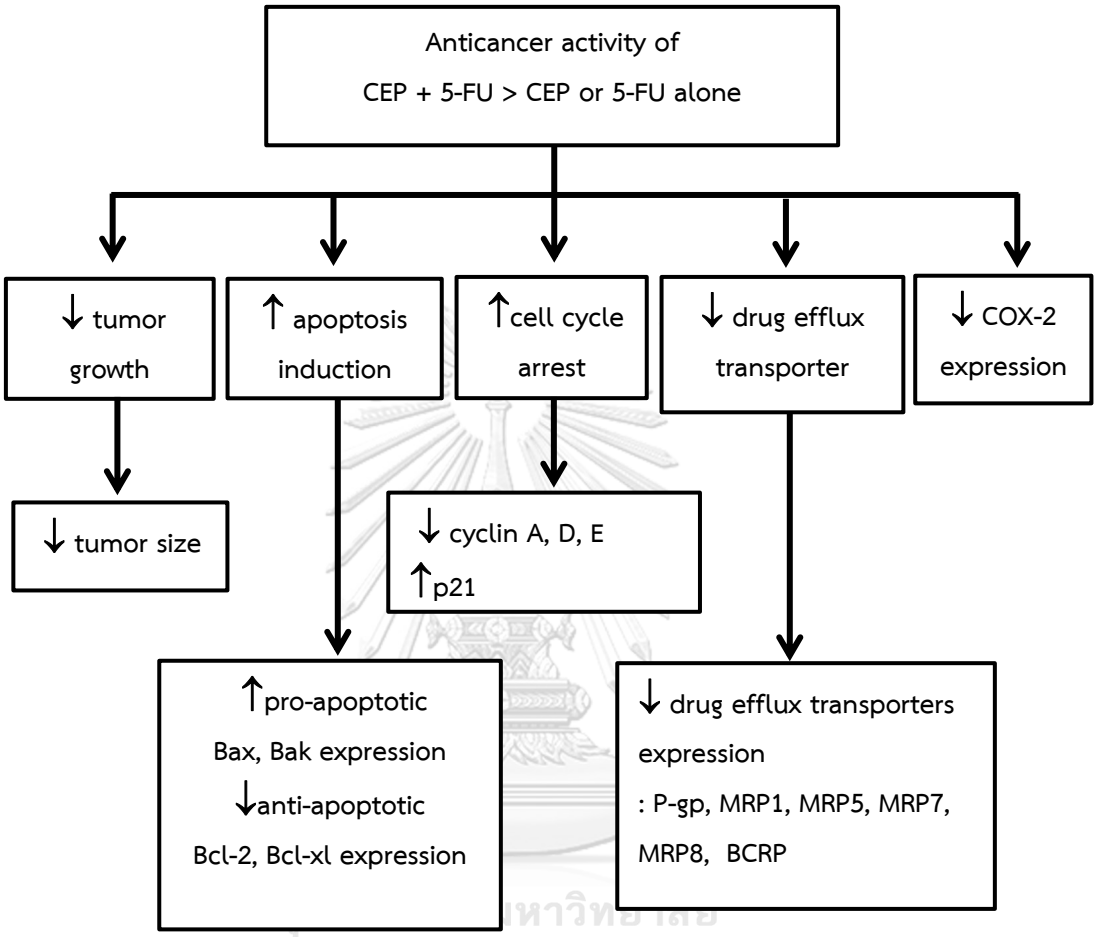
1.3.2 To investigate mechanism(s) underlying the synergistic effect of CEP on antitumor effect of 5-FU in a human colon cancer xenograft model.

1.4 Hypothesis

1.4.1 CEP can enhance the antitumor effect of 5-FU in a mouse xenograft model.

1.4.2 CEP can modify the expression of Bcl-2 family members, cell cycle regulators and efflux transporters as well as COX-2 in a mouse xenograft model.

1.5 Conceptual framework



CHAPTER II

LITERATURE REVIEWS

2.1 Colon cancer

Cancer is the abnormal growth of cells that have the ability to invade or spread to other parts of the body. Colon cancer is the development of cancer from the colon. The types of colon cancer are adenocarcinoma, carcinoid tumors, lymphomas, sarcomas, and gastrointestinal stromal tumors (GISTs). The most common one is adenocarcinoma which account for about 90% to 95%. In 2016, the American Cancer Society reported that colon cancer was the third most common cancer in both man and woman in the United States[4]. Similarly, The National Cancer Institute of Thailand revealed in 2014 that colon cancer was the first most common cancer in men and the third most common in women (after breast and cervix cancer) with a prevalence of 17.21% and 8.78%, respectively[5]. Colon cancer begins as a benign adenomatous polyp, which then develops into an advanced adenoma with high dysplasia and later progresses to an invasive cancer. Common signs and symptoms of this disease include blood in the stool (either bright red or very dark), change in bowel habits (diarrhea or constipation), weight loss, and feeling tired constantly[42, 43]. There are several risk factors that might increase chance of developing colon cancer, which are age over 50, colon polyps, family history of colon cancer, smoking, heavy alcohol consumption, lack of exercise, poor diet (such as red meat and low fiber) and patient with the history of inflammatory bowel disease (IBD)[44, 45]. Moreover, it was reported that inactivation of tumor suppressor genes such as adenomatous polyposis coli (*APC*) and tumor protein p53 (*TP53*) has been the most common genetic changes detected in colon cancer patients[46, 47]. Previous studies demonstrated that inactivation of APC allowed translocation

of the β-catenin to nucleus, leading to the over transcription of oncogenes[48, 49]. In addition to APC, p53, another important tumor suppressor, has been shown to be involved in induction of cell cycle arrest and apoptosis in response to DNA damage. Loss of p53 function also reduced cellular sensitivity to 5-FU by preventing colon cancer cells to undergo apoptosis[6, 50]. Besides inactivation of tumor suppressor genes, activation of oncogenes such as *RAS* and *BRAF* has played important roles in the progression of colon cancer. Additionally, growth factors such as epidermal growth factor (EGF) as well as cyclooxygenase 2 (COX-2) are related to colon cancer progression (Figure1)[51].

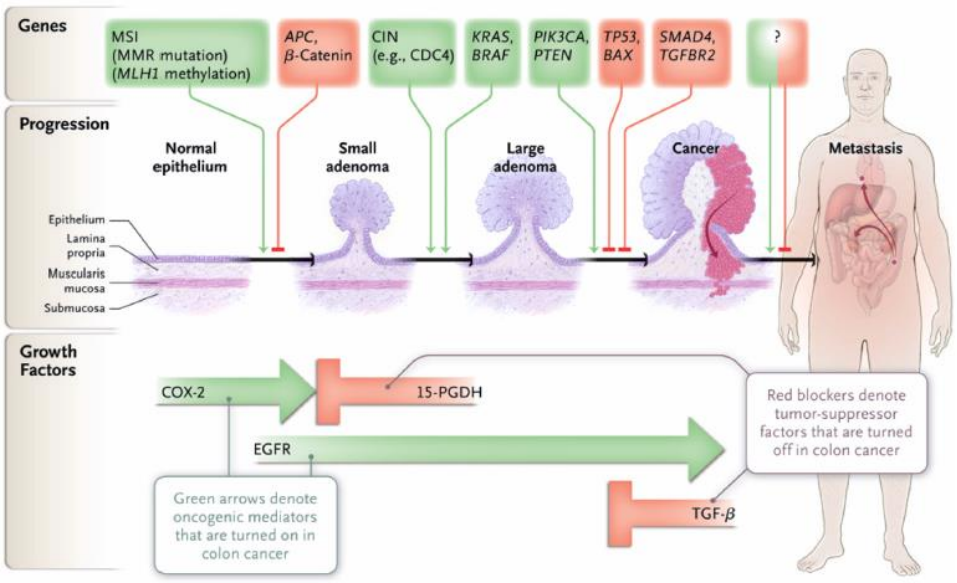


Figure 1. Genes and growth factor pathways involved in progression of colon cancer[51]

2.1.1 Stages of colon cancer

Colon cancer has been classified into 5 stages ranging from 0 to IV. The stages are based on the T, N, and M scores[52]. Cancer stages for colon cancer are defined as follows:

Stage 0: These cancers are also called carcinoma *in situ* of the colon. The cancer has not grown beyond the first layer of the colon wall. It is a non-invasive cancer.

Stage I: The cancer has grown into either the second or third layer of the colon wall. There is no cancer in nearby or distant sites.

Stage II: The cancer has grown into the fourth layer of or outside the colon wall. There is no cancer in nearby or distant sites.

Stage III: The cancer has spread from the colon to nearby lymph nodes or there are tumor deposits.

Stage IV: The cancer has spread to distant organs that is not near the colorectal such as the liver, lung, or ovary, or to a distant lymph node

Currently, there are many types of treatment for colon cancer such as surgery, radiotherapy, chemotherapy and targeted therapy. Surgery is the most common treatment options for colon cancer. After surgery, chemotherapy and/or radiation therapy are commonly used to improve the effectiveness of the treatment and prevent recurrence[53]. Chemotherapeutic drugs that are commonly used in colon cancer patients include 5-fluorouracil (5-FU), oxaliplatin, irinotecan and capecitabine[52]. Additionally, targeted therapies such as bevacizumab, cetuximab and panitumumab have been approved for treatment of patients with metastatic colon cancer[54]. Of all anticancer agents, 5-FU has long been the mainstay drug for treatment of patients with colon cancer[54].

2.2 5-Fluorouracil

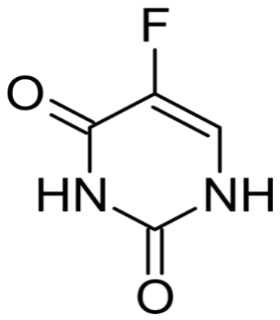


Figure 2. Chemical structure of 5-fluorouracil ($C_4H_3FN_2O_2$)[55]

5-Fluorouracil (5-FU), an analogue of uracil with a fluorine atom at the C-5 position in place of hydrogen (Figure 2)[55], is a chemotherapeutic agent widely used in the treatment of several cancers, including colon cancer, gastric cancer, pancreatic cancer, breast as well as head and neck cancer[56] Once inside the cells, the drug is converted into fluorouridine monophosphate (FUMP) by orotate phosphoribosyl transferase (OPRT)[57]. FUMP is then phosphorylated to fluorouridine diphosphate (FUDP), which can be further phosphorylated to the active metabolite, fluorouridine triphosphate (FUTP). Incorporation of FUTP into RNA instead of uridine triphosphate (UTP) causes alterations in RNA processing. FUDP can also be converted by ribonucleotide reductase enzyme into fluorodeoxyuridine diphosphate (FdUDP), which is either phosphorylated into fluorodeoxyuridine triphosphate (FdUTP) or dephosphorylated into fluorodeoxyuridine monophosphate (FdUMP). FdUTP then gets incorporated into DNA whereas FdUMP can inhibit thymidylate synthase (TS), a key enzyme involved in DNA synthesis, causing DNA damage (Figure 3)[6, 58-60].

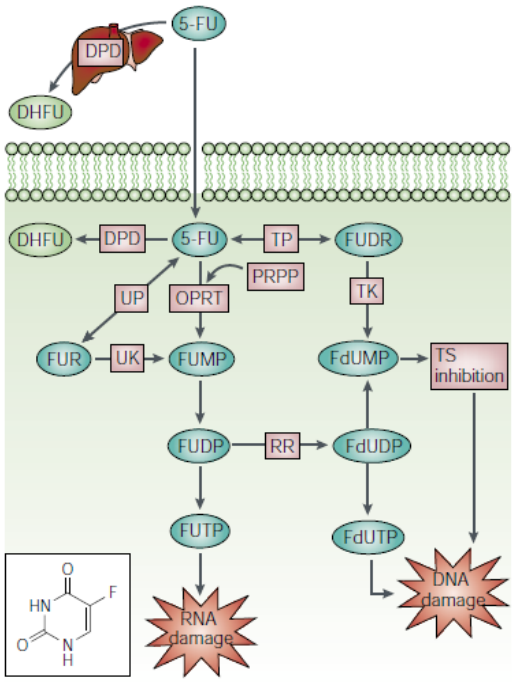


Figure 3. Metabolism and mechanism of action of 5-fluorouracil[6]

5-FU is commonly administered with leucovorin, a reduced folate, to stabilize 5-FU's interaction with TS enzyme. The most common side effects of the drug are hand-foot syndrome, mucositis and alopecia (hair loss). Occasionally, it can cause cardiotoxicity. In addition to severe side effects, its clinical application has been limited due to drug resistance[6, 61]

2.2.1 5-FU resistance

Drug resistance is a major problem for cancer treatment. There are several mechanisms underlying 5-FU resistance, including alteration of drug influx and efflux, enhancement of drug inactivation, evasion of apoptosis, and overexpression of drug targets such as TS[61].

2.2.2 Drug efflux transporters

Over-expression of ATP-binding cassette (ABC) transporters is mainly responsible for resistance by extruding chemotherapeutic agent out of the cell[62, 63]. The ABC transporters consist of 49 members and can be divided into seven families (called ABC A–G), based on amino acid sequences[63, 64]. The ABC transporters commonly contain two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs); however, members of the ABCC group contain two NBDs and two TMDs, and an additional third TMD (TMD0) with five transmembrane segments and an extra N-terminus. Conversely, members of the ABCG2 group have only 1 TMD and 1 NBD.

Several members of drug efflux transporters have been shown to confer drug resistance in cancer therapy, including P-glycoprotein (P-gp/MDR1), multidrug resistance associated protein 1 (MRP1), and breast cancer resistance protein (BCRP). Previous studies have report that P-gp, encoded by *ABCB1 gene*, conferred resistance to many chemotherapeutic agents including 5-fluorouracil[12, 24, 65]. Recently, suppression of P-gp via inhibition of transient receptor potential channel 5 (TrpC5) reversed the resistance of human colon cancer HCT118 and LoVo cells to 5-FU[14]. A previous study also found that over-expression of MRP1 (ABCC1) was common in human colon carcinogenesis. It has been reported that long-term exposure of 5-FU can induce multidrug resistance (MDR) in the hepatocellular carcinoma cell line by increasing the activity or expression of MRP1[66]. In addition, a study of Cao et al revealed that MRP1 knockdown can increased response of colorectal cancer cells to 5-FU and oxaliplatin both *in vitro* and *in vivo*[67]. Several studies also found that overexpression of BCRP mRNA conferred resistance to many anticancer drugs such as doxorubicin[68], imatinib[69]and 5-FU[16]. Furthermore, silencing of BCRP using RNA interference

(RNAi) could result in accumulation of 5-FU, inducing DNA damage in breast cancer cells[16]. In addition to the most important drug efflux transporters, MRP5 (encoded by *ABCC5* gene) and MRP8 (encoded by *ABCC11* gene) have been shown to be involved in 5-FU resistance by extruding its metabolites[15, 70].

2.2.3 Cyclooxygenase

Cyclooxygenase (COX) enzymes, also known as prostaglandin-endoperoxide synthases (PTGS), catalyze the conversion of arachidonic acid, released from membrane phospholipid by phospholipase A_2 (PLA_2), into prostaglandin G_2 (PGG_2), an unstable intermediate that is rapidly converted to PGH_2. PGH_2 is then converted to different prostaglandins (PGs) including PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and thromboxane A_2 (TXA_2) by specific prostaglandin synthase enzymes (Figure 4)[71, 72]. COX enzymes can be divided into 3 isoforms including COX-1, COX-2 and COX-3. COX-1, a constitutive enzyme that expresses in most tissues in the body, is involved in homeostasis such as gastric mucosa production, platelet aggregation and renal blood flow regulation. Conversely, COX-2 is an inducible enzyme expressing in response to various stimuli such as pro-inflammatory cytokines (tumor necrosis factor-α [TNF-α], interleukin -1α [IL-1α] and interleukin-1β [IL-1β][73, 74], several growth factors (endothelial growth factor [EGF], vascular endothelial growth factor [VEGF], fibroblast growth factor [FGF2]), and tumor promoters (v-sac, v-Ha-ras, HER-2/neu, and Wnt)[74]. COX-3, an alternative splice variant of COX-1, is a new isoform of the COX family has been recently identified. It is abundantly expressed in the canine cerebral cortex; however, its role in human is not fully understood[75, 76].

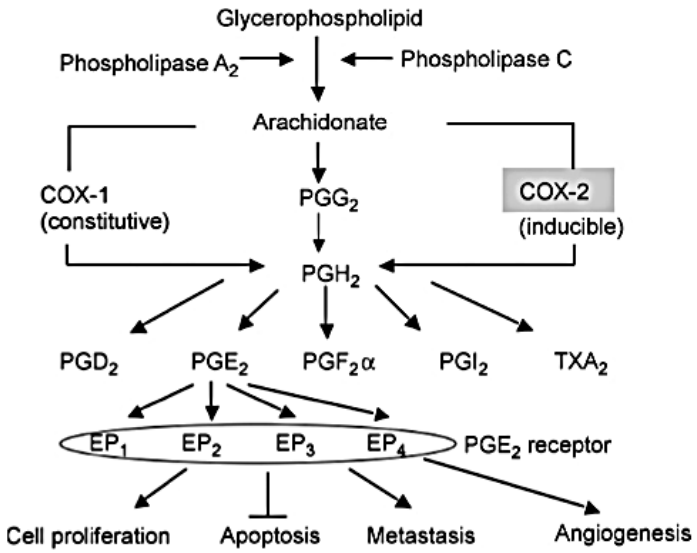


Figure 4. Prostaglandin biosynthesis pathway[72]

COX-2 plays an important role in cancer development. Over-expression of COX-2 has been detected in many cancers such as breast, prostate, esophagus and colon[77]. Roelofs *et al.* showed that COX-2 mRNA was overexpressed approximately in 80% human colon carcinomas, compare with normal colon mucosa[78]. Overexpression of COX-2 led to elevated PGE_2 level, which was correlated with cell proliferation, survival and angiogenesis. The action of PGE_2 is mediated by binding with different membrane receptors called prostaglandin E receptors (EP receptors) including EP1, EP2, EP3, and EP4, which are G protein-coupled receptors[17, 18, 79]. In 2005, Castellone *et al*. reported that EP2 receptor was associated with development of cancer by activating phosphoinositide3-kinase (PI3K)/Akt and β-catenin pathways, leading to transcriptional activation of T cell factor (TCF) (Figure 5)[80]. Furthermore, overexpression of COX2/PGE_2, which is mediated through EP4 receptor, could suppress apoptosis via activation of the Ras/MAPK/ERK pathway and transcription of CREB, resulting in elevated Bcl-2 expression[81]

(Figure 5). In addition, the PEG2 derived from COX-2 overexpression could cause resistance to many chemotherapeutic agents such as cisplatin and 5-FU by inducing the expression of MRP1[20]. Currently, the Food and Drug Administration (FDA) has approved the use of celecoxib (celebrex), a COX-2 inhibitor, for treatment of familial adenomatous polyposis (FAP), which highly express COX-2. However, COX-2 inhibitors may increase the risk of cardiovascular and thrombotic effects in clinic. Therefore, using natural compounds that can inhibit COX-2 and drug efflux transporters in combination with conventional chemotherapeutic agents is a promising approach to improve the effectiveness and reduce side effects associated with chemotherapeutic drugs.

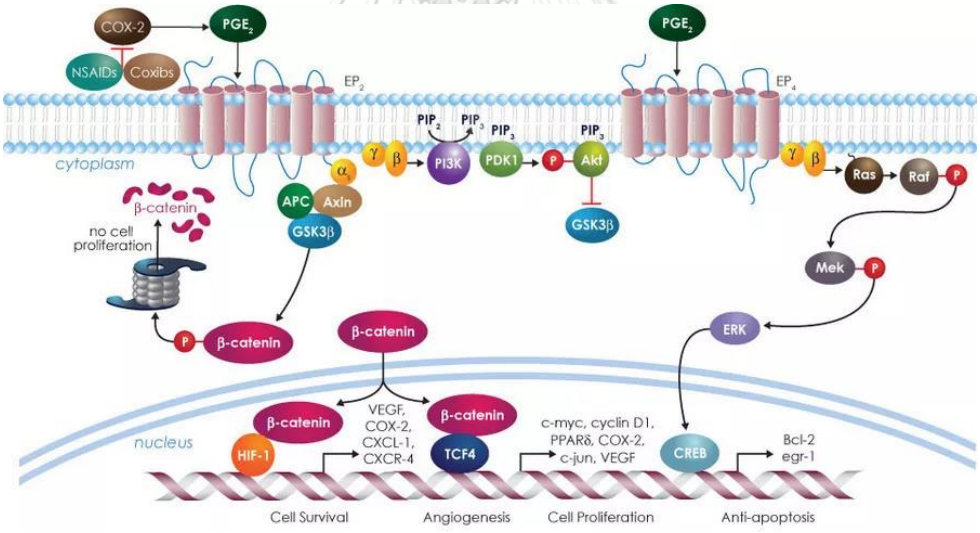


Figure 5. PGE2 signaling in colorectal cancer[81]

2.3 Cepharanthine (CEP)

Cepharanthine (CEP) (Figure 6) is a biscoclaurine alkaloid extracted from the roots of *Stephania cepharantha* Hayata. It can also be found in *Stephania suberusa* L.L forman and *Stephania erecta* Craib in Thailand. It is approved

by Japanese Ministry of Health for the treatment of radiation-induced leukopenia, alopecia areata and alopecia pityrodes[22]. CEP has also been commonly used to treat a wide variety of acute and chronic diseases such as venomous snakebites[82], malaria[83] and septic shock[84]. Additionally, several preclinical studies revealed that CEP possess various pharmacological activities, including inhibition of plasma membrane lipid peroxidation[85], inhibition of histamine release[86], anti-inflammatory effect[87], anti-allergic effect[88], multidrug resistance-reversing effect[89], inhibition of platelet aggregation[37] as well as antitumor[29, 31, 35, 36, 90-92]. CEP is currently available in both powder and tablet forms. Once absorbed, it is mainly distributed to the liver, spleen, kidney and lung[24]. Although it is widely used, serious side effects have never been reported. Previous studied report that in a total of 3,556 clinical cases, 28 cases (approximately 0.79%) receiving 10-20 mg CEP per day experienced very mild side effects such as headaches, stomach discomfort and dizziness[84].

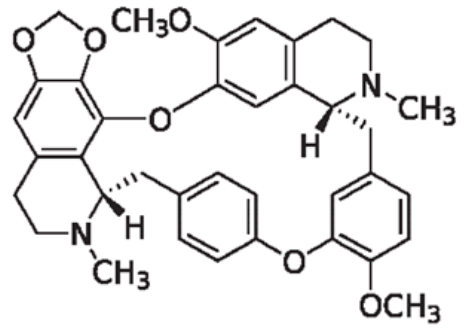


Figure 6. Chemical structure of cepharanthine (CEP)[24]

2.3.1 Anti-cancer effect

Previous studies found that CEP displayed anticancer activity against several different types of cancer including leukemia, lymphoma, lung carcinoma, myeloma, cholangiocarcinoma, oral squamous cell carcinoma

and hepatocellular carcinoma[29, 30, 32, 91]. CEP was shown to trigger apoptosis in non-small lung cancer cells via upregulation of Bax, downregulation of Bcl-2 and activation of caspase-3 and PARP[93]. CEP could also inhibit the growth of myeloma by inducing cell cycle arrest and apoptosis through increased free radicals and enhanced Bax[31]. In hepatocellular carcinoma (HuH-7) cell line, CEP was found to induce apoptosis via activation of JNK1/2, downregulation of Akt and induction of reactive oxygen species production[29]. Additionally, CEP could induce apoptosis in human adenosquamous cell carcinoma (TYS cell line) by activating caspase 3 and inducing cell cycle arrest at the G1 phase through the expression of p21[37]. CEP could also suppress angiogenesis and the growth of oral squamous cells carcinoma by inhibiting expression of vascular endothelial growth factor (VEGF) and interleukin (IL)-8 through blockade of NF-ϰB activity[94]. In 2010, Seubwai *et al* found that CEP was able to inhibit the growth of cholangiocarcinoma cells without toxicity in an animal model. Mechanistic studies illustrated that CEP inhibit NF-ϰB activity and activate caspase enzymes[32]. It was also shown to increase sensitivity of the oral squamous cell carcinoma to radiation by inhibiting DNA repair process and inducing apoptosis via activation of caspases[95]. In addition, CEP was shown to induce sensitization of human cervical adenocarcinoma (HeLa cell line) to radiation through inhibition of STAT3 signaling pathway and COX-2 expression both *in vitro* and *in vivo*[36]. Remarkably, Rattanawong have demonstrated that CEP was more effective in controlling the growth of human colon cancer cell line, HT-29 cells, which are relatively resistant to commonly used chemotherapeutic agents, when compared to COLO-205 cells[96]. *In vivo* study also showed that CEP could inhibit the growth of primary effusion lymphoma through inhibition of NF-ϰB activity[33]. Moreover, it was reported

that CEP induced leukemia cells that were resistant to doxorubicin to undergo apoptosis by increasing free radicals and up-regulating Fas-antigen expression in mice[22]. Harada *et al* also reported the potentiation effect of CEP on anticancer activity of S-1 in animal implanted with human oral squamous cell carcinoma. Further mechanistic studies revealed that CEP induced apoptosis, reduced the expression of TS and dihydropyrimidine dehydrogenase (DPD) enzymes and increased the expression of orotate phosphoribosyl transferase (OPRT) enzyme[30]. Similarly, CEP was shown to enhance anticancer effects of adriamycin and doxorubicin in mice with Ehrlich ascites tumor and leukemia, respectively[89, 97]. Recently, *Sukanya et al* has reported that CEP enhanced the anticancer effect of 5-FU in human colon cancer cells *in vitro*[41].

2.3.2 Anti-inflammatory effect

CEP was shown to exert anti-inflammatory effect through inhibition of NF-κB by inhibiting IKK pathway, resulting in reduced expression of IL-6, tumor necrosis factor-α (TNF-α), and nitric oxide (NO) in lipopolysaccharide (LPS)-induced rats[98]. In addition, CEP could inhibit the inflammatory response in the acute lung injury (ALI) by suppressing activation of NF-κB, degradation of IκB-α, and phosphorylation of ERK, JNK, and p38[99].

2.3.3 Anti-oxidant effect

It was reported that CEP could inhibit formation of free radicals, including 1, 1-diphenyl-2-picryl-hydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), N, N-dimethyl-p-phenylenediaminedihydrochloride (DMPD), superoxide anion ($O_2^{\bullet -}$), and hydrogen peroxide (H_2O_2) and inhibit lipid peroxidation[100]. Furthermore,

Halicka *et al* found that CEP decreased levels of constitutive ATM activation (CAA) and histone H2AX phosphorylation (H2AX), preventing free radicals-induced DNA damage[101].

2.4 Cell cycle

The cell cycle, an important process for multicellular species, involves in cell growth, DNA replication and cell division, resulting in two daughter cells. The cell cycle can mainly be divided into 2 phases; interphase and mitotic. Interphase involves the accumulation of the substances required for cell division. It is further divided into three phases, including G1, S and G2 (Figure 7). In the G1 phase or pre-synthetic phase, the cells synthesize mRNA and proteins which are required for DNA synthesis. During the S phase, the cells synthesize DNA, resulting in DNA replication. The cells then enter the G2 phase or pre-mitotic phase, by which the cells check on the integrity and completion of DNA before cell division. Mitotic phase or M phase, consisting of mitosis and cytokinesis, is the phase by which cells divide into two identical daughter cells [102].

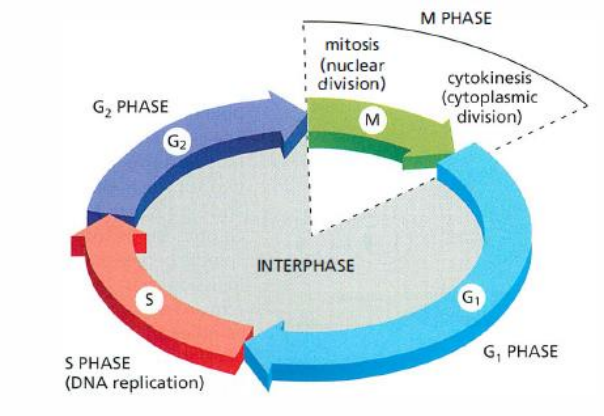


Figure 7. Four stages of cell cycle[102]

The cell cycle progression is mainly controlled by cyclin-dependent kinases (CDKs) (Figure 8). The CDKs such as Cdk1, Cdk2, Ckd4 and Ckd6 are a family of serine/threonine protein kinases. Their kinase activity is dependent on the presence of activating subunits called cyclins such as cyclin D, E, A and B [(103)]. The cyclin/CDK complex, is in a partial activation state and can be fully activated by cdk-activating kinase (CAK). Normally, cyclin D binds to CDK4 and 6, controlling cell cycle progression from G0 to G1 phase whereas binding of cyclin E to CDK2 is necessary for the progression from the G1 to the S phase. Similarly, transition of the cells from the S phase into the G2 phase and the G2 phase into the M phase are regulated by cyclin A/CDK2/1 and cyclin B/CDK1, respectively[(104)].

The work of cyclin/CDK complex is tightly regulated by CDK inhibitors (CKIs), which are divided into two families, including Ink4 family and the Cip/Kip family. The Ink4 family members, including $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$, inhibit the action of cyclin/CDK complex by binding to CDKs, changing conformation of cyclin-binding site and preventing binding of cyclin to CDKs. The Ink4 family members primarily target Cdk4 and Cdk6 whereas the Cip/Kip family members, including, $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$, are more promiscuous and broadly interfere with the activities of cyclin/CDK complex [(105, 106)].

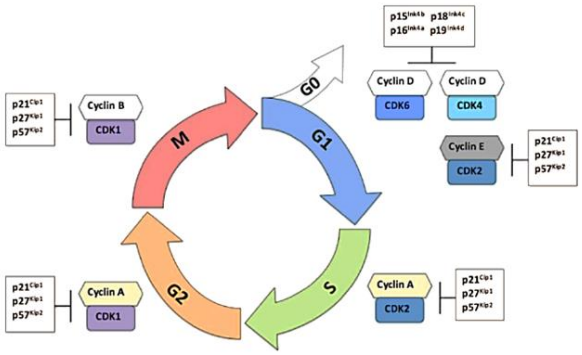


Figure 8. Cell cycle control[(106)]

2.5 Apoptosis

Apoptosis or program of cell death is a process occurring in multicellular organisms, which regulates normal development and homeostasis. It can be characterized by membrane blabbing, cell shrinkage, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation [107]. There are two main apoptotic pathways, including the extrinsic pathway and the intrinsic pathway (Figure 9) [108].

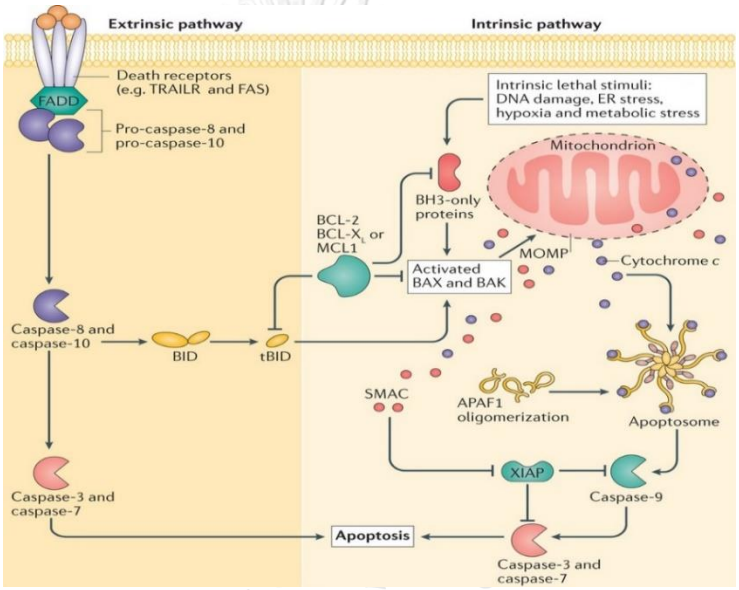


Figure 9. Apoptosis pathway[108]

2.5.1 Extrinsic apoptosis pathways

The extrinsic pathway involves the interaction of death receptors that are members of the tumor necrosis factor (TNF) receptor family such as TNF receptor (TNF-R) and death receptor 3 (DR3) to extracellular proteins such as TNF-α and TNF-related apoptosis inducing ligand (TRAIL)[109]. Binding of ligand to its receptor can recruit adapter proteins such as Fas-associated death domain (FADD) or TNF receptor-associated death domain (TRADD) [110], which then associates with an initiator caspase such as procaspase-8 and

procaspase-10, forming a death-inducing signaling complex (DISC). The formation of DISC results in auto-catalytically activation of procaspase-8, further stimulating executioner caspases such as caspase-3 and caspase-7, leading to apoptotic cell death[111].

2.5.2 Intrinsic apoptosis pathway

The intrinsic apoptotic pathway starts with various types of stimuli, such as DNA damage, hypoxia, viral infections and oxidative stress. This pathway is tightly regulated by Bcl-2 family proteins, which can be divided into three groups, including pro-apoptotic Bcl-2 family proteins such as Bak and Bax; anti-apoptotic Bcl-2 family proteins such as Bcl-2 and Bcl-xl and BH3-only pro-apoptotic Bcl-2 family proteins such as BIM, PUMA and NOXA [112]. The BH3-only proteins contribute to the pro-apoptotic function of Bax and Bak by triggering their oligomerization and translocation to outer membrane of mitochondria, leading to pore formation and release of important proteins from the intermembrane space into the cytosol such as second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (Smac/DIABLO), high-temperature requirement protein A2 (HtrA2) and cytochrome C. The cytochrome C then binds to Apaf-1 and procaspase-9, forming an “apoptosome”. The clustering of procaspase-9 in this manner leads to caspase-9 activation. The activated caspase 9 then stimulates executioner caspases such as caspase-3 and caspase-7, resulting in apoptosis. On the other hand, the anti-apoptotic proteins such as Bcl-2 and Bcl-xl can bind with pro-apoptotic proteins, preventing mitochondrial pore formation and suppressing apoptotic signaling cascade[108].

CHAPTER III

MATERIALS AND METHODS

3.1 Equipments

- Autopipette (Gilson, USA)
- Biohazard laminar flow hood (Labconco, USA)
- C-DiGit Blot Scanner (Licor, US)
- Centrifuge (Eppendrof, Germany)
- Controller pipette (Gilson, USA)
- CO_2 incubator (Thermo Scientific, USA)
- Gel electrophoresis (Hoefer, USA)
- Homogenizer (IKA laboratory technology, China)
- Light microscope (Nikon, Japan)
- PCR thermal cycler (Eppendorf, Germany)
- pH meter (Mettler Toledo, Switzerland)
- StepOnePlus™ Real-Time PCR system (Applied Biosystems, USA)
- TE 22 Mini Tank Transfer Unit (Hoefer, USA)
- Vernier caliper (Vernier Software & Technology, USA)
- Volumetric pipette (BRAND, Germany)
- Vortex mixer (Scientific Industries, USA)

3.2 Materials

- 0.1 ml Low profile polypropylene thin wall PCR tube strips 0.1 ml (Corning Life Sciences Life Sciences, USA)
- 75 cm^2 Rectangular cell culture flask (Corning Life Sciences Life Sciences, USA)
- 0.5 ml Syringe (BD Biosciences, USA)
- 29 gauge Needle (BD Biosciences, US)

- 15 ml Conical tubes (Corning Life Sciences Life Sciences., USA)

3.3 Reagents

- Ammonium persulfate (Sigma-Aldrich-Aldrich, USA)
- Bromophenol blue (Sigma-Aldrich-Aldrich, USA)
- ß-mercaptoethanol (Sigma-Aldrich-Aldrich, USA)
- Bio-Rad Protein Assay Kit (Bio-Rad, USA)
- Cepharanthine (Abcam, UK)
- Dimethyl sulfoxide (DMSO) (Sigma-Aldrich-Aldrich,USA)
- Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA)
- Fetal bovine serum (FBS) (Gibco, USA)
- 5-Fluorouracil (Sigma-Aldrich-Aldrich, USA)
- Glycerol (Sigma-Aldrich-Aldrich, USA)
- Glycine (Sigma-Aldrich-Aldrich, USA)
- Improm-II reverse transcription system (Promaga, USA)
- Isoflurane (Piramal, USA)
- Matrigel (Corning Life Sciences Life Sciences, USA)
- Methanol (Merck, Germany)
- N,N,N',N'-Tetramethylethylenediamine (TEMED) (Sigma-Aldrich-Aldrich, USA)
- Penicillin-streptomycin (Gibco, USA)
- Protogel (National Diagnostic, USA)
- Primary antibody (Cell Signaling Technology, U.S)
    - P21 (12D1) Rabbit monoclonal antibody (21 kDa)
    - Bak (D2D3) Rabbit monoclonal antibody (25 kDa)
    - ß-actin (13E5) Rabbit monoclonal antibody (45 kDa)

- Protease inhibitors (Sigma-Aldrich, USA)
- Pyronin Y (Sigma-Aldrich, USA)
- RIPA lysis buffer (Thermo sciencetific, USA)
- Polyvinylidene difluoride (PVDF) membrane (Merck, Germany)
- Secondary antibody (Cell Signaling Technology, U.S)
    - Anti- IgG, HRP-linked antibody
- Sodium dodecyl sulfate polyacrylamide (SDS) (EM science, U.S)
- SYBR-Green qPCR supermix universal (Life Technologies, USA)
- Trypsin/EDTA (Gibco, USA)
- 0.4% Trypan blue dye (Sigma-Aldrich-Aldrich, USA)
- TRIzol Reagent (Invitrogen, USA)
- Tris base (Vivantis, USA)

3.4 Methods

3.4.1 Chemicals

Cepharanthine (CEP, Abcam, UK [purity > 95%]) and 5-fluorouracil (5-FU, Sigma-Aldrich, USA) P was dissolved 100% dimethyl sulfoxide (DMSO) and stored at 4ºC. The normal saline or DMSO (Sigma-Aldrich) was used as control.

3.4.2 Cell culture

Human colon cancer cell line HT-29 was purchased from American Type Culture Collection (ATCC, USA). The cells were cultured in Dulbecco's modified eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C in 5% CO_2 incubator.

3.4.3 Animal

All animal procedures were performed in accordance with the guideline established by the National Institutes of Health for humane treatment of animals. The animal protocol was approved by Animal Ethics Committee of the Faculty of Medicine, Chulalongkorn University. BALB/cMlac-nu mice (~ 4-6 weeks, National Laboratory Animal Center, Mahidol University, Thailand) were kept in the 12:12 light-dark cycle at 25±2 °C at the Faculty of Medicine, Chulalongkorn University's animal laboratory facilities. Mice were provided with sterile water and food *ad libitum* and allowed an acclimatization period of at least 7 days before the experiments. BALB/cMlac-nu mice were subcutaneously inoculated with HT-29 cells ($1x10^7$cells/mouse) suspended in 100 µl matrigel (Corning Life Sciences, USA) in the right flank of mice using a 29-gauge needle (BD Biosciences, US). Treatment was initiated when the tumors tumors were approximately 100-150 mm^3 (7 days after implantation)

3.4.4 Treatment protocol

The mice were randomly divided into 5 groups (4-5 mice/group) and treated with 20 µl of normal saline or DMSO (Sigma-Aldrich, USA), 10 mg/kg of 5-FU (Sigma-Aldrich, USA), 20 mg/kg of CEP (Abcam, UK) and 10 mg/kg of 5-FU in combination with 20 mg/kg of CEP. All animals received drugs by intraperitoneal injection every other day for 21 days. Mice were observed for clinical signs daily and body weights were recorded once every other day. Tumor size was measured in two perpendicular diameters using a caliper every other day and tumor volume was calculated based on the following equation: tumor

volume (mm^3) = 1/2 [length x $(width)^2$][113]. Twenty-three days after treatment, mice were sacrificed and the tumors were dissected out, weighed and snap-frozen in liquid nitrogen for further study.

3.4.5 Quantitative real-time RT-PCR

The mRNA levels of pro-apoptotic (Bax and Bak), anti-apoptotic (Bcl-2 and Bcl-xl) Bcl-2 family members, cell cycle regulators (cyclin A, D, E, p21) and COX-2 in tumor tissues were analyzed by real-time RT-PCR using SYBR Green as a probe. The SYBR Green dye binds the minor groove of double-stranded DNA. Therefore, the intensity of the fluorescence increases as the PCR products accumulate. Breifly, total cellular RNA was isolated from tumor tissue using TRIzol reagent (Invitrogen, USA). Isolated RNA was then converted into cDNA using Improm-II™ Reverse Transcription system (Promega, USA). Amplification of target genes was carried out using SYBR Green qPCR super mix universal (Life Technologies, USA) with the specific primers listed in Table 1. GAPDH was used as an internal control. Real-time reactions were run on StepOnePlus™ 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 30s, and 72°C for 30s. The fold change in mRNA level normalized to GAPDH was calculated using the $2^{-\Delta\Delta_{CT}}$

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

Target Gene	Primer sequences
GAPDH	Forward: 5'- AAGGTCGGAGTCAACGGATTTGGT-3' Reverse: 5'- ATGGCATGGACTGTGGTCATGAGT-3'
BAX	Forward: 5'-GACGAACTGGACAGTAACATG-3' Reverse: 5'-AGGAAGTCCAATGTCCAGCC-3'
BAK	Forward: 5'-AGCTGCCATGGTAATCTAACTCA-3' Reverse: 5'-GATGTGGAGCGAAGGTCACT-3'
Bcl-2	Forward: 5'-TCATGTGTGTGGAGAGCGTCAA-3' Reverse: 5'-CTACTGCTTTAGTGAACCTTTTGC-3'
Bcl-xl	Forward: 5'-TTGGACAATGGACTGGTTGA-3' Reverse: 5'-GTAGAGTGGATGGTCAGTG-3'
Cyclin A	Forward: 5'-CTGCTGCTATGCTGTTAG-3' Reverse: 5'-TGTTGGAGCAGCTAAGTCAAAA-3'
Cyclin D	Forward: 5'-TTGTTGAAGTTGCAAAGTCCTGG-3' Reverse: 5'- ATGGTTTCCACTTCGCAGCA-3'
Cyclin E	Forward: 5'-TCCTGGATGTTGACTGCCTT-3' Reverse: 5'-CACCACTGATACCCTGAAACCT-3'
p21	Forward: 5'-CCTGTCACTGTCTTGTACCCT-3' Reverse: 5'-GCGTTTGGAGTGGTAGAAATCT-3'
P-gp	Forward: 5'-TGTTCAAACTTCTGCTGCTCCTGA-3' Reverse: 5'-CCCATCATTGCAATAGCACG-3'
MRP1	Forward: 5'-AGCTTTATGCCTGGGAGCTGG-3' Reverse: 5'-CGGCAAATGTGCACAAGGCCA-3'
MRP5	Forward: 5'-GCTTGTTTTGCTGCAGGGC-3' Reverse: 5'-AGTGCTGGTTCTCTCCCTCA-3'

MRP7	Forward: 5'-CATGCAAGCCACGCGGAACG-3' Reverse: 5'-AAGCTGGGCTGGTGGAGGGT-3'
MRP8	Forward: 5'-AGTATGATGCTGCCTTGA-3' Reverse: 5'-GGTGAGGTAGGAGAACAG-3'
BCRP	Forward: 5'-CACAACCATTGCATCTTGGCTG-3' Reverse: 5'-TGAGAGATCGATGCCCTGCTTT-3'
COX-2	Forward: 5'-CCCTGAGCATCTACGGTTTG-3' Reverse: 5'-TCGCATACTCTGTTGTGTTCC-3'

3.4.6 Western blot analysis

Tumor tissue lysates were prepared by homogenizing tumor tissue in RIPA lysis buffer (Thermo scientific, USA) containing protease inhibitors (Sigma-Aldrich, USA). Protein concentration of supernatants was determined by a microplate assay with the Bio-Rad DC Protein assay reagents (Bio-Rad, USA) using bovine serum albumin as a standard. Twenty micrograms of protein lysate were separated on an 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a PVDF membrane. The membrane was blocked in non-fat dry milk (NFDM) and then incubated with antibodies against Bak (dilution 1:1000), p21 (dilution 1:2000), and ß-actin (dilution 1:2000) (Cell Signaling, USA) overnight at 4 °C. The membrane was then washed and incubated with HRP-linked secondary antibody (dilution 1:2000) for 1 h at room temperature. Bands were visualized using chemiluminescence detection system (Pierce, USA). ß-actin was used as the internal control for protein normalization.

3.4.7 Statistical analysis

All data are presented as mean ± standard error of mean (SEM). Statistical analysis of data was performed by one-way analysis of variance (ANOVA) followed by Bonferroni/Dunn post hoc using SPSS statistics 21 software (IBM Corporation, USA). The difference is considered significant if $P<0.05$.

CHAPTER IV

RESULT

4.1 The growth-inhibitory effects of 5-FU and CEP in a colon cancer xenograft model

CEP was previously found to enhance anticancer effect of 5-FU against colon cancer *in vitro*[41]. Therefore, the present study was to evaluate whether CEP could increase the antitumor activity of 5-FU in a colon cancer xenograft mouse model. HT-29 tumor bearing mice were treated with either 5-FU or CEP alone or 5-FU in combination with CEP. As shown in **Fig.10A**, 5-FU alone or CEP alone could significantly delay tumor growth when compared with the control group whereas DMSO (vehicle control) did not have any effect on the growth of tumor with respect to the control group. Remarkably, the combination treatment of 5-FU and CEP was more effective in controlling the growth of tumor when compared with either CEP or 5-FU alone. Notably, there were no significant changes in body weight in any treatment group, indicating that the single treatment with either 5-FU or CEP alone and the combination treatment of 5-FU and CEP are safe **(Fig.10B).**

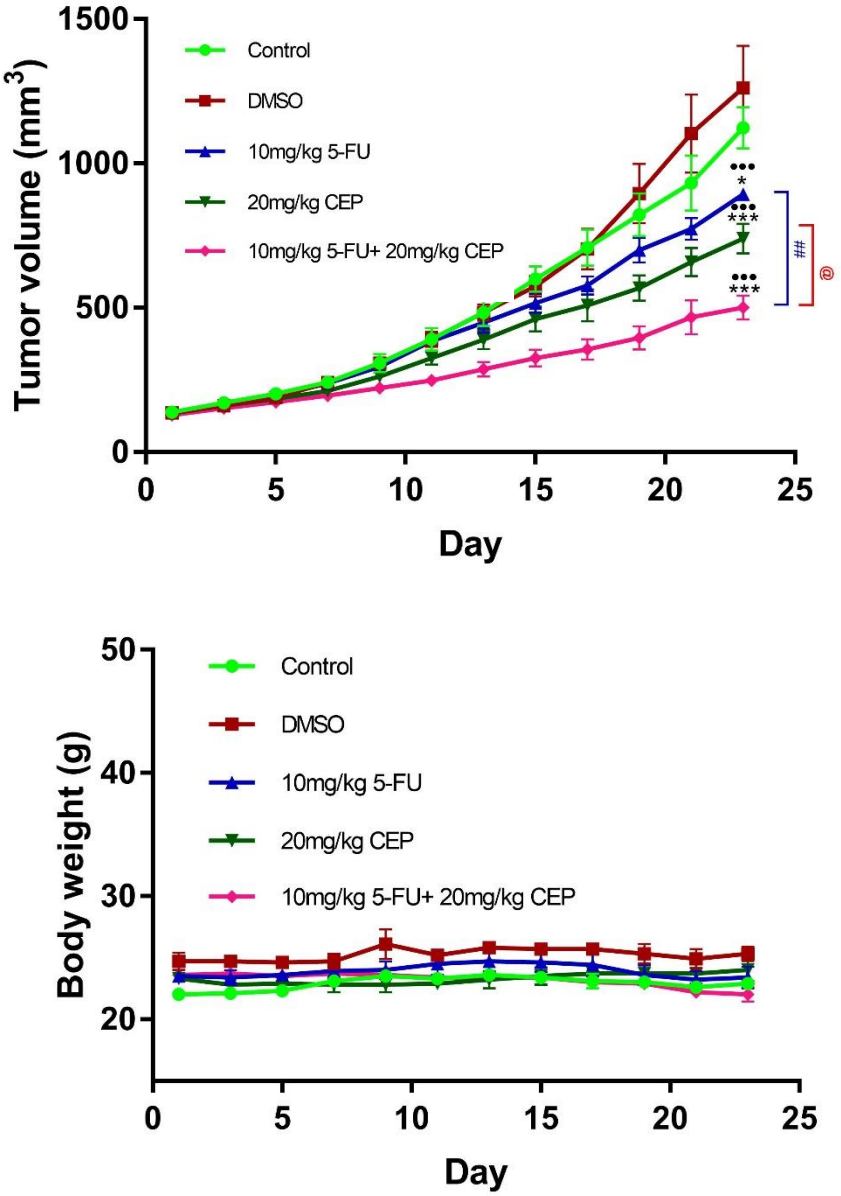


Figure 10. The anticancer effects of 5-FU or CEP alone and 5-FU in combination with CEP in a colon cancer xenografted mouse model. (A) Tumor volume and (B) body weight were measured every other day. Results of the control and DMSO groups (n = 4) and CEP or 5-FU and combination groups (n=5) were presented as means ± SEM. *p≤0.05, **p≤0.01 compared with control, $^{•••}$p ≤ 0.001 compared with DMSO, $^{\#\#}$ p≤0.01 compared with 5-FU, $^{@}$ p≤0.05 compared with 5-FU.

4.2 The effects of 5-FU and CEP on the expression of Bcl-2 family members mRNAs in a mouse xenograft model

Chemotherapy induces cancer cell to undergo apoptosis, primary through the intrinsic pathway, which is tightly regulated by Bcl-2 family proteins[114, 115]. Thus, the mRNA levels of Bcl-2 family proteins including pro-apoptosis (Bak and Bax) and anti-apoptosis (Bcl-2 and Bcl-xl) were measured in tumor tissues using quantitative real-time RT-PCR. As shown in **Fig.11A**, CEP alone significantly up-regulated Bak mRNA expression when compared with the control group. Interestingly, the combination treatment of 5-FU and CEP significantly increased mRNA level of Bak when compared with the control ($p \leq 0.01$) and 5-FU alone groups ($p \leq 0.05$). It however should be noted that the mRNA levels of Bax, another pro-apoptotic member of Bcl-2 family, were not altered in any treatment group **(Fig.11B).** Although treatment with either 5-FU or CEP alone did not affect the levels of anti-apoptotic Bcl-xl mRNA with respect to the control group, the combination treatment of 5-FU and CEP significantly down-regulated the expression of Bcl-xl mRNA when compared with the 5-FU alone group (**Fig.11C**; $p \leq 0.05$). Notably, no significant changes in Bcl-2 mRNA level were detected in any treatment group (**Fig.11D**). Taken together, these results suggest that CEP may enhance anticancer activity of 5-FU by upregulating the expression of Bak.

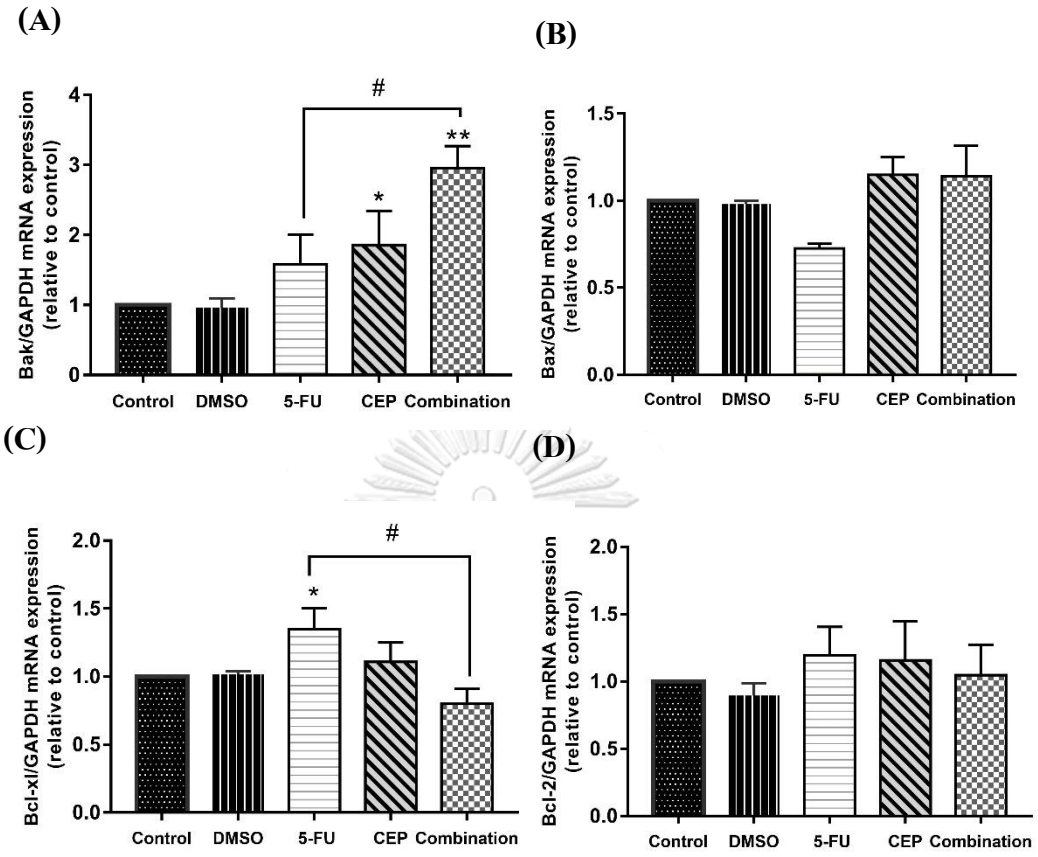


Figure 11. The effects of 5-FU or CEP alone and 5-FU in combination with CEP on the expression of pro-apoptotic (Bak and Bax) and anti-apoptotic (Bcl-2 and Bcl-xl) mRNAs in a colon cancer xenograft mouse model. HT-29 tumor bearing mice were treated with either 5-FU or CEP alone or 5-FU in combination with CEP and the mRNA expression levels of pro-apoptotic (A) Bak, (B) Bax and anti-apoptotic (C) Bcl-xl, (D) Bcl-2 in tumor tissues were analyzed by quantitative real-time RT-PCR. The values were normalized to GAPDH. Results of the control and DMSO groups (n = 4) and CEP or 5-FU and combination groups (n=5) were presented as means ± SEM. *$p \leq 0.05$, **$p \leq 0.01$ compared with control, $^{\#}p \leq 0.05$ compared with 5-FU.

4.3 The effects of 5-FU and CEP on Bak protein expression in a mouse xenograft model

To further confirm whether CEP could upregulate the expression of Bak, western blotting was used to determine the effect of the treatments on protein levels of Bak in tumor tissues. Similar to the mRNA levels, CEP alone significantly increased Bak protein levels when compared with the control group (**Fig.12;** $p \leq 0.05$). Moreover, a significant increase in Bak protein was detected in animals receiving 5-FU in combination with CEP with respect to either the control ($p \leq 0.001$) and 5-FU alone ($p \leq 0.01$) groups.

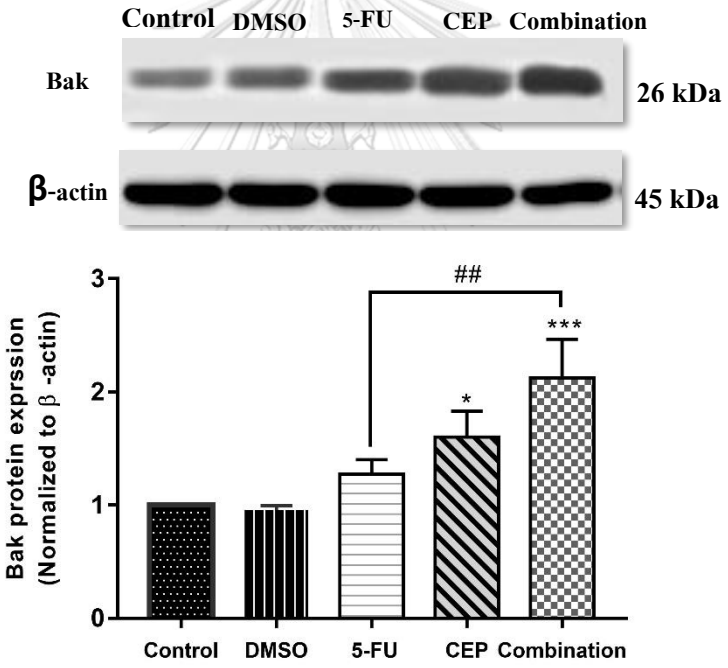


Figure 12. The effects of 5-FU or CEP alone and 5-FU in combination with CEP on Bak protein expression in a colon cancer xenograft mouse model. Bak protein levels in tumor tissues were detected by western blotting using β-actin as an internal control. Results of the control and DMSO groups (n = 4) and CEP or 5-FU and combination groups (n=5) were presented as means ± SEM. *$p \leq 0.05$, ***$p \leq 0.001$ compared with control, $^{\#\#}p \leq 0.01$ compared with 5-FU.

4.4 The effects of 5-FU and CEP on cell cycle regulators in a mouse xenograft model

Previous *in vitro* and *in vivo* studies have found that CEP could decrease the expression of cyclin E and D mRNA levels and increased the expression of p21 mRNA level in adenosquamous cell carcinoma and osteosarcoma cell lines[35, 37], therefore the effects of 5-FU or CEP alone or 5-FU combined with CEP on the expression of cyclin A, D, E and p21 mRNAs were evaluated in this study. As shown in **Figs.13A-C**, treatment with either 5-FU or CEP alone or 5-FU in combination with CEP did not significantly alter the levels of cyclin A, D as well as cyclin E mRNAs. Conversely, the expression of p21 mRNA in tumor tissues of animals treated with CEP alone was significantly higher than that of control animals ($p \leq 0.05$). Moreover, treatment of 5-FU combined with CEP significantly increased p21 mRNA expression when compared with control ($p \leq 0.01$) or 5-FU alone ($p \leq 0.01$) groups (**Fig.13D**).

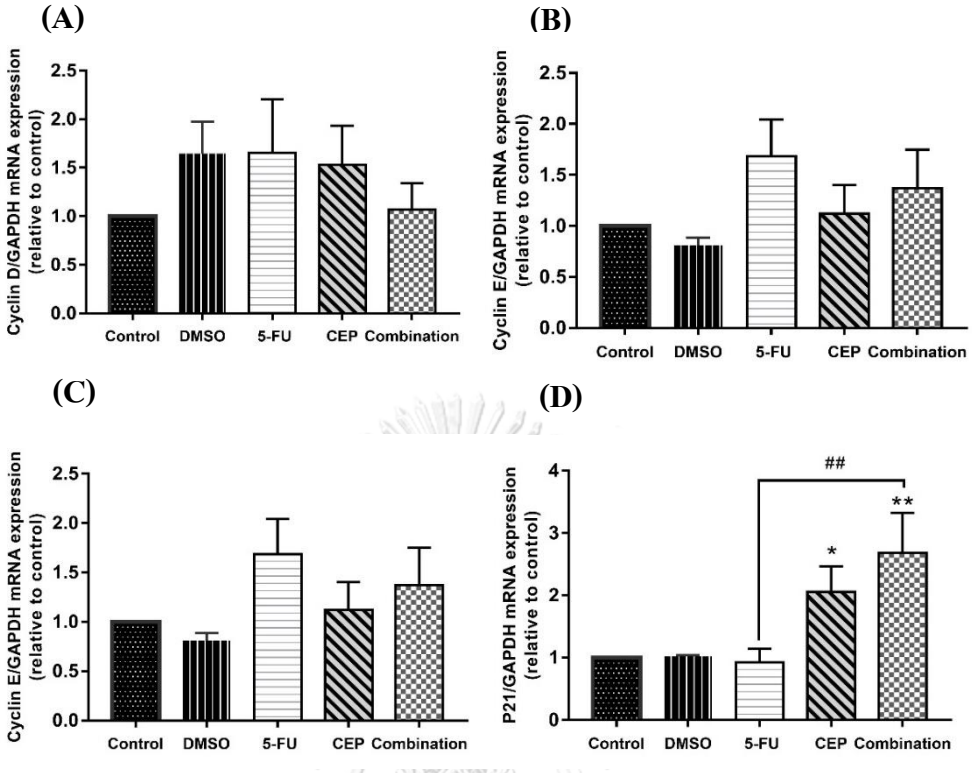


Figure 13. The effects of 5-FU or CEP alone and 5-FU in combination with CEP on the expression of cell cycle regulators (cyclins A, D and E and a CDK inhibitor, p21) mRNAs in a colon cancer xenograft mouse model. HT-29 tumor bearing mice were treated with either 5-FU or CEP alone or 5-FU in combination with CEP and the mRNA expression levels of cell cycle regulators, including (A) cyclin D, (B) cyclin E, (C) cyclin A and (D) p21 in tumor tissues were analyzed by quantitative real-time RT-PCR. The values were normalized to GAPDH. Results of the control and DMSO groups (n = 4) and CEP or 5-FU and combination groups (n=5) were presented as means ± SEM. *$p \leq 0.05$, **$p \leq 0.01$ compared with control, $^{\#\#}p \leq 0.01$ compared with 5-FU.

4.5 The effects of 5-FU and CEP on P21 protein expression in a mouse xenograft model

To further confirm the real-time RT-PCR analysis results, the protein levels of p21 in tumor tissues were measured by western blotting. As shown in **Fig.14**, CEP alone significantly upregulated the expression of p21 protein when compared with the control group ($p \leq 0.01$). Interestingly, a significant increase in p21 protein level was found in tumor tissues of mice receiving the combination of 5-FU and CEP with respect to the control ($p \leq 0.001$) and 5-FU alone ($p \leq 0.01$) groups. Therefore, it is likely that the synergistic effects of CEP on anticancer activity of 5-FU partly through upregulation of p21.

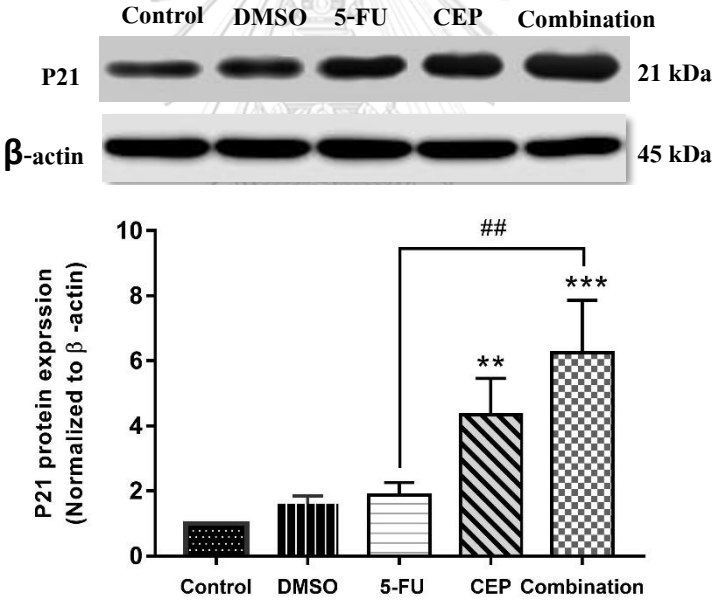


Figure 14. The effects of 5-FU or CEP alone or 5-FU in combination with CEP on p21 protein expression in a colon cancer xenograft mouse model. p21 protein level in tumor tissue were detected by western blotting using β-actin as an internal control. Results of the control and DMSO groups (n = 4) and CEP or 5-FU and combination groups (n=5) were presented as means ± SEM. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ compared with control, $^{\#\#}p \leq 0.01$ compared with 5-FU.

4.6 The effects of 5-FU and CEP on drug efflux transporters in a mouse xenograft model

It is well-known that upregulation of drug efflux transporters could reduce intracellular drug accumulation, leading to treatment failure[116]. Therefore, the mRNA levels of the most important drug efflux transporters, including p-gp, MRP1, BCRP, MRP5, MRP7 and MRP8 in tumor tissues of mice receiving treatments were measured by quantitative real-time RT-PCR. As shown in **Fig.15A** the results showed that either 5-FU or CEP alone or 5-FU combination with CEP did not change the expression of p-gp mRNA expression. However, 5-FU alone significantly induced expression of MRP1and BCRP mRNAs when compared with the control group **(Figs.15B&C)**. In contrast to 5-FU, treatment of CEP alone significantly decreased BCRP mRNA expression with respect to the control group **(Fig.15B;** $p \leq 0.05$) Although the combination treatment of 5-FU and CEP did not significantly alter the expression of MRP1 and BCRP when compared with the control group, their combination significantly down-regulated the expression of MRP1 and BCRP mRNA when compared with the 5-FU alone group **(Figs.15B&C).** Conversely, treatment with either 5-FU or CEP alone or combination of 5-FU and CEP did not affect the mRNA levels of the MRP5, MRP7 and MRP8 (**Figs.15D-F**). Collectively, it is likely that CEP may enhance anticancer activity of 5-FU by downregulating the expression of BCRP mRNA efflux transporter.

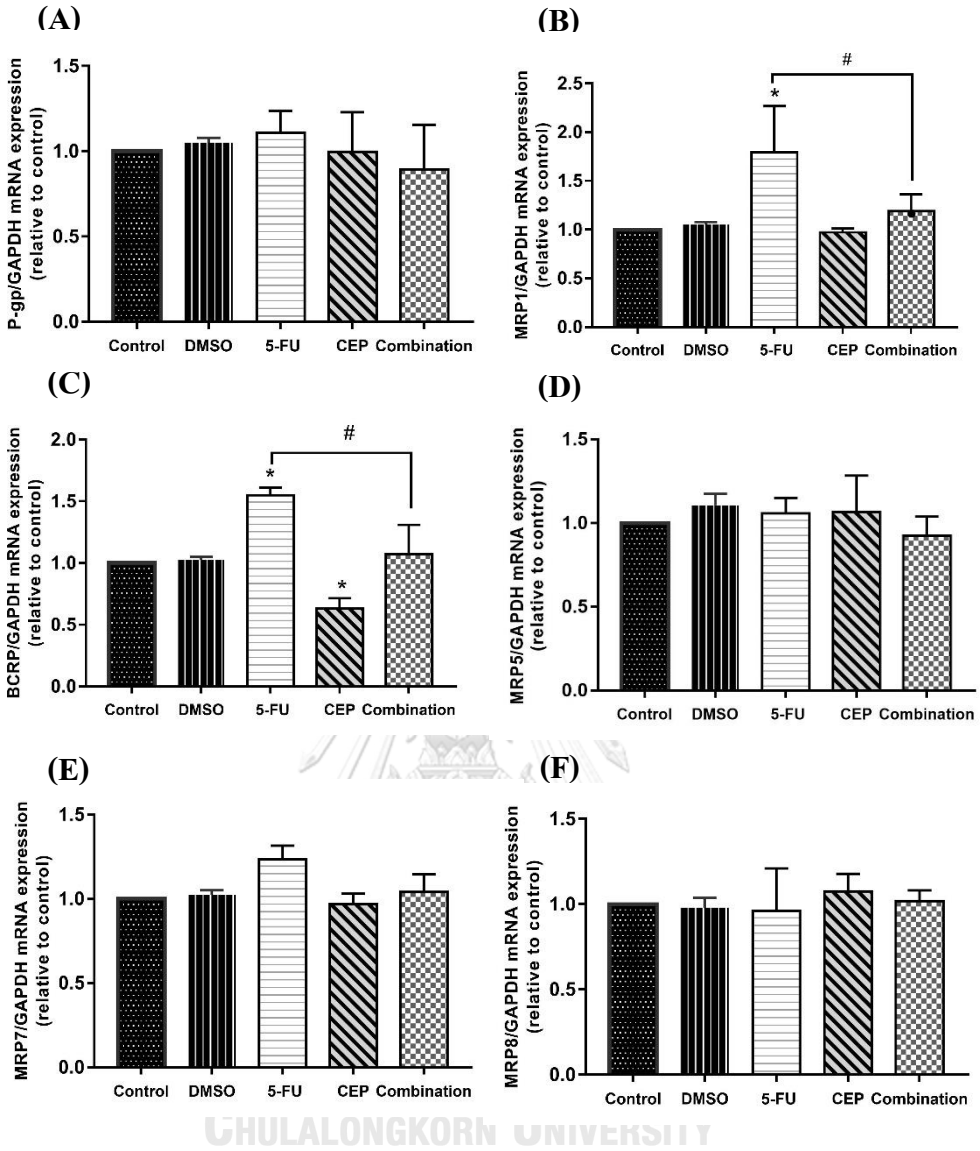


Figure 15. The effects of 5-FU or CEP alone and 5-FU in combination with CEP on efflux transporter mRNA levels in a colon cancer xenograft mouse model. The expression of (A) P-gp, (B) MRP1, (C) BCRP, (D) MRP5, (E) MRP7 and (F) MRP8 mRNAs in tumor tissues were analyzed by quantitative real-time RT-PCR. The values were normalized to GAPDH. Results of the control and DMSO groups (n = 4) and CEP or 5-FU and combination groups (n=5) were presented as means ± SEM. *$p \leq 0.05$ compared with control, $^{\#}p \leq 0.05$ compared with 5-FU.

4.7 The effects of 5-FU and CEP on COX-2 mRNA expression in a mouse xenograft model

COX-2 has played an important role in inflammation-promoted cancer by inducing cell proliferation, angiogenesis and metastasis and inhibiting apoptosis [81]. Therefore, the levels of COX-2 mRNA in tumor tissues were measured by quantitative real-time RT-PCR**. As shown in Fig.16**, CEP alone significantly downregulated the COX-2 mRNA expression when compared with control group ($p \leq 0.05$) whereas treatment with 5-FU alone did not significantly alter the expression of COX-2 mRNA with respect to the control group. Remarkably, the COX-2 mRNA levels in tumor tissues of animals receiving 5-FU in combination with CEP were significantly lower than those of the control ($p \leq 0.01$) or 5-FU-treated ($p \leq 0.001$) mice **(Fig.16).** Therefore, it is possible that CEP-induced COX-2 downregulation is involved in the synergistic anticancer effect of 5-FU and CEP.

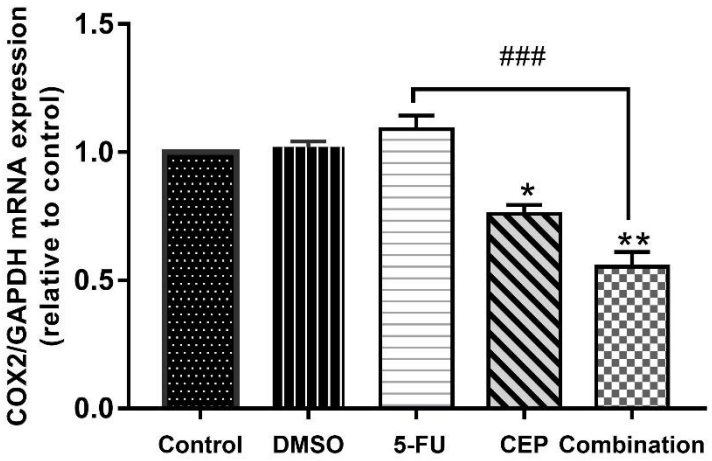


Figure 16. The effects of 5-FU or CEP alone and 5-FU in combination with CEP on the expression of COX-2 mRNA levels in a colon cancer xenograft mouse model. HT-29 tumor bearing mice were treated with either 5-FU or CEP alone or 5-FU in combination with CEP and the mRNA levels of COX-2 in tumor tissues were analyzed by quantitative real-time RT-PCR. The values were normalized to GAPDH. Results of the control and DMSO groups (n = 4) and CEP or 5-FU and combination groups (n=5) were presented as means ± SEM. *$p \leq 0.05$; **$p \leq 0.01$ compared with control, $^{###}p \leq 0.001$ compared with 5-FU.

CHAPTER V

DISCUSSION AND CONCLUSION

Colon cancer is the third most common type of cancer and the leading cause of cancer-associated mortality worldwide[3, 4]. In Thailand, the incidence of colorectal cancer has increased steadily over the past several years[5]. Chemotherapeutic agents, including 5-fluorouracil (5-FU), oxaliplatin, irinotecan and capecitabine, are commonly used in patients with colon cancer. Although 5-FU has been a mainstay in the treatment of colon cancer for many years, its clinical application has been hampered due to serious side effects and drug resistance[6]. Therefore, the approach of using natural compounds in combination with 5-FU is highly promising because the natural compounds may enhance the potency of 5-FU and minimize side effects associated with the chemotherapeutic agent.

Cepharanthine (CEP) has been approved by the Japanese Ministry of Health for the treatment of radiation-induced leukopenia, alopecia areata and alopecia pityrodes. Although it is widely used, its serious side effects have never been reported[24]. In addition, many *in vitro* and *in vivo* studies demonstrated that CEP possess various pharmacological activities, including anti-allergic, anti-oxidant, anti-malaria, immunomodulatory, multidrug resistance reversal as well as anticancer[24, 27]. Previously, CEP was shown to exert its anticancer activity against several types of cancer such as leukemia, lymphoma, lung carcinoma, myeloma, cholangiocarcinoma, oral squamous cell carcinoma, and hepatocellular carcinoma[23, 29, 30, 32, 33, 35-37, 89, 94, 117]. Remarkably, it was reported that CEP was more effective in controlling the growth of the p53 mutant HT-29 colon cancer cell line, which were relatively resistant to commonly used chemotherapeutic agents when compared to the p53 wild-type COLO-205 colon cancer cell line[96]. Additionally, CEP has

recently been found to enhance the anticancer effect of 5-FU in human HT-29 colon cancer cells *in vitro*[41]. Therefore, the present study was to evaluate the effects of CEP alone and CEP in combination with 5-FU in a colon cancer xenograft mouse model. The results of this study showed that treatment with either 5-FU or CEP alone significantly inhibited the growth of colon cancer *in vivo*. Notably, the combination treatment of 5-FU and CEP was more effective in controlling tumor growth with respected to treatment with either one of them alone. It however should be noted that there was no significant weight loss in animals receiving either 5-FU, CEP or their combination, indicating that all treatments may not be toxic to the mice. In agreement with the results of this study, safety of CEP has been demonstrated in animal models of leukemia, hepatocellular carcinoma and primary effusion lymphoma[33, 39, 89]

A previous study reported that the ratio of Bcl-xl and Bax was associated with chemosensitivity to 5-FU[118]. Similarly, it has been shown that overexpression of Bcl-xl could induce 5-FU resistance in colon cancer cell lines[119]. In addition, long-term exposure of 5-FU resulted in drug resistance in colon cancer cells which was mediated through upregulation of Bcl-2 and Bcl-xl and downregulation of Bax[120]. Although the results of this study demonstrated that treatment with 5-FU alone did not affect the mRNA levels of Bcl-2 family proteins, treatment of CEP alone significantly upregulated Bak at both mRNA and protein levels. Notably, combination treatment of CEP and 5-FU could significantly induce the expression of Bak mRNA and protein levels and downregulate Bcl-xl mRNA level. Previously, several studies indicated that CEP inhibited tumor growth in many cancers by modulating Bcl-2 family proteins[35, 36, 93]. CEP was shown to upregulate Bax, downregulate Bcl-2, activate caspase-3 and poly (ADP-ribose) polymerase (PARP) cleavage,

leading to apoptosis in non-small-cell lung cancer[93]. In addition, CEP could inhibit STAT signaling pathway, resulting in downregulation of target genes, including Bcl-x, c-Myc and cyclin D1 in osteosarcoma cell line[35]. CEP was also shown to enhance sensitivity of cervical adenocarcinoma cells to radiation by downregulating Bcl-2, c-Myc and COX-2[36]. Taken together, upregulation of Bak and downregulation of Bcl-xl may partly be responsible for the synergistic effects of CEP on anticancer activity of 5-FU against human colon cancer.

Many chemotherapeutic agents exert their anticancer activity via blocking cell cycle progression[121, 122]. Cell cycle is regulated by the cooperation of two important proteins, cyclin and cyclin-dependent kinase (CDK) which is further regulated by CDK inhibitors such as p21 and p27[121]. Previously, CEP was shown to suppress tumor growth by inducing G1 arrest through downregulation of cyclin E and upregulation of p27[95]. Additionally, CEP disrupted cell cycle progression by suppressing expression of cyclin D1, CDK-6, c-myc and upregulation of p21 in adenosquamous cell carcinoma[37]. Furthermore, it was reported that CEP inhibited the growth of myeloma cell line by triggering cell cycle arrest via upregulation of p21 and induced apoptosis through increased free radicals and enhanced Bax[31]. In a manner similar to other findings, upregulation of p21 expression at both mRNA and protein levels in tumor tissues following treatment with either CEP alone or CEP in combination with 5-FU were observed in this study. Previously, it was shown that vitamin D analogs (PRI-2191 and PRI-2205) could potentiate antitumor activity of 5-FU through increased p21 expression and decreased thymidylate synthase (TS) expression[123]. Similarly, upregulation of p21 and down-regulation of glucose transporter 1 (Glut-1) were shown to be involved in the synergistic inhibition of genistein and 5-FU on HT-29 colon cancer cell

growth[124]. Collectively, it is possible that CEP enhances the anti-cancer activity of 5-FU by increasing expression of p21.

Upregulation of the ABC efflux transporters such as P-glycoprotein (P-gp), multidrug resistance proteins (MRPs) and breast cancer resistance protein (BCRP) are primarily associated with drug resistance[125, 126]. A previous study demonstrated that long-term exposure of 5-FU led to multidrug resistance (MDR) by increasing the activity or expression of MRP1, Bcl-xl, thymidylate synthase (TS) and E-cadherin (E-cad) in the hepatocellular carcinoma cell line [66] and knockdown of MRP1 could increase response of MDR colorectal cancer cells to 5-FU and oxaliplatin both *in vitro* and *in vivo*[67]. In addition to MRP1, RNA interference (RNAi)-based knockdown of BCRP could enhance 5-FU-induced DNA damage in breast cancer cells, highlighting that overexpression of MRP1 and BCRP is associated with resistance to 5-FU[16, 127]. In agreement with other studies, the results of this study showed that treatment with 5-FU alone significantly induced expression of MRP1 and BCRP mRNAs in tumor tissues. In contrast to 5-FU, treatment with CEP alone significantly decreased BCRP mRNA expression with respect to the control group. Remarkably, significant decreases in MRP1 and BCRP mRNAs were noted in tumor tissues of animals receiving 5-FU in combination with CEP with respected to the 5-FU alone group, suggesting that CEP could prevent upregulation of MRP1 and BCRP caused by 5-FU. Recently, potentiating effect of epigallocatechin gallate on anticancer activity of 5-FU has been shown to be related to downregulation of MRP1 and BCRP, leading to higher intracellular 5-FU concentrations[128]. Therefore, it is likely that treatment of 5-FU induced MRP1 and BCRP expression, resulting in a decrease in drug accumulation in tumor tissues. However, CEP could prevent this phenomenon.

Cyclooxygenase-2 (COX-2) is often found to be overexpressed in many types of cancers, including breast, prostate and colon[77]. Increased COX-2 expression was associated with resistance to apoptosis, increased angiogenesis, and increased tumor invasiveness in various types of cancer, including colon cancer[81]. It was previously reported that the genetic inactivation of COX-2 suppressed intestinal adenoma development in mice[129]. Similarly, it was shown that nonsteroidal anti-inflammatory drugs (NSAIDs) targeting COX-2 could directly shrink colon adenomas in some patients [130]. Moreover, combination of celecoxib, a selective COX-2 inhibitor, with 5-FU was shown to effectively inhibit the growth of tumor in colorectal cancer (HT-29) xenograft model by downregulating COX-2 and inducing cytochrome C, caspase-3 and caspase-9[131]. Previously, it was reported that anti-inflammation effect of CEP was mediated thought inhibition of COX-2 expression at both mRNA and protein levels in a rodent model of limb ischemia–reperfusion[132]. Similarly, CEP suppressed the expression of COX-2 and inducible nitric oxide synthase (iNOS) and production of nitric oxide (NO), leading to attenuation of inflammation in RAW264.7 cells[133]. In addition to anti-inflammatory activity, anticancer effect of CEP has been reported to be partly associated with a decrease in COX-2 expression. Fang et al. reported that CEP enhanced sensitivity of human cervical adenocarcinoma cells to radiation through downregulation of COX-2[36]. Similarly, this study found that CEP alone significantly decreased the expression of COX-2 mRNA in tumor tissues. Moreover, a significant downregulation of COX-2 was also noted in tumor tissues of mice receiving CEP in combination with 5-FU when compared with the 5-FU alone treatment group. This is in agreement with other studies which demonstrated that the combination of genistein and 5-FU abolished the up-regulation of COX- 2 and secretion of prostaglandin

caused by 5-FU treatment in HT-29 colon cancer cells[23] and treatment of 5-FU in combination with curcumin could suppress COX-2 expression at both mRNA and protein levels, leading to improved effectiveness of 5-FU in colon cancer[36]. Taken together, it is likely that CEP-induced COX-2 downregulation is associated with the synergistic anticancer effect of 5-FU.

CONCLUSION

This study clearly illustrated that treatment with either CEP alone or CEP in combination with 5-FU effectively controlled the growth of colon cancer in a mouse xenograft model. Mechanistic studies revealed that combination of CEP and 5-FU resulted in upregulation of a CDK inhibitor, p21, and a proapoptotic regulator, Bak, at both mRNA and protein levels and downregulation of antiapoptotic Bcl-xl mRNA. Additionally, the combination also prevented 5-FU-induced MRP1 and BCRP mRNAs expression in tumor tissues. Moreover, treatment with either CEP alone or in combination with 5-FU downregulated the expression of COX-2 mRNA in HT-29 colon cancer mouse xenograft model. Therefore, this investigation suggests that CEP can potentially be used either alone or in combination with other chemotherapeutic agents for colon cancer treatment. However, further elucidation and verification of these observations are still warranted.

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APPENDIX

APPENDIX

PREPARATION OF REAGENTS

1. Incomplete DMEM medium stock solution (1 L)

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

Mix and stir until dissolve

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

Adjust final volume to 1 L with ddH_2O

Sterilize medium by filtering through a 0.2 sterile membrane filter

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

2. Complete DMEM medium (100 ml)

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

3. 1X Phosphate Buffered Saline (PBS) (1 L)

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

Mix and stir until dissolve

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

Adjust final volume to 1 L with ddH_2O

Transfer into cell culture bottle and sterilize by autoclaving and Store at room temperature

4. Separating buffer (500 ml)

Tris base	45.43 g

Mix and stir until dissolve

Adjust the pH to 8.8 using 1N NaOH or 1N HCl while stirring

Adjust final volume to 500 ml with ddH2O

5. Stacking buffer (500 ml)

Tris base	15.14 g

Mix and stir until dissolve

Adjust the pH to 6.8 using 1N NaOH or 1N HCl while stirring

Adjust final volume to 500 ml with ddH2O

6. Sample diluting buffer(SDB) (225 ml)

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

Mix all the ingredients., adjust final volume to 225 ml with ddH2O

7. 10x leamli buffer (1L)

Tris base	30.25 g
Glycine	144 g
Sodium dodecyl sulfate (SDS)	10 g

Mix and stir until dissolve

Adjust final volume to 1L with ddH2O

8. 1x leamli buffer (1L)

100 ml 10X leamli buffer in 900 ml ddH2O

9. 10x Tris-Buffered Saline (TBS) (1L)

Tris base	12.1 g
NaCl	87.5 g

Mix and stir until dissolve

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

Adjust final volume to 1L with ddH2O

10. 1x Tris-Buffered Saline(TBS) (1L)

100 ml 10x Tris-Buffered Saline (TBS) in 900 ml ddH2O

11. 1x Tris-Buffered Saline(TBS)/Tween buffer (1L)

0.5 ml Tween 20 to 1L Tris-Buffered Saline(TBS)

12. 10X transfer buffer (1L)

Tris base	30 g
Glycine	144 g
Sodium dodecyl sulfate (SDS)	1 g

Mix and stir until dissolve

Adjust final volume to 1L with ddH2O

13. 1x Transfer buffer(1L)

100 ml 10x transfer buffer and 200ml methanol to 1L with ddH2O

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